Optimization of postharvest processing for hops (*Humulus lupulus*) and cannabis (*Cannabis sativa*)

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List of acronyms

1-deoxy-D-xylulose-5-phosphate	DXP
2,2-diphenyl-1-picrylhydrazine	DPPH
2-C-methyl-D-erythritol-4-phosphate	MEP
3-hydroxy-3-methyl-glutaryl-CoA	HMG-CoA
4-diphosphocytidyl-2-C-methyl-D-erythritol	CDP-ME
Cannabichromene	CBC
Cannabichromenenic acid	CBCA
Cannabichromevarin	CBCV
Cannabichromevarinic acid	CBCVA
Cannabidiol	CBD
Cannabidiolic acid	CBDA
Cannabidivarin	CBDV
Cannabidivarinic acid	CBDVA
Cannabigerol	CBG
Cannabigerolic acid	CBGA
Cannabigerovarin	CBGV
Cannabigerovarinic acid	CBGVA
Cannabinol	CBN
Cannabivarin	CBV
Central composite rotatable statistical design	CCRD
D-glyceraldehyde-3-phosphate	G3P
Dimethylallyl pyrophosphate	DMAPP
Divarinolic acid	DA
Ferric reducing ability of power	FRAP
Freeze-drying	FD
Gas chromatography-tandem mass spectrometer	GC-MS/MS
Geranyl diphosphate	GPP
Isopentenyl pyrophosphate	IPP
Liquid chromatography-tandem mass spectrometer	LC-MS/MS
Microwave-assisted hot-air drying	MAHD

Nuclear magnetic spectroscopy	NMR
Olivetolic acid	OA
Quality Assurance and Quality Control for Cannabis	QAQCC
Tetrahydrocannabinol	THC
Tetrahydrocannabinolic acid	THCA
Tetrahydrocannabivarin	THCV
Tetrahydrocanabivarinic acid	THCVA
Trolox equivalent antioxidant activity	TEAC

Abstract

Differences in cannabis (*Cannabis sativa*) plant chemistry between accessions are influenced by genetics, plant growth and development, and environmental conditions. Resulting secondary metabolite profiles are further altered post-harvest during storage, drying and extraction, all of which present sizable challenges to licensed producers of food- and pharmaceutical-grade products in Canada and elsewhere. This thesis focused on improving cannabis biomass drying and extraction methods suitable for scale-up in the cannabis industry. Compiling new data for this novel research field, with few studies given the new regulatory framework, will help fill the knowledge gaps. Factors affecting the drying and extraction kinetics for the different systems were evaluated and optimized to improve the quality of dried biomass and extracts. Preliminary studies were first conducted with biomass from another Cannabaceae family member, hops (*Humulus lupulus*), to determine the effect of post-harvest processing on drying kinetics and oil extraction. Fresh and pre-frozen hops inflorescences at -80˚C were subjected to freeze-drying, hot air and microwave-assisted hot air drying (MAHD). The effects of drying temperature (35˚C, 50˚C, and 65˚C), with different microwave power (100 W and 200 W) were evaluated. Results showed that pre-freezing caused structural damage to the lupulin glands of hops. Irrespective of the drying condition for hops, pre-freezing reduced drying time by 0.17% to 85.9% by increasing the effective moisture diffusion coefficient. Moisture diffusion coefficient increased with higher drying temperature and microwave power, ranging between 5.9×10^{-10} m^2 s^{-1} and 2.4×10^{-7} m^2 s^{-1}. Knowledge acquired with hops was then applied to cannabis biomass from three cannabis accessions, Qrazy Train, Qrazy Apple, and Qrazy Angel. The relationship between sample mass reduction and relative humidity during freeze-drying and the effects of pre-freezing and freeze-drying temperature on cannabis drying kinetics, trichome structure, and color, in addition to cannabinoid and terpene concentrations were investigated. Cannabis samples were dried at 10˚C, and 20˚C, with different pre-freezing conditions (-20˚C and -40˚C). Data logged by the three relative humidity sensors (A, B, and C) showed that only sensor C recorded the closest to the actual changes in relative humidity during the entire drying process and can be attributed to placing the sensor near a representative cannabis bud in the center of the drying tray. Modelling studies showed that the rational regression model best explains the relationship between mass reduction and relative humidity during drying. Pre-freezing rates of 0.13˚C min^{-1} - 0.15˚C min^{-1} were recorded for pre-freezing at -20˚C and significantly ($p < 0.05$) increased by 71.2% - 73.5% when

inflorescence was pre-frozen at -40˚C. Freeze-drying increased [CBDA], [CBGA], and [CBG] in dried samples ranging from 0.45 mg g^{-1} to 0.38 mg g^{-1}, 2.87 mg g^{-1} to 4.91 mg g^{-1}, and 0.57 mg g^{-1} to 1.33 mg g^{-1}, respectively, when compared to fresh, undried samples. Irrespective of the pre-freezing condition or cannabis accession, drying at 20˚C reduced drying time by 10.4% to 31.9%. An increase in cannabinoid content caused by pre-freezing and freeze drying were further evaluated by analysing the antioxidant content in cannabis and hops. Antioxidant activity in extracted inflorescence using the 2,2-diphenyl-1-picrylhydrazine (DPPH) reduction and ferric reducing ability of power (FRAP) assays showed that pre-freezing significantly increased antioxidant values by 13% (DPPH) and 29.9% (FRAP) for hops, and by 7.7% (DPPH) and 19.4% (FRAP) for cannabis. Freeze-drying and MAHD significantly ($p < 0.05$) reduced antioxidant activity in hops by 79% and 80.2% [DPPH], respectively, and 70.1% and 70.4% [FRAP], respectively, when compared to antioxidant activity in extracts obtained from pre-frozen, undried hops. DPPH assay showed that both freeze-drying and MAHD significantly ($p < 0.05$) reduced antioxidant activity of cannabis by 60.5% compared to the pre-frozen samples, although there was no significant ($p < 0.05$) reduction in the antioxidant activity using the FRAP method. Extraction studies were conducted using cold ethanol, ultrasound-assisted, and microwave-assisted extraction to maximize yield and concentration of cannabinoids and terpenes in the extracts. Optimal conditions for ultrasound-assisted and microwave-assisted extraction had sample-to-solvent ratios of 1:15 and 1:14.4, respectively, for 30 min at 60°C. Ultrasound-assisted extraction yielded 14.4% and 14.2% more oil and terpenes, respectively, compared with microwave-assisted extracts. Optimal conditions at different extraction temperatures for cold ethanol extraction were a cannabis-to-ethanol ratio of 1:15 and a 10 min extraction time. Yields with cold ethanol extraction were 18.2, 19.7, and 18.5 g 100 g dry $matter^{-1}$ for −20°C, −40°C and room temperature, respectively. Considering reference ground samples, [THCA] increased from 17.9 to 28.5 and 20 g 100 g dry $matter^{-1}$ for ultrasound-assisted and microwave-assisted extraction, respectively. For cold ethanol extraction, compared to the reference ground sample, [THCA] changed from 17.9 g 100 g dry $matter^{-1}$ to 15, 17.5, and 18.3 for −20°C, −40°C, and room temperature, respectively. Data affirm that pre-freezing plant material prior to drying shortens postharvest processing times, and this can potentially be applied to other industrial crops. Assay-specific determination of antioxidant activity in medicinal plants may provide added value to extracts. Findings are of

industrial relevance for improving cannabis post-harvest extraction while ensuring quality of this regulated crop.

Résumé

Les différences dans la chimie des plantes de cannabis (*Cannabis sativa*) entre accessions sont influencées par la génétique, la croissance et le développement des plantes, et les conditions environnementales. Les profils des métabolites secondaires qui en résultent sont encore modifiés après la récolte pendant l'entreposage, le séchage et l'extraction, ce qui présente des défis de taille pour les producteurs autorisés de produits de qualité alimentaire et pharmaceutique au Canada et ailleurs. Cette thèse s'est concentrée sur l'amélioration des méthodes de séchage et d'extraction de la biomasse de cannabis qui conviennent à une mise à l'échelle dans l'industrie du cannabis. La compilation de nouvelles données pour ce nouveau domaine de recherche qui compte peu d'études, compte tenu du nouveau cadre réglementaire, aidera à combler des lacunes dans les connaissances. Les facteurs affectant la cinétique de séchage et d'extraction pour les différents systèmes ont été évalués et optimisés afin d'améliorer la qualité de la biomasse séchée et des extraits. Des études préliminaires ont d'abord été menées avec la biomasse d'un autre membre de la famille des Cannabaceae, le houblon (*Humulus lupulus*), afin de déterminer l'effet du traitement post-récolte sur la cinétique de séchage et d'extraction de l'huile. Des inflorescences de houblon fraîches et pré-congelées à -80°C ont été soumises à la lyophilisation, au séchage à l'air chaud et au séchage à l'air chaud assisté par micro-ondes (MAHD). Les effets de la température de séchage (35°C, 50°C et 65°C), avec différentes puissances de micro-ondes (100 W et 200 W) ont été évalués. Les résultats ont montré que la précongélation a causé des dommages structurels aux glandes de lupuline du houblon. Indépendamment des conditions de séchage du houblon, la précongélation a réduit le temps de séchage de 0.17% à 85.9% en augmentant le coefficient de diffusion de l'humidité. Le coefficient de diffusion de l'humidité augmentait avec la température de séchage et la puissance des micro-ondes, entre 5.9×10^{-10} $m^2 s^{-1}$ et 2.4×10^{-7} $m^2 s^{-1}$. Les connaissances acquises avec le houblon ont ensuite été appliquées à la biomasse de cannabis provenant de trois accessions de cannabis, Qrazy Train, Qrazy Apple et Qrazy Angel. Les relations entre la réduction de la masse de l'échantillon et l'humidité relative pendant la lyophilisation et les effets de la pré-congélation et de la température de lyophilisation sur la cinétique de séchage du cannabis, la structure des trichomes et la couleur, ainsi que les concentrations en cannabinoïdes et en terpènes ont été étudiés. Les échantillons de cannabis ont été séchés à 10°C et 20°C, avec différentes conditions de pré-congélation (-20°C et -40°C). Les données enregistrées par les trois capteurs d'humidité relative (A, B et C) ont montré que seul le

capteur C a enregistré les changements les plus proches de l'humidité relative réelle pendant tout le processus de séchage, ce qui peut être attribué au fait qu'il a été placé près d'un bourgeon de cannabis représentatif au centre du plateau de séchage. Les études de modélisation ont montré que le modèle de régression rationnelle explique le mieux la relation entre la réduction de masse et l'humidité relative pendant le séchage. Des taux de pré-congélation de 0.13°C min^{-1} – 0.15°C min^{-1} ont été enregistrés pour la pré-congélation à -20°C et ont significativement ($p < 0,05$) augmenté de 71.2% – 73.5% lorsque l'inflorescence a été pré-congelée à -40°C. La lyophilisation a augmenté [CBDA], [CBGA], et [CBG] dans les échantillons séchés de 0.45 mg g^{-1} à 0.38 mg g-1, de 2.87 mg g^{-1} à 4.91 mg g^{-1}, et de 0.57 mg g^{-1} à 1.33 mg g^{-1}, respectivement, par rapport aux échantillons frais non séchés. Indépendamment de la condition de précongélation ou de l'accession de cannabis, le séchage à 20°C a réduit le temps de séchage de 10.4% à 31.9%. L'augmentation de la teneur en cannabinoïdes causée par la pré-congélation et la lyophilisation a été évaluée plus en détail en analysant la teneur en antioxydants du cannabis et du houblon. L'activité antioxydante dans les inflorescences extraites à l'aide des tests de réduction de la 2,2-diphényl-1-picrylhydrazine (DPPH) et le pouvoir réducteur ferrique (FRAP) a montré que la pré-congélation augmentait significativement les valeurs antioxydantes de 13% (DPPH) et 29.9% (FRAP) pour le houblon, et de 7.7% (DPPH) et 19.4% (FRAP) pour le cannabis. La lyophilisation et la DHMV ont réduit de manière significative ($p < 0.05$) l'activité antioxydante du houblon de 79% et 80.2% [DPPH], respectivement, et de 70.1% et 70.4% [FRAP], respectivement, par rapport à l'activité antioxydante des extraits obtenus à partir de houblon pré-congelé et non séché. Le test DPPH a montré que la lyophilisation et la MAHD ont réduit de manière significative ($p < 0.05$) l'activité antioxydante du cannabis de 60.5% par rapport aux échantillons pré-congelés, bien qu'il n'y ait pas eu de réduction significative ($p < 0.05$) de l'activité antioxydante par la méthode FRAP. Des études d'extraction ont été menées en utilisant l'éthanol froid, l'extraction assistée par ultrasons et l'extraction assistée par micro-ondes pour maximiser le rendement et la concentration des cannabinoïdes et des terpènes dans les extraits. Les conditions optimales pour l'extraction assistée par ultrasons et par micro-ondes avaient des rapports échantillon-solvant de 1:15 et 1:14,4, respectivement, pendant 30 min à 60°C. L'extraction assistée par ultrasons a donné 14.4% et 14.2% d'huile et de terpènes en plus, respectivement, par rapport aux extraits assistés par micro-ondes. Les conditions optimales à différentes températures d'extraction pour l'extraction à l'éthanol froid étaient un rapport

cannabis/éthanol de 1:15 et une durée d'extraction de 10 minutes. Les rendements de l'extraction à l'éthanol froid étaient de 18.2, 19.7 et 18.5 g à 100 g de matière sèche^{-1} pour -20°C, -40°C et la température ambiante, respectivement. En ce qui concerne les échantillons de référence moulus, le [THCA] a augmenté de 17.9 à 28.5 et 20 g 100 g de matière sèche^{-1} pour l'extraction assistée par ultrasons et par micro-ondes, respectivement. Pour l'extraction à l'éthanol froid, par rapport à l'échantillon de référence, [THCA] est passé de 17.9 g 100 g de matière sèche^{-1} à 15, 17.5 et 18.3 pour -20°C, -40°C et la température ambiante, respectivement. Les données confirment que la congélation préalable du matériel végétal avant le séchage raccourcit les temps de traitement après la récolte, et cette méthode peut potentiellement être appliquée à d'autres cultures industrielles. La détermination spécifique de l'activité antioxydante des plantes médicinales peut apporter une valeur ajoutée aux extraits. Les résultats sont d'une importance industrielle pour améliorer l'extraction post-récolte du cannabis tout en assurant la qualité de cette culture réglementée.

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Contribution to original knowledge

In this work, earlier postharvest technologies for medicinal plants and other food crops were initially reviewed while considering preservation of important secondary metabolites, including cannabinoids and terpenes, within Cannabaceae family members. From an industry perspective, it was important to optimize the postharvest activities to increase the extraction yield and concentration of secondary metabolites in extracts while improving product shelf life. From a plant's perspective, the effect of postharvest parameters on quality attributes and metabolite profiles was examined using a wide range of pre-freezing, drying and extraction temperatures, microwave and ultrasound power densities, and storage conditions. Together, this work has contributed new knowledge to this scientific field that is applicable to the global transitioning cannabis industry. Specifically, our understanding of the effect of pre-freezing, drying, and extraction conditions on cannabis trichomes and extracts have been expanded in the following manner:

1. Pre-freezing is an important additional step for optimizing extraction of essential oils and drying of biomass. Scanning electronic microscopy analyses indicate that pre-freezing causes structural damage to lupulin glands for hops and cannabis trichomes making oils more readily available for extraction, thereby reducing extraction time and increasing yield.
2. Pre-freezing plant material prior to drying can shorten postharvest processing of hops and cannabis drying times by 0.2% - 85.9%. This method can potentially be applied to other industrial crops.
3. Pre-freezing biomass before drying can be used to improve metabolite profiles and potentially add value to cannabis biomass. Pre-freezing significantly increased antioxidant values by 13% (DPPH) and 29.9% (FRAP) for hops, and by 7.7% (DPPH) and 19.4% (FRAP) for cannabis. Results show a strong correlation between antioxidant activity and cannabinoid concentrations during storage and drying.
4. This work offers comprehensive and detailed information on the optimization of the freeze-drying methodology using relative humidity sensors to help with the real-time determination of the end of the drying process. This innovative method can prevent insufficient drying and has the potential to be used in other enclosed drying systems,

provided that the relative humidity sensors are placed near a representative bud in the center of the drying tray.

5. This work provides new insight into microwave-assisted hot air drying, freeze drying, and the impact of changing the operating parameters (pre-freezing, shelf temperature, and microwaves) on the drying behaviour of medicinal plants.
6. Freeze-drying can increase concentrations of CBDA, CBGA, and CBG in all dried samples which increased by 73.3% to 87.7%, 23.4% to 42.4%, and 6.9% to 51.9%, respectively, compared to their respective fresh, undried samples.
7. Although both microwave-assisted hot air drying (MAHD) and freeze-drying systems showed loss in secondary metabolites, freeze-drying has a higher metabolite retention compared to MAHD.
8. Increasing the shelf temperature of both drying systems will significantly decrease drying times by significantly increasing drying kinetics.
9. Drying kinetic modelling for hops and cannabis can be best described using the predictive Page and Logarithmic mathematical models which can be used for industrial scale-up purposes.
10. This work offers new comprehensive and detailed information on the extraction conditions for cannabis biomass using cold ethanol, ultrasound-assisted, and microwave-assisted extraction techniques. The data improve the extraction efficiencies of cannabis companies thereby reducing the high concentration of cannabinoids in their waste biomass.
11. Optimal conditions for ultrasound-assisted and microwave-assisted extraction were sample-to-solvent ratios of 1:15 and 1:14.4, respectively, for 30 min at 60°C. Ultrasound-assisted extraction yielded 14.4% more oil, compared with microwave-assisted extracts.
12. Optimal conditions at different cold ethanol extraction temperatures were shown at cannabis-to-ethanol ratio of 1:15 and 10 min extraction time, yielding high extraction efficiencies (83.6-102.1%).

Contribution of authors

Following the McGill Guidelines for a Manuscript-Based Thesis, the contributions made by the candidate and the co-authors to the completion of this work are described here.
Philip Wiredu Addo is the principal author of this work, supervised by Dr. Mark Lefsrud from the Department of Bioresource Engineering, McGill University, Sainte-Anne-de-Bellevue, Quebec, Canada.

Dr. Mark Lefsrud, the supervisor and director of the thesis, co-authored all manuscripts and provided scientific guidance in the planning and execution of the work, as well as co-editing and reviewing manuscripts.

Dr. Valérie Orsat and Dr. Vijaya Raghavan co-supervised this thesis, co-authored five chapters (3, and 5 – 7) and made valuable comments to improve the manuscripts.

Dr. Sarah MacPherson reviewed all manuscripts, made valuable comments to improve the manuscripts and co-authored all manuscripts.

Mr. Vincent Desaulniers Brousseau and Mr. Victorio Morello reviewed the literature reviewed manuscript and co-authored the chapter 2.

Mr. Tristan Chauvin-Bossé assisted in the coding of the sensors used for the thesis and co-authored chapter 4.

Dr. Maxime Paris provided funding assistance for the thesis and supervised studies conducted at EXKA Inc. He co-authored 3 chapters (2, 4, and 5)

Mrs. Nicole Taylor co-supervised the analytical analyses of cannabinoids and terpenes for the thesis and co-authored three chapters (3 – 5).

Mr. Sai Uday Kumar Reddy Sagili, Mr. Samuel Eichhorn Bilodeau, Mr. Frederick-Alexandre Gladu-Gallant, Mr. Douglas A. MacKenzie, Mrs. Jennifer Bates, Mr. Garnet McRae co-authored chapters 6 and 7. They provided the needed laboratory training and assistance needed to complete the thesis.

Journal papers

1. **Addo, P.W**., Brousseau, V.D., Morello, V., MacPherson, S., Paris, M., and Lefsrud, M., 2021. Cannabis chemistry, post-harvest processing methods and secondary metabolite profiling: A review. *Industrial Crops and Products*. 170:1-12. CHAPTER 2
2. **Addo, P.W.,** Taylor, N., MacPherson, S., Raghavan, V., Orsat, V., and Lefsrud, M., 2022. Impact of pre-freezing and microwaves on drying behavior and terpenes in hops (*Humulus lupulus*). *Journal of Applied Research on Medicinal and Aromatic Plant*. 100436:1-11. CHAPTER 3
3. **Addo, P.W.,** Chauvin-Bossé, T., Taylor, N., MacPherson, S., Paris, M., and Lefsrud, M., 2022. Freeze-drying of *Cannabis sativa* using real-time relative humidity monitoring and mathematical modeling for the cannabis industry (submitted to *Industrial Crops and Products*). CHAPTER 4
4. **Addo, P.W.,** Poudineh, Z., Taylor, N., MacPherson, S., Paris, M., Raghavan, V., Orsat, V., and Lefsrud, M., 2023. Correlation between total antioxidant capacity, cannabinoids, and terpenoids in hops and cannabis (submitted to *Plants*). CHAPTER 5
5. **Addo, P.W.,** Sagili, S.U.K.R., Bilodeau, S.E.; Gladu-Gallant, F.-A.; MacKenzie, D.A., Bates, J., McRae, G., MacPherson, S., Paris, M., Raghavan, V., Orsat, V., and Lefsrud, M., 2022. Cold ethanol extraction of cannabinoids and terpenes from cannabis using response surface methodology: optimization and comparative study. *Molecules*. 27(24):1-19. CHAPTER 6
6. **Addo, P.W.,** Sagili, S.U.K.R., Bilodeau, S.E.; Gladu-Gallant, F.-A.; MacKenzie, D.A., Bates, J., McRae, G., MacPherson, S., Paris, M., Raghavan, V., Orsat, V., and Lefsrud, M., 2022. Microwave- and ultrasound-assisted extraction of cannabinoids and terpenes from cannabis using response surface methodology. *Molecules*. 27(24):1-23. CHAPTER 7

Organization of thesis

Chapter 1 is the introduction of the thesis. The introduction explains the rationale behind the project. Chapter 2 encompasses the literature review and brief discussion of topics involved in this research. Chapter 3 describes the preliminary studies conducted using hops to help understand the effect of different postharvest handling/processing parameters. Chapters 4 to 7 describe the research and experiments that fulfilled each research objective. Between each chapter, connecting texts provide the transition and rationale between each study. Chapter 8 discuss the limitations of the thesis and practical aspects of the findings. Chapter 9 provides a summary of each study, describing the significant contributions to knowledge and suggesting further studies on the research topic. References and appendix follow. International System of Units (SI) are used throughout.

Chapter 1: Introduction

General introduction

This chapter provides background information and the rationale that lead to the development of this research project. The hypothesis and the objectives of this research are stated.

Background

The cannabis plant is one of the world's oldest plant sources for food and textile fibre (Ren et al., 2019). Believed to have originated in China, it is now mainly cultivated for its medicinal and psychotropic properties (Li, 1973; Ren et al., 2019). The different uses of this plant, as a raw material or as an entheogen, in conjunction with its introduction in a wide range of climates and environments, has resulted in a vast array of biotypes (Clarke and Merlin, 2016). Cannabis is classified as one taxonomical species, *Cannabis sativa*, but has been listed as three species (*Cannabis sativa, Cannabis indica,* and *Cannabis ruderalis*) by some scholars (Salentijn et al., 2015; Small, 2015). The phytochemistry of *Cannabis sativa,* the hemp type, exhibits a high cannabidiol (CBD) to tetrahydrocannabinol (THC) ratio containing less than 0.3% THC, while the opposite is true for *Cannabis indica* with a THC content greater than 1% (Fischedick et al., 2010). *Cannabis ruderalis* is normally ignored as it contains the lowest levels of THC and CBD (Bilodeau et al., 2019; Fischedick et al., 2010; Small, 2015). Hobbyists and scientists have hybridized, back-crossed, and propagated these plants for desirable characteristics, amounting to greater than 2,300 different strains or "chemovars" (Salentijn et al., 2015). With this, some researchers now classify cannabis strains according to their relative cannabinoid concentrations, accession for THC, CBD, and cannabigerol (CBG) (Aizpurua-Olaizola et al., 2016; Fournier et al., 1987; Pacifico et al., 2008).

The traditional use of cannabis as a medicinal crop has been substantiated with recent studies highlighting its potential use in oncology (Birdsall et al., 2016; Chandra et al., 2017), the management of schizophrenia (Deiana, 2013), epilepsy (Maa and Figi, 2014), chronic pain (Boehnke et al., 2016; Häuser et al., 2018), and other diseases (Campeny et al., 2020; Kosiba et al., 2019; Wilsey et al., 2013; Wilsey et al., 2008). In the US, cannabis is considered a Schedule 1 drug that is still highly prohibited and regulated in many States (Mead, 2017; Whiting et al., 2015). The cannabis industry in Canada is controlled by the Cannabis Act; this Act permits the sale of cannabis in the form of fresh or dry leaves, plants, and seeds for research or adult use

(Cox, 2018). A growing number of countries have now legalized the use of medical cannabis, changing the stigma around the contemporary criminal nature of this plant (Abuhasira et al., 2018).

New laws surrounding cannabis have spurred a renewed interest in cannabis research (Burnett et al., 2022). THC has been the hallmark cannabinoid since its isolation and synthesis in 1964 (Gaoni and Mechoulam, 1964), yet changes to the cannabis research paradigm have enabled the study of other cannabinoids, such as cannabinol (CBN), cannabigerol (CBG), and other classes of secondary metabolites, particularly terpenes. Studies of these molecules have given rise to the "entourage effect" concept, whereby it is hypothesized that the medical potential of cannabis stems from a synergistic combination of many cannabinoids (estimated at over 100) and terpenes, rather than just one molecule (Aizpurua-Olaizola et al., 2016; Fowler, 2003; Russo, 2011; Russo, 2018). Our current understanding is that cannabinoids alter brain activity by acting on cannabinoid receptors (Howlett, 2002; Ranganathan and D'Souza, 2006; Van Sickle et al., 2005; Volkow et al., 2016). Two major cannabinoid receptors, CB_1 and CB_2, reportedly affect neuronal processing and immunomodulation, respectively (Howlett, 2002). Although this simplified model appears inadequate, as some evidence suggests that therapeutic cannabinoids could work in conjunction with other receptors (McHugh et al., 2010) and synergistically with terpenes via the "entourage effect" (Russo, 2011; Russo, 2018). More recently, several recent animal studies indicate that major terpenes in cannabis do not directly activate the CB_1 or CB_2 receptors (Ferber et al., 2020; Harris et al., 2019; Santiago et al., 2019).

The emerging era of medical cannabis and cannabinoid research requires optimal post-harvesting processes that maintain consistency, coupled with complete secondary metabolite profiling for different cannabis strains. Drying and extraction techniques that conserve the chemical composition and integrity of pharma-grade cannabis extracts with different profiles are of the utmost importance (Challa et al., 2021). As the demand for medical and adult use (recreational) cannabis increases, new advances in nutraceutical drying and extraction may be adopted and applied to the cannabis plant to diminish the time needed for efficient drying and extraction, increase extract yield, add value to by-products, minimize solvent consumption, and standardize extract quality (Wang and Weller, 2006).

Connecting text

In this review, a summary of cannabis chemistry and biosynthesis of secondary compounds is provided, and post-harvest processing practices occurring along the cannabis product value chain that might affect cannabis phytochemistry, potency, and volatility are presented. An emphasis was placed on improved drying and extraction methods for plant material suitable for the cannabis industry.

This section was published as a literature review manuscript as follows:

Addo, P.W., Brousseau, V.D., Morello, V., MacPherson, S., Paris, M., Lefsrud, M., 2021. Cannabis chemistry, post-harvest processing methods and secondary metabolite profiling: A review. *Industrial Crops and Products.* 170:1-12.

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Supervision - Mark Lefsrud and Maxime Paris

Chapter 2: Cannabis chemistry, post-harvest processing methods and secondary metabolite profiling: A review

Literature review

2.1 Cannabis plant anatomy and cannabinoid-containing structures

The cannabis plant belongs to the Cannabaceae family that includes flowering plants such as hops (*Humulus lupulus*) and hackberries (*Celtis occidentalis*). Main similarities among plants in this family include palmate leaves and dioecious flowers grouped into inflorescences (or buds) (Small, 2015). Cannabis plants have palmate leaves with serrate leaflets (Bernstein et al., 2019) and green cylindrical stems support inflorescences. Smaller inflorescences are present on lower branches whilst the largest apical inflorescence, or cola, forms at the top (Feeney and Punja, 2015).

Aerial parts of the cannabis plant are covered with non-glandular trichomes and glandular trichomes (Desaulniers Brousseau et al., 2021). Non-glandular trichomes are found on stems, leaves, petioles, stipules, and bracts of the plant. They regulate the climate surrounding the tissue and hence, protect plant tissues against biotic and abiotic stresses (Giordano et al., 2020). Glandular trichomes are the primary structures for cannabinoid and terpenoid production and storage (Livingston et al., 2020). Glandular trichomes can be divided into three groups based on their shape: capitate-stalked, capitate-sessile, and bulbous. Capitate-stalked trichomes are large and globular; they are mostly found in cannabis flowers during the flowering stage (Happyana et al., 2013). Capitate-sessile trichomes are mostly found in abaxial leaf surfaces, petioles, and young stems during the vegetative and flowering stages (Livingston et al., 2020). Bulbous trichomes are the smallest type of glandular trichome and are balloon-shaped (Happyana et al., 2013; Raman et al., 2017). Studies show that cannabinoid and terpene biosynthesis mostly occur in the capitate-stalked and capitate-sessile trichomes (Happyana et al., 2013; Namdar et al., 2018).

2.2 Cannabinoid and terpenoid biosynthesis

2.2.1 Cannabinoids in cannabis

Medically relevant cannabinoids may be grouped into three categories: endocannabinoids, phytocannabinoids and synthetic cannabinoids (Maurya and Velmurugan,

2018). Endocannabinoids are produced by almost every organism in the Animalia kingdom (Ueda et al., 2015). They are endogenous lipids that are predominantly found in the brain, interacting with cannabinoid receptors and affecting physiological functions (Maurya and Velmurugan, 2018). Phytocannabinoids are naturally produced by the cannabis plant (Wang et al., 2016; Yamaori et al., 2010). They are formed through decarboxylation of their respective 2-carboxylic acids, a process that is catalyzed by heat, light, or alkaline conditions (Wang et al., 2016). The four major phytocannabinoids are THC, CBD, CBG, and CBN. While THC is mainly psychoactive (Morrison et al., 2009; Wachtel et al., 2002), CBD is mainly used for pain relief (Burstein, 2015; Whiting et al., 2015), and CBG has reportedly demonstrated antibacterial properties (Appendino et al., 2008). Cannabinol is the primary product of THC degradation. Cannabinol concentrations increase after harvest, during storage, extraction of secondary metabolites, and exposure of cannabis plants to light and air (Wianowska et al., 2015). Although cannabinol causes psychological and behavioral effects, it is less potent than THC (Maurya and Velmurugan, 2018).

Synthetic cannabinoids are compounds that have the same binding affinity as phytocannabinoids for human cannabinoid receptors (Shevyrin and Morzherin, 2015). For instance, dronabinol is an FDA-approved synthetic form of THC, while other synthetic cannabinoids are cautioned against for unknown toxic effects and abuse potential (Cooper, 2016). Interestingly, the complete biosynthesis of major cannabinoids was recently reported in yeast (*Saccharomyces cerevisiae*) (Luo et al., 2020).

2.2.1.1 Cannabinoid biosynthesis

Cannabinoids are produced when olivetolic acid (OA) or divarinolic acid (DA) is prenylated by geranyl diphosphate (GPP) (Raharjo et al., 2004). Olivetolic acid is geranylated to form cannabigerolic acid (CBGA), which is converted by oxidocyclase enzymes to Δ9-tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), cannabichromenenic acid (CBCA) and CBG. Cannabigerovarinic acid (CBGVA), produced from the geranylation of divarinolic acid, is a biosynthetic precursor of Δ9-tetrahydrocanabivarinic acid (THCVA), cannabidivarinic acid (CBDVA), cannabichromevarinic acid (CBCVA), and cannabigerovarin (CBGV). These undergo non-enzymatic decarboxylation to yield Δ9-tetrahydrocannabivarin (THCV), cannabidivarin (CBDV), and cannabichromevarin (CBCV), respectively (Wang et al.,

2016). Decarboxylation is necessary to convert precursor molecules, such as THCA, CBDA, CBCA, and CBGA, into the pharmaceutically active compounds THC, CBD, cannabichromene (CBC), and CBG (Citti et al., 2018; Taschwer and Schmid, 2015; Wang et al., 2016).

2.2.2 Terpenes in cannabis

Plant essential oils may contain terpenes, polyketides, alkaloids, and lipids that are produced via secondary metabolism, and they are not directly associated with the plant's growth and development (Andre et al., 2016). Terpenes are aromatic hydrocarbon compounds that are used by plants to deter herbivores (Andre et al., 2016; War et al., 2012). Isoprene (2-methy-1,3-butadiene), a 5-carbon unit, serves as the building block for all terpenes (Ashour et al., 2018). It has been suggested that both cannabinoids and terpenes may be used as chemotaxonomic markers to distinguish between different cannabis strains (Elzinga et al., 2015).

2.2.2.1 Terpene biosynthesis

Although terpenes are often used interchangeably with terpenoids, terpenoids are modified terpenes that contain an oxygen-containing functional group (Pichersky and Raguso, 2018). Terpenoids are produced via the mevalonic acid pathway or the non-mevalonate pathway (Zhu et al., 2014). Both of these pathways comprise two phases: the synthesis of the isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) molecules and condensation of these molecules to yield various terpenes (Hunter, 2007). The first three steps of the mevalonic acid pathway are known as the upper pathway. It involves the condensation of two acetyl-CoA molecules to produce an acetoacetyl-CoA molecule (Zhu et al., 2014). The acetoacetyl-CoA molecule undergoes condensation to yield 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). Mevalonate is produced by a reduction reaction of HMG-CoA (Lefer et al., 2001; Schönbeck and Libby, 2004). The lower mevalonic acid pathway involves the conversion of the mevalonate into IPP and DMAPP (Hunter, 2007; Zhu et al., 2014).

The non-mevalonate pathway begins with the condensation of pyruvate and D-glyceraldehyde-3-phosphate (G3P) to 1-deoxy-D-xylulose-5-phosphate (DXP) (Odom 2011). The compound, DXP, undergoes a reduction reaction to yield 2-C-methyl-D-erythritol-4-phosphate (MEP) which is converted to 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) in the cytidine triphosphate (CTP) dependent reaction (Kuzuyama 2002). The metabolite, CDP-

ME, undergoes phosphorylation to produce 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate (CDP-ME2P). Isopentenyl pyrophosphate, the terminal product of phase one of the non-mevalonic pathway, is produced from CDP-ME2P. Enzymes required for the terpenoid sysnthesis are attractive targets for the development of drugs and herbicides (Shi et al., 2019; Umeda et al., 2011). For example, DXP reductoisomerase is inhibited by fosmidomycin, a natural herbicidal product that interferes with carotenoid synthesis (Corniani et al., 2014).

2.3 Drying techniques in the cannabis industry

Different drying techniques have been used to preserve cannabis plant material. Oven drying increases plant shelf life, yet the limited application of oven drying and the high risk of quality changes has led to the development of alternate drying technologies (Prakash and Kumar, 2014; Vijayavenkataraman et al., 2012). Modern drying techniques, such as microwave-assisted hot-air drying and freeze-drying, have improved drying rates and quality deterioration (Kwaśnica et al., 2020; Orsat et al., 2007). In the cannabis industry, hot-air oven drying is commonly used because of its ease of operation and low cost. However, some studies show that this technique causes the evaporation of some essential secondary metabolites (Argyropoulos and Müller, 2014; Muñoz-López et al., 2018). Kwaśnica et al. (2020) showed that essential oil, myrcene, and humulene yields from hemp reduced by 25%, 23%, and 37% respectively when the oven temperature was increased from 50°C to 70°C. To mitigate this problem, the selection of a drying technique for cannabis should be determined by the strain's chemical profile, drying behaviour, and the end product required. As of yet, at least one manufacturer of a medical-grade cannabis products has reported the use of a forced-air dryer for large scale drying and a simple laboratory oven for smaller samples (Chandra et al., 2017). In this section, modern drying technologies that could be considered appropriate for the cannabis industry are reviewed.

2.3.1 Vacuum freeze-drying

Vacuum freeze-drying (lyophilization) is the removal of water molecules by sublimation and surface desorption (Tsinontides et al., 2004). This technology is based on the phase behaviour of water at the triple point, the temperature, and pressure at which all three phases of water coexist in an equilibrium (Ishwarya et al., 2015; Tsinontides et al., 2004). Water exists as a solid in the ABD zones, as a liquid in the DBC zones, and as a vapor in the ABC zones (Figure

2.1). All thermal motions of water molecules cease, and the sublimation, melting, and vaporization curves of water meet at the triple point. This occurs at 0.01°C and 0.61 kPa. Freeze drying occurs below the triple point of water, as water is converted directly from the solid phase to the gaseous phase.

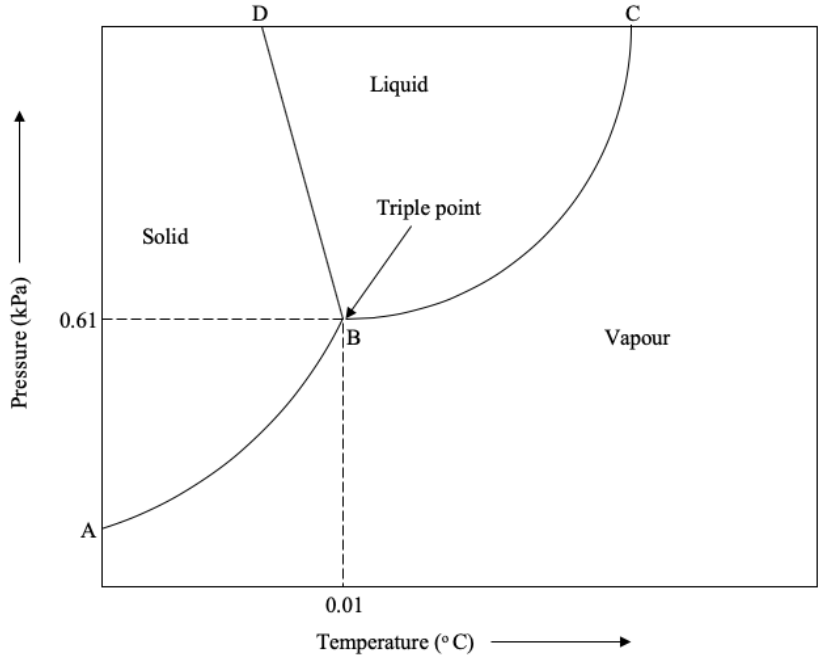

Figure 3.1. Triple point of water based on pressure and temperature.

The freeze dryer consists of a drying chamber with an airtight door and a quick-locking arrangement. The dryer is connected to a vacuum system to reduce the pressure of the system and a condenser to remove vapors sublimed off the product. Freeze-drying is carried out in three steps; freezing, primary drying, and secondary drying (Tang and Pikal, 2004). During freezing, sample temperature is reduced to approximately -40°C, thus converting most of the water present into ice. Freezing the product before drying is a critical step as it determines the final quality of the product. This step prevents the formation of water foam when the vacuum is applied (Kasper and Friess, 2011).

Primary drying is the sublimation of ice present in the frozen plant material. It is a slow process conducted at cooler temperatures, safely below the product's critical collapse temperature. Primary drying is based on the temperature and pressure gradients between the

sample and the system; this is achieved by keeping the system pressure constant below the triple point of water and increasing the temperature (Figure 2.1). Drying is complete when the sample's temperature is the same as the set system temperature (Patel et al., 2010). Secondary drying is performed to remove residual moisture present in the sample (Tang and Pikal, 2004). This is done at an appropriate higher temperature that does not destroy heat-liable compounds such as proteins and lipids present in the sample.

Vacuum freeze-drying has served as the benchmark for the production of high-quality dried substances (Jangam, 2011). Analyses of two tomato (*Lycopersicon esculentum*) varieties showed that freeze-drying decreased ascorbic acid content by 10%, while hot air drying decreased ascorbic acid content by 56% (Chang et al., 2006). Phytochemical analyses of *Aloe vera*, a medicinal plant, showed no significant differences between the freeze-dried and fresh samples (Ng et al., 2020). This shows the potential use of the technology in the cannabis industry. The low temperature employed in freeze-drying inhibits microbial and enzymatic activities. This maintains the sample quality and structure (Ratti, 2001). The main disadvantage of vacuum freeze-drying is the high initial capital and operational cost. It only permits drying in batches and requires higher energy (Bantle et al., 2011; Rahman and Mujumdar, 2008).

2.3.2 Atmospheric freeze-drying

Atmospheric freeze-drying is advantageous because the drying process is run under atmospheric pressure and a continuous system may be designed (Claussen et al., 2007). The diffusion of water vapor from the drying boundary through the dried surface of a material is based on the vapor pressure gradient between the material and the cold air, and not the absolute pressure on the system (Meryman, 1959). Vapor pressure is directly proportional to temperature, and the low temperature of cold air results in low vapor pressure (Meryman, 1959; Yang et al., 2012). This serves as the driving force for the removal of water from frozen material. Atmospheric freeze-drying is achieved by circulating cold dry air over the frozen material below -3°C to -10°C, to maintain the frozen nature of the material and improve the mass transfer of the water (Bantle et al., 2011; Claussen et al., 2007; Rahman and Mujumdar, 2008).

Atmospheric freeze-drying can be carried out in a tunnel dryer, fluidized bed dryer, or a spray freeze dryer. In the fluidized bed dryer, cold air is introduced at atmospheric pressure through the perforated bed and mechanical shaking is used to create the fluidized effect (Iheonye

et al., 2020). Frozen materials are lifted from the bottom and suspended in a stream of air. Heat transfer is accomplished by direct contact between the frozen material and cold air, and mass transfer is achieved by the sublimation of the frozen water from the material. Compared to tunnel dryers, the heat and mass transfer rates are better in fluidized bed dryers. Tunnel dryers can avoid size reduction problems caused by mechanical shaking. Spray freeze-drying is a combination of spray drying and freeze-drying. It involves the atomization of the material, freezing, and drying (Claussen et al., 2007; Ishwarya et al., 2015). Volatile retention analyses using a gas spectrometer proved that spray freeze-drying has higher retention (93%) compared to freeze-drying (77%) and conventional hot air spray drying (57%) (Ishwarya et al., 2015).

The main goal of atmospheric freeze-drying is to reduce freeze-drying time and maintain the quality of the dried material. One study evaluated the general acceptability of dehydrated apple (*Malus domestica*) samples using organoleptic assessment. High scores were acquired for both vacuum freeze-drying and atmospheric freeze-drying, confirming no significant differences between the two processes concerning sensory quality (Reyes et al., 2011).

2.3.3 Hot air drying

Hot air drying is a food preservation method based on heat, mass, and momentum transfer principles in thermodynamics (Devahastin and Niamnuy, 2010). It involves the evaporation of water or any other solvent by the movement of hot air around the material to be dried (Wankhade et al., 2013). Hot air drying can be used to enhance the shelf-life of products over one year, if products are well packaged and if water activity is reduced to levels that prevent microbial growth (Tapia et al., 2008).

Hot air drying can adversely affect the physical, biochemical, and quality attributes of fruits and vegetables resulting from thermal degradation of functional, nutritional, and organoleptic properties (Argyropoulos and Müller, 2014; Kotwaliwale et al., 2007; Muñoz-López et al., 2018; Yanyang et al., 2004). Argyropoulos and Müller (2014) studied the effects of convective hot air drying temperature on the essential oil of lemon balm (*Melissa officinalis*). The study showed the essential oils reduced by 16%, 23%, 65%, and 73% at 30°C, 45°C, 60°C and 75°C drying temperatures, respectively when compared to undried samples. In a Mexican plum (*Spondias purpurea*) study performed in a pilot-scale vertical tray dryer, the evaporation of water caused deformation and brown pigmentation on the fruit's surface. This pigmentation

resulted from high temperature, prolonged drying, and reactions such as non-enzymatic browning (Muñoz-López et al., 2018). To reduce these effects and increase the drying rate using a hot air dryer, pre-treatment has been recommended. Blanching with potassium metabisulphite or ascorbic acid can reduce the drying time by 12.5% at 55°C and 65°C (Demir et al., 2004; Kingsly et al., 2007). Moreover, it has been demonstrated that pre-treatment with 0.5% sodium metabisulfite or 0.5% calcium chloride significantly preserves the overall quality of dried tomato samples, as lycopene, total phenolic compounds, and β-carotene were best retained when compared to oven drying (Mwende et al., 2018). The evaporation temperature of volatile terpenes may be of concern for cannabis extracts, and the addition of food preservatives remains pertinent to labelling of edible cannabis products. Nonetheless, the limitations of hot air drying can be overcome by combining this method with ultrasound, infrared, and microwave technologies (Alibas, 2007; Hebbar et al., 2004; Liu et al., 2015b).

2.3.4 Microwave-assisted hot air drying

Microwaves are non-ionizing electromagnetic waves found between the radio and infrared wavelengths on the electromagnetic spectrum (Chandrasekaran et al., 2013). Microwaves are located within the frequency band of 300 MHz to 300 GHz. For most industrial applications, 915 MHz is considered most useful because of its greater penetration depth. A frequency of 2,450 MHz is mostly used in domestic microwave ovens and commercial units designed for analytical chemistry studies (Chandrasekaran et al., 2013; Zhang et al., 2006).

Microwave-assisted hot air drying is based on volumetric heating, which ensures uniform distribution of heat (Wray and Ramaswamy, 2015). It enhances the drying rate through the excitation of the electric dipoles of water molecules. Microwave energy increases the kinetic and intermolecular energies of the water molecules (Zielinska et al., 2019). At the beginning of the drying process, microwaves create porous structures in the material to enhance the flow of moisture from the inner part to the surface (Zhang et al., 2006). Microwave energy heats the water molecules to the evaporation temperature, creating a temperature gradient. This promotes the movement of the water molecules to the outer layers of the material (Feng et al., 2012). Microwaves, during the final stages of the drying process, help with the removal of the bound water present in the material (Andrés et al., 2004).

Various studies have demonstrated that air drying coupled with microwaves significantly improves the preservation of nutrient contents, microstructure, and color of the dried sample (Dev et al., 2011; Pham et al., 2018). Garlic (*Allium sativum*) drying time was reduced by 80%, with superior quality when microwaves at 0.4 W g^{-1} were combined with hot air at 60–70°C (Sharma and Prasad, 2001). In addition, 2,450 MHz microwave-assisted hot air drying at 50°C significantly reduced the loss of volatile and bioactive compounds in fresh drumstick fruits (*Moringa oleifera*) pods when compared to conventional hot air drying, while drying time and energy usage were reduced (Dev et al., 2011).

To maintain drying temperature, drying rate, and avoid the risk of overheating, microwaves are applied intermittently throughout the drying process (Li et al., 2010). Specifically, drying of plant material can be done at a lower temperature for a shorter time (Li et al., 2010; Orsat et al., 2007). It may be possible to apply this drying technique in the cannabis industry to help reduce the effect of drying on the quality and potency of cannabis. Ultimately, techniques that preserve terpenes and cannabinoids should be considered.

2.4 Stability of secondary metabolites in cannabis during storage

Various factors related to post-harvest cannabis storage conditions, such as microbial activity, moisture content, room temperature, duration, and light affect the quality and potency of cannabis (Backer et al., 2019; Grafström et al., 2019; Mazzetti et al., 2020; Morello et al., 2022; Raut et al., 2020b; Taschwer and Schmid, 2015). After harvest, the biosynthesis of the secondary metabolites is disrupted and chemical composition is altered (ElSohly et al., 2017; Taschwer and Schmid, 2015). Storage studies performed by Grafström et al. (2019) over four years showed that CBD is not prone to oxidative degradation and stable over time. However, decarboxylation of THCA to THC in the stored plant material is increased by the presence of oxygen and an increase in temperature (Wang et al., 2016). Specifically, THC concentrations markedly increase from 1.5% to 2.1%, 12.3%, and 12.8% when stored at 50°C, 100°C, and 150°C, respectively, due to THCA decarboxylation (Taschwer and Schmid, 2015). Cannabinol (CBN) is not present in fresh plant material; rather, CBN is formed by the decarboxylation of THC during extended storage, mostly over 24 h, and at temperatures over 50°C (Taschwer and Schmid, 2015; Wang et al., 2016).

Cannabis can be successfully stored in an air-tight bag in darkness in a cold room with no secondary metabolite degradation (Grafström et al., 2019). This system, termed "curing" by some cannabis growers, is done to preserve volatile compounds (terpenes), and promote the decarboxylation of THCA to THC under controlled storage conditions. This process inhibits the activity of pathogenic and spoilage microorganisms (McPartland and McKernan, 2017). As favourable nutrient levels, moisture content, water activity, and temperature can promote microbial growth, cannabis plant material can host a variety of microorganisms that can affect the concentration of the secondary metabolites (McKernan et al., 2015; Winston et al., 2014). Poor drying and storing under humid conditions encourage the formation of microbial spores, and harmful microbial toxins such as aflatoxin, as well as powdery mildew and botrytis (Chandra et al., 2017; Charoux et al., 2019). Generally, storing plants at a water activity level below 0.3 and 11% moisture content can inactivate microbial activity (McKernan et al., 2015; McPartland and McKernan, 2017).

2.5 Particle size reduction of cannabis

Particle size reduction (grinding) is required to efficiently extract secondary metabolites from cannabis (Patel et al., 2017). Grinding improves surface area contact between plant materials and extraction solvents. Grinding is different from powdering as powdered materials have a more homogenized and smaller particle size, leading to better surface area contact with extraction solvents (Kamble et al., 2019; Pegoraro et al., 2019). Deciding between dry and wet grinding is one of the critical problems in the grinding. Studies show that dry grinding requires more energy and time compared to wet grinding (Mani et al., 2004; Moiceanu et al., 2019).

Variables to consider in the design of the grinder are the feed rate, rotor speed of the equipment, temperature, and moisture content of the cannabis (Kamble et al., 2019; Mani et al., 2004), as these factors are important for conserving secondary metabolites and minimizing energy consumption. In cannabis, elevated temperatures can cause cannabinoid decarboxylation and the evaporation of terpenoids (Pegoraro et al., 2019; Wang et al., 2016). In sample preparation described for laboratory studies, a mortar and pestle are commonly used to grind cannabis (Lewis-Bakker et al., 2019). This method is not feasible for commercial production and the current practice in most industry settings is the use the cryogenic grinding technology (Atkins, 2019; Chandra et al., 2017). This involves cooling of the plant material with liquid

nitrogen or dry ice beyond its glass state before milling to help conserve secondary metabolites (Atkins, 2019; Balasubramanian et al., 2012). If dry ice is used, dry ice mass should be three times that of the cannabis sample; this improves grinding into finer particulates (Patel et al., 2017).

2.6 Cannabinoid and terpene extraction

Optimal extraction of essential oils containing medically relevant bioactive compounds is paramount. Variations in essential oil quality and quantity are due to several factors, including geographical location, vegetative plant phases, environmental factors, and extraction methods (Heikal, 2017; Naghdi Badi et al., 2004). Commonly used extraction methods include distillation, conventional Soxhlet extraction, ultrasound-assisted extraction, and microwave-assisted extraction (Albero et al., 2019; Brighenti et al., 2017; Pegoraro et al., 2019). Most studies have reported that modern extraction methods, such as microwave-assisted extraction and ultrasound-assisted extraction, are comparably efficient to traditional solvent methods (Carro et al., 2013; Chemat et al., 2017; Pegoraro et al., 2019; Péres et al., 2006; Schaneberg and Khan, 2002). Methods for extracting oils from cannabis are based on the solvent polarity effects on the secondary metabolite under investigation (Dilworth et al., 2017; Jiang et al., 2017). Polar solvents such as ethanol and hexane are mostly preferred to non-polar solvents, as they have a higher affinity for cannabinoids and terpenes (Schaneberg and Khan, 2002). The concentration of cannabinoids and terpenoids decrease with the position of the inflorescence from top to bottom of the flowering stem regardless of the extraction method (Namdar et al., 2018).

2.6.1 Distillation

Distillation is commonly used to extract plant essential oils (Baydar et al., 2008; Mejri et al., 2010). It is based on the principle of isotropic distillation, as desired secondary metabolites form an azeotropic mixture with water (Ma et al., 2019). Distillation is done by heating a mixture of water and plant material in a flask, followed by the condensation of the vapor (El Asbahani et al., 2015). A basic setup comprises a heat transfer medium, a condenser and a decanter to collect the condensate (Jeyaratnam et al., 2016). To increase efficiency and minimize the losses of oxygenated components, a cohobating tube can be added to the extraction system. Cohobating

ensures that there is adequate water in the boiling system by returning the water from the condensation and decantation to the boiling water (Dilworth et al., 2017; Mahfud et al., 2017).

Distillation methods may be classified into three categories based on the heating medium: steam, water, or a combination of water and steam (Azmir et al., 2013). With water distillation, the plant material is completely immersed in boiling water. To prevent thermal degradation and settling of dense materials at the bottom, plant materials must be kept in constant motion (Dilworth et al., 2017). This helps with the even distribution of heat (Azmir et al., 2013; Dilworth et al., 2017). Steam distillation involves the use of steam to heat plant material, causing evaporation of the essential metabolites (Azmir et al., 2013). This method is widely employed because the system can be easily controlled, but compared to water distillation, it involves a higher setup capital. The main difference between water distillation and combined water and steam distillation is that the plant material does not come in direct contact with water. Instead, it is placed on a solid support above the boiling water so that steam can directly pass through the plant material (Pateiro et al., 2018). This extraction method gives greater oil yield, and the process is quicker compared to water distillation (Baydar et al., 2008).

Water distillation has proven more effective than steam distillation for essential oil extraction. A study on terpineol, the main component of bangle rhizomes (Z*ingiber purpureum)* essential oil, showed that metabolite concentration was higher (26.8%) when extracted by water distillation compared to that (22.5%) obtained by steam distillation (Mahfud et al., 2017). The extraction period influences yield and extract composition, yet extending the extraction period beyond 120 min does not significantly change yield and is not economical as it increases energy consumption (Baydar et al., 2008).

2.6.2 Conventional Soxhlet extraction

Soxhlet extraction traditionally involves a flask, an extraction chamber, and a condenser (De Castro and Priego-Capote, 2010). The extractor design dates back to 1879, and the process is based on the principle that the desired compounds are highly soluble in the solvent used, while impurities are insoluble (Redfern et al., 2014). This process is normally used for fragile plant materials that cannot tolerate the heat of the distillation method (Azmir et al., 2013; Tongnuanchan and Benjakul, 2014). During Soxhlet extraction, the plant material is placed in a thimble, a porous bag made from cellulose or a filter paper before it is fitted into the extraction

chamber (De Castro and Priego-Capote, 2010). As the extraction solvent heats from the bottom of the flask, the vapor travels up the sidearm and into the condensing chamber. The condenser ensures that the solvent vapor is cooled and drips into the thimble containing the plant material. Desired metabolites dissolve in the solvent. As the extraction chamber fills up, it is emptied by siphoning the solvent into the flask (De Castro and Priego-Capote, 2010). Extraction time typically ranges from 6–24 h (De Oliveira et al., 2013).

More recent apparatuses have been designed to accommodate higher temperatures and pressure that accelerate the kinetics underlying the procedure. Compared to traditional Soxhlet extraction, much less solvent and time are needed to achieve similar results (Wang and Weller, 2006). Accelerated solvent extraction apparatuses usually use organic solvents but pressurized hot water or carbon dioxide (CO_2) can be used. In the case of CO_2, polar modifiers must be added if the extraction of polar compounds is desired. The choice of solvent depends greatly on the target metabolites in cannabis and the extract composition can be dramatically altered based on the solvent used.

2.6.3 Ultrasound-assisted extraction

Ultrasound-assisted extraction is a rapid, simple, and eco-friendly method for extracting bioactive metabolites from plants, with reduced initial and production costs due to the low energy and time required (Moradi et al., 2018). Ultrasound-assisted extraction improves the extraction process by increasing solvent penetration into plant cells through cavitation, preventing degradation of thermally unstable compound; this significantly reduces the extraction time required in traditional extraction systems (Vilkhu et al., 2008).

The system consists of an ultrasound generator and a probe that introduces waves into the solvent containing the plant material to generate disturbances (Albero et al., 2019). Repetition of this disturbance causes expansion and compression cycles in the molecules of the medium, leading to the formation and collapse of gas bubbles. Gas bubble implosion generates changes in temperature and pressure that enhance the penetration of a solvent into the plant matrix. This results in an increased mass transfer of the analytes into the solvent. Extraction rates are increased by macroturbulence, high velocity inter-particle collisions that are caused by the implosion of the gas bubbles (Ji et al., 2006; Rastogi, 2011). Various studies have indicated that extraction rate coefficients are four times higher in the presence of ultrasound compared to

systems without ultrasound (Huang et al., 2015; Rege et al., 2004; Vinatoru, 2001). Ultrasound intensity has a significant effect on the quantity of essential oil extracted (Li et al., 2004; Rastogi, 2011). After 3 h of extraction, an ultrasound intensity of 47.6 W cm^{-2} increased oil yield from soybeans (*Glycine max*) by 2.4% (Li et al., 2004).

2.6.4 Microwave-assisted extraction

Various advances in microwave-assisted extraction have improved the extraction and quality of essential oils. The principle behind this technology is that the microwaves excite molecules in the essential oil, thereby increasing the rate of extraction. Microwaves may be used in conjunction with solvent extraction, Soxhlet extraction, and distillation (Luque-Garcıa and De Castro, 2004; Routray and Orsat, 2012; Stashenko et al., 2004). Importantly, microwave-assisted extraction is a safe and environmentally friendly method, as it reduces solvent and energy consumption, along with various environmental hazards such as chemical wastes. Process costs are generally lower than most conventional extraction techniques. Microwave-assisted extraction is the only technique that can be used without any solvent (Lucchesi et al., 2004; Lucchesi et al., 2007). Essential oils extracted by solvent-free microwave-assisted extraction have no significant differences in quantity (yield) or quality (aromatic profile) compared to distillation extraction, and solvent-free microwave-assisted extraction time is markedly shorter than conventional distillation (30 min vs. 4.5 h) (Lucchesi et al., 2004).

Microwaves are mostly used together with solvent extraction in industries. In this system, the solvent used must be able to absorb microwaves and dissipate the energy in the system. The ability of a solvent to absorb microwave energy partly depends on the dissipation factor (tan δ) of the solvent and this is governed by Maxwell's equation (Routray and Orsat, 2012). The dissipation factor is the ratio of the solvent's dielectric loss (ε″) and dielectric constant (ε′). Dielectric loss is a measure of the efficiency of converting microwave energy into heat. The dielectric constant is a measure of the polarity of a solvent. A solvent of higher polarity is preferred for microwave-assisted extraction.

Microwave-assisted extraction using methanol at 109°C and microwave power of 375 W achieved the highest extraction yields of total cannabinoids in hemp compared with traditional extraction methods (Chang et al., 2017). However, decarboxylated forms of cannabinoids were higher with microwave-assisted extraction than with conventional extraction techniques. This

may be attributed to increased energy provided to the molecules by the microwaves, resulting in acid decarboxylation (Brighenti et al., 2017).When flavonoids from the roots of *Astragalus propinquus* were extracted with microwave-assisted extraction, maximum yield occurred at 110°C for 25 min using 90% ethanol. Compared to Soxhlet extraction, there was no significant difference in the percentage of flavonoids extracted, although microwave-assisted extraction used less time (Xiao et al., 2008).

2.6.5 Supercritical fluid extraction

The food and pharmaceutical industries are continually searching for innovative extraction techniques to obtain high purity and high quality essential oils. The high cost of organic solvents and increasing public awareness of the environmental outcomes, health, and toxic residues associated with the use of organic solvents have increased the need for novel and clean processing technologies. Supercritical fluid extraction (SFE) as a "green" technology has many advantages over traditional methods, particularly in the preservation of thermosensitive compounds using low temperatures (Machado et al., 2013).

Supercritical fluid extraction uses supercritical fluids, fluids at pressure and temperature above their critical value, as the solvent and explores the solvation power of the fluids (Sahena et al., 2009). A solvent in a supercritical state has intermediate physio-chemical characteristics similar to liquid and gas, which increases its density and diffusion properties (Machado et al., 2013; Sahena et al., 2009). High density increases the solvation, penetrating, and extracting powers of the solvent. Several studies have evaluated the importance of supercritical fluids, expressly CO_2, in the cannabis industry (Brighenti et al., 2017; Da Porto et al., 2012; Devi and Khanam, 2019; Moreno et al., 2020b). The low critical pressure and temperature of CO_2 (Table 2.1) make it suitable for extracting heat-sensitive compounds such as lipids. Da Porto et al. (2012) showed that the highest cannabis oil yield was 22% with supercritical CO_2 extraction, corresponding to a 72% recovery. This was obtained at 30 MPa and 40°C or 40 MPa and 80°C, with a solvent-sample ratio of 40 kg CO_2 kg^{-1} hemp seeds. Similar results were observed by Devi and Khanam (2019) with a hemp seed oil yield of 36.3% at 40°C and 35 MPa.

Table 3.1. Critical temperature and pressure of some pure compounds.

Compound	Critical Temperature (°C)	Critical Pressure (kPa)
Carbon dioxide	31.1	7380
Ethanol	240.9	6140
Methanol	239.6	8090
Acetone	235.1	4700
Water	374.1	2206

However, the low polarity of CO_2 presents limitations for supercritical extraction of polar compounds, including THCA and CBDA. The addition of a polar solvent such as ethanol to supercritical CO_2 can circumvent this problem and increase the extraction of polar compounds (Ahmadkelayeh and Hawboldt, 2020; Devi and Khanam, 2019; Grijó et al., 2019). Although other alternatives exist, ethanol has been the most used modifier in the presence of supercritical CO_2, due to its low toxicity. Although the technique struggles with the cost-effectiveness for low volume products, supercritical CO_2 is overcoming this limitation more effectively with technological optimization and providing an attractive alternative to conventional extraction and refining methods for the food processing and pharmaceutical industries. However, a winterization step is required after supercritical CO_2 extraction to remove undesirable heavier compounds such as waxes.

2.7 Cannabis secondary metabolite profiling

Precise secondary metabolite profiling of cannabis products is necessary to comply with regulations outlined by governmental agencies where cannabis use has been legalized. Improvements in secondary metabolite profiling methods for cannabis have garnered increasing interest in recent years, and various profiling studies have been conducted using spectrometry and spectroscopy (Brown et al., 2019; Lacey et al., 2001). Analytical methods for cannabis should aim for the greatest accuracy, at the lowest cost, with a short runtime. However, no single technology can provide complete coverage of all chemical compounds present, as metabolites of interest have different chemical properties. Over 100 secondary metabolites have been identified in the cannabis plant using different analytical techniques (Andre et al., 2016). Gas

chromatography coupled with flame ionization detector and liquid chromatography with an ultraviolet detector are mostly used in the cannabis industry (Borille et al., 2017).

Mass spectroscopy coupled with chromatography displays a higher sensitivity, detects a broad number of metabolites, and is faster compared to other spectroscopy techniques (Kaklamanos et al., 2016). Nuclear magnetic spectroscopy (NMR) is used to determine metabolite structure, which is an important aspect of cannabis profiling (Lacey et al., 2001). NMR is based on the principle that hydrogen, carbon, nitrogen, phosphorus, and oxygen present in the metabolites have magnetic properties (Addo et al., 2022a). The use of different types of chromatography techniques, including liquid chromatography, gas chromatography, thin-layer chromatography, or paper chromatography are dependent on the chemical compound under investigation (Jin et al., 2017).

In gas chromatography, the mobile phase is usually a gas and the stationary phase is a solid or liquid held in a column (Bartle and Myers, 2002). Depending on whether the stationary phase is a solid or a liquid, the technique is accordingly termed as gas solid chromatography or gas liquid chromatography (Scalbert et al., 2009). The sample is introduced as a gas at the column head. Components having finite solubility in the stationary phase distribute themselves between the stationary and mobile phases, according to the distribution law (Al-Rubaye et al., 2017). Elution occurs when an inert gas (e.g. nitrogen or helium) is forced through the column. Rate of movement of various components along the column depends on their tendency to be dissolved in the stationary phase (Coskun, 2016). Components having negligible solubility in the liquid phase move rapidly through the flame (Al-Rubaye et al., 2017; Bartle and Myers, 2002). High temperatures required for sample vaporization before injection can result in cannabinoid decarboxylation to their corresponding neutral forms, and the thermal degradation of other cannabinoids (Citti et al., 2018). This is problematic when quantifying individual cannabinoids and thermal stress may be reduced by a derivatization step before gas chromatography analysis (Borille et al., 2017; Kumirska et al., 2013). Derivatization techniques include alkylation, acylation, and silylation; this step increases the thermal stability and volatility of the compounds (Kumirska et al., 2013).

Liquid chromatography is useful for separating ions or molecules according to their affinity for the liquid mobile phase (Heckel and Dombek, 2009). The principle of the technique is based on the interactions of the sample with the mobile and stationary phases. There are

different types of liquid chromatography: normal-phase, reversed-phase, size exclusion, and bio-affinity. Essential components of the liquid chromatography device are the solvent depot, high-pressure pump, a commercially prepared column, detector, and a recorder (Coskun, 2016). Liquid chromatography does not cause thermal stress preventing cannabinoid decarboxylation (Jin et al., 2017). Acidic and neutral cannabinoids are detected using liquid chromatography with no need of a derivatization step. High-pressure liquid chromatography is the most widely used analytical liquid chromatography technique. It employs a stationary phase that can either be a liquid or a solid, coupled with a liquid mobile phase. High pressure liquid chromatography coupled with an ultraviolet diode array detector has demonstrated superiority over other chromatographic methods for cannabinoid analyses by reducing the run time (< 10 min) with improved resolution of CBD and CBG peaks (Citti et al., 2016; Patel et al., 2017). However, it is difficult to separating major cannabinoids as peaks overlap (Borille et al., 2017).

2.8 Implications for the evolving cannabis industry

The legalization and depenalization of the cannabis industry have intensified cannabis production and driven sales of cannabis and cannabis products for medical and adult use. Increased medical cannabis use is supported by evidence demonstrating the therapeutic potential of cannabis in different phytochemically-focused formulations such as Epidiolex® (CBD), Marinol® (THC), and Sativex® (THC/CBD), which are indications (Abrams, 2019; Chandra et al., 2017). A growing number of clinical trials investigating the safety and efficacy of novel cannabinoid-focused formulations predict that this pharmaceutical sector will expand shortly. With this, regulatory bodies, for medical and adult cannabis use alike, will necessarily prioritize quality and safety.

Inconsistency, safety, and reliability of finished products can be related to the lack of standardized quality control and assurance procedures in the cannabis industry. Quality assurance focuses on providing confidence that quality requirements of the industry are developed while quality control deals with the fulfillment of these quality requirements (Pusiak et al., 2021; Sarma et al., 2020). These principles are governed by Good Production Practices and Good Manufacturing Practices across the cannabis value chain. Contamination and inconsistency are a public health concern and postharvest processing parameters (storage, drying, and extraction conditions) need to be closely monitored so that finished products analyzed by third

party licensed laboratories meet acceptable limits set by regulatory bodies. High humidity during postharvest processing sometimes provides ideal conditions for microbial growth. Drying at elevated temperatures (> 60°C) can improve the drying rate and time, while killing any microbes present however, it can have adverse effects on cannabinoid profiles (Kwaśnica et al., 2020).

International committees should partner more readily with industry and academic researchers to hasten the development of global standards for cannabis. Although some commercial entities have developed efficient processing parameters to improve the safety and potency of cannabis, most of these novel procedures are proprietary in this highly competitive market, thus slowing standardization. Important differences in the cannabis regulations among countries have stressed the need for standardized analytical protocols that accurately measure cannabinoid content in cannabis and cannabis products, as well as microbial and chemical contaminants (Sarma et al., 2020).

Validated methods are required by licensed analytical laboratories, protocols are proprietary, and varying phytochemical profiles from different laboratories for the same product do not instill confidence at present. Although attempts have been made to produce standardized protocols, they face the challenge of the natural cannabinoid in trichomes by their position on the same plant, the absence of blank cannabis matrix, and conversion of acidic cannabinoids to neutral forms during sample preparation and analysis (Burgel et al., 2020; Mahlberg and Kim, 2004). Cryogenic grinding to obtain sample homogeneity for cannabinoids before analyses and cannabis certified reference material shows promise for solving some of these issues (McRae and Melanson, 2020).

Validated analytical protocols that confirm the presence of synthetic cannabinoids are needed (Assi et al., 2020; Darke et al., 2020). A quality study by Assi et al. (2020) showed that synthetic cannabinoids induced adverse psychedelic effects including alteration in perception, anxiety, paranoia, psychosis, depression, and death. Vaping cannabis products containing terpene additives have raised valid concerns around toxicity and regulation of these synthetic or added compounds is eminent (Meehan-Atrash et al., 2019; Meehan-Atrash et al., 2017). The rapid determination of cannabinoids and contaminants needs to be developed as current analytical processes such as high-pressure liquid chromatography, are costly and require large amounts of solvents. Near and mid infrared technology has, over the years, been developed to assist cannabis producers with the rapid determination of cannabinoids. However, the calibration of this system

is tedious and requires a large sample size to produce a robust model, over 1000 samples (Addo et al., 2022a).

Another major challenge facing large cannabis production operations is a work force leftover from the illicit cannabis trade, where training in natural science and engineering principles underlying plant production systems and controlled environment agriculture may be lacking. Operations that rely on trial-and-error methodologies to improve production without this knowledge can result in lower productivity, inconsistencies in finished products, and increased operational costs that serve as a deterrent for investors. The emergence of diploma and university training programs such as the Quality Assurance and Quality Control for Cannabis (QAQCC) that produce highly qualified personnel, integrate research that provides solutions to industry challenges, and meet existing or future legislation will prove valuable in this evolving industry. Smart agricultural practices, including energy-efficient plant production and automated nutrient tracking systems, will surely help reduce production and labor costs, all geared toward improving the quality of cannabis and cannabis products.

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Connecting text

Chapter 2 reviewed the current state and challenges of postharvest handling/processing in the cannabis industry. Potential drying systems and storage conditions for the cannabis industry were described. Yet, optimal conditions to improve the shelf-life, drying rate and preserve the secondary metabolites have not been reported. Therefore, Chapter 3 shows the effect of pre-freezing on the drying behavior of hops (*Humulus lupulus*) when subjected to freeze-drying, hot air drying, and microwave-assisted hot air drying. Hop was used as a model plant for cannabis because they both belong to the same family, Cannabaceae and have similar physical and chemical traits.

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Chapter 3: Impact of pre-freezing and microwaves on drying behavior and terpenes in hops (*Humulus lupulus*)

Abstract

Hop buds (*Humulus lupulus*) are paramount to bittering, flavoring, and microbiological stability in beer. To optimize post-harvest processing, fresh and pre-frozen hops were subjected to freeze-drying, hot air and microwave-assisted hot air drying. Pre-freezing occurred at -80˚C, prior to drying at 35˚C, 50˚C, and 65˚C, with different microwave power (0 W, 100 W and 200 W, where 0 W represented conventional hot air drying). Results show that hops drying kinetics can be described using the predictive Page and Logarithmic mathematical models. Obtained R^2, SSE, and RMSE values ranged between 0.999 to 0.982, 0.035 to 0.001, and 0.058 to 0.004, respectively. Irrespective of the drying condition, pre-freezing reduced drying time by 0.17% to 85.9%. Pre-freezing hop buds increases the effective moisture diffusion coefficient, and it increases with higher drying temperature and microwave power, ranging between 5.9×10^{-10} m^2 s^{-1} and 2.4×10^{-7} m^2 s^{-1}. SEM analyses indicate that pre-freezing causes structural damage to lupulin glands. The average concentration of myrcene, limonene, caryophyllene, and humulene for fresh hops were 15.1 mg g^{-1}, 0.3 mg g^{-1}, 3.1 mg g^{-1}, and 6.5 mg g^{-1} respectively. For the dried samples under the various conditions, the concentration ranged from 12.2 mg g^{-1} to 0.5 mg g^{-1} (myrcene), 0.3 mg g^{-1} to 0.1 mg g^{-1} (limonene), 1.5 mg g^{-1} to 0.3 mg g^{-1} (caryophyllene), and 2.7 mg g^{-1} to 0.5 mg g^{-1} (humulene). Results affirm that pre-freezing plant material prior to drying can shorten postharvest processing times, and this method can potentially be applied to other industrial crops. This study highlights the importance of controlled postharvest processing to ensure industrial crop quality.

3.1 Introduction

Hops (*Humulus lupulus*) is an essential raw material used in the brewing industry (Rodrigues Arruda et al., 2021). Secondary metabolites present in hops improve the microbiological stability of beer, in addition to impacting aroma and flavor (Vaughan et al., 2005). The practice of preserving hops with different drying methods dates back many centuries (Moir, 2000). Sun and solar drying technologies increase plant shelf life, yet these applications are limited in temperate regions and the risk of contamination is high, leading to the development of alternate drying technologies (Prakash and Kumar, 2014; Vijayavenkataraman et al., 2012). Hot air drying is a food preservation method based on heat, mass, and momentum transfer principles in thermodynamics, involving the evaporation of water by the movement of hot air around the plant material (Devahastin and Niamnuy, 2010). Hot air drying can adversely affect the physical, biochemical, and quality attributes of industrial crops via thermal degradation of functional, nutritional, and organoleptic properties (Argyropoulos and Müller, 2014; Dev et al., 2011; Muñoz-López et al., 2018). These limitations may be reduced when combined with other techniques such as ultrasound, infrared, and microwave technologies (Alibas, 2007; Hebbar et al., 2004; Liu et al., 2015b).

Microwave-assisted hot air drying reduces drying time as it is based on volumetric heating that ensures uniform distribution of heat (Orsat et al., 2007). Hot air drying coupled with microwaves significantly improves nutrient preservation, microstructure, and dried sample color (Dev et al., 2011; Pham et al., 2018). For most industrial applications, 915 MHz is considered most useful because of its greater penetration depth. A 2,450-MHz frequency is mostly used in domestic microwave ovens and commercial units designed for analytical chemistry studies (Chandrasekaran et al., 2013; Zhang et al., 2006).

Vacuum freeze-drying has served as the benchmark for the production of high-quality dried substances (Jangam, 2011) in both the food and pharmaceutical industries. Vacuum freeze-drying, known as lyophilization, is based on the phase behavior of water at the triple point, the temperature, and pressure at which all three phases of water coexist in an equilibrium (Ishwarya et al., 2015; Tsinontides et al., 2004). Water molecules are removed during freeze-drying by sublimation and surface desorption processes. The low temperature employed in freeze-drying not only inhibits microbial and enzymatic activities but maintains the sample quality and structure (Ratti, 2001).

Various studies have explored the potential of pre-freezing for improving drying rate, maintaining the nutritional and sensory quality of products, and modifying food structure (Ando et al., 2016; Peng et al., 2018; Tatemoto et al., 2016). Dandamrongrak et al. (2013) showed that pre-freezing at temperatures of -34°C and 0°C before drying reduces drying time by 45.9% and 7.5%, respectively. Ando et al (2016) demonstrated a significantly high drying rate of frozen-thawed carrots (*Daucus carota*) is caused by ice crystal formation, which leads to cellular breakdown, facilitating water migration thus enhancing mass transfer.

Thin-layer drying models are widely used for many agricultural products and they explain the relationship between the changes in moisture content as a function of drying time (Ertekin and Firat, 2017). Thin-layer drying models can be classified as theoretical, semi-empirical, and empirical models (Babalis et al., 2006). Theoretical models are based on Fick's second law of diffusion and thermodynamic heat and mass transfer laws (Castro et al., 2018). Although theoretical models are difficult to compute and apply, they provide a better understanding of the transport processes occurring during the falling rate period during drying (Castro et al., 2018; Ertekin and Firat, 2017). Semi-empirical and empirical models are based on the diffusion theory, a simplified form of the Fick's law (Benseddik et al., 2018). However, empirical models are mostly favored depending on experimental conditions, and may provide a better fit to experimental data (Babalis et al., 2006).

Various empirical models have been developed for different agricultural products. The Page model is an empirical modification of the Newton (Lewis) model that includes a dimensionless empirical constant (n). It has been used to determine the drying kinetics of shelled corn (*Zea mays*) and other agricultural products (Ertekin and Firat, 2017; Simpson et al., 2017). Following this, three main modifications were made. Modified Page model describes the drying of soybeans (*Glycine max*) (Simpson et al., 2017), while Henderson and Pabis improved the drying model using Fick's second law of diffusion (Ekechukwu, 1999; Ertekin and Firat, 2017). This model effectively predicts the drying rate at the beginning of the drying process but is less efficient for the last stages; as the slope of this model, "k", is related to effective moisture diffusivity when the drying process takes place only in the falling rate period and moisture diffusion controls the drying system. The logarithmic model is also based on Fick's second law of diffusion and has been used to successfully describe the drying behavior of green bell peppers (*Capsicum annuum*); this model is the logarithmic form of the Henderson and Pabis model, with

an empirical term addition (Doymaz and İsmail, 2010). Lastly, Wang and Singh created an empirical model for intermittent drying of rough rice (*Oryza sativa*) (Ertekin and Firat, 2017). Since various studies show that both the Page and logarithmic models are the best drying models for agricultural products, they were used for this study (Hu et al., 2017; Palamanit et al., 2020; Raut et al., 2020b).

The objective of this research was to understand the effects of pre-freezing on the drying behavior of hops when subjected to freeze-drying, hot air drying, and microwave-assisted hot air drying. Specific study objectives were the following: i) Determine the effect of pre-freezing, drying temperature, and microwave power on hops and their drying kinetics, color, moisture diffusivity, and terpene concentration; ii) Develop mathematical models for drying hops that could be applied to scaled-up operations and used for other industrial crops.

3.2 Materials and methods

3.2.1 Hops cultivation

Hops (Brewer's gold) were cultivated outdoors at McGill University's Macdonald Campus farm in Sainte-Anne-de-Bellevue, QC, Canada. The setup consisted of five wooden beams spaced 2.74 m apart, creating four plots. The top ends of the five wooden beams were connected with a nylon rope from which 12 nylon ropes, 3 ropes/plot, were tied to provide support for plants, spaced 1 m apart. Hops were planted on May 3, 2020, and harvested from mid-September to the end of October 2020. Preliminary tests were conducted using a split plot design to show no significant differences between hops harvested from the different plots.

3.2.2 Hot air and microwave assisted hot air drying of samples

Harvested hop buds were divided into two groups, untreated and pre-frozen. The untreated group was dried and analyzed immediately after harvest and the pre-frozen group was frozen at -80°C for 24 h prior to drying and analysis. In each experiment, approximately 100 g hop buds were placed in a nylon mesh sample holder tray (diameter = 0.21 m). The plant material was spread in one layer and placed inside the microwave cavity. Drying was performed at temperatures of 35°C, 50°C, and 65°C, with varying microwave power levels at 0 W, 100 W, and 200 W. A microwave density of 0 W represented the conventional hot air drying method. Drying was performed until the sample reached a dry basis moisture content of 12%. Dried

samples were transferred into a plastic bag and stored in a refrigerator at 5°C before analyses. Drying was performed in triplicate under each condition.

Hot air drying and microwave-assisted hot air drying were conducted in an automated laboratory-scale microwave oven (Figure 3.1A). The main components were a 2,450-MHz microwave generator (Gold Star 2M214, Seoul, South Korea) with adjustable power (0 to 750 W), waveguides, a three-port circulator, a manual three-stub tuner to match the load impedance, microwave couplers to measure forward and reflected power, a carbon load to absorb reflected power, and a microwave cavity made of brass (0.47 × 0.47 × 0.27 m) in which the samples were processed. The microwave generator produced microwaves with varying power densities based on the supplied power. Generated microwaves were guided to the microwave cavity using a waveguide. The manual three-stub tuner was used to adjust the reflected power, thereby keeping it at the minimum possible value (<10% of the incident power). Temperatures were measured using fiber-optic probes (Nortech EMI-TS series, Quebec, QC, Canada) connected to a data acquisition unit (Agilent 34970A, Santa Clara, CA, US), which was connected to a computer. The entire setup was continuously monitored and controlled using HP-VEE (Agilent, Santa Clara, CA, US) object-oriented programming language. The setup was equipped with an electronic balance to automatically record sample mass at 5 min intervals.

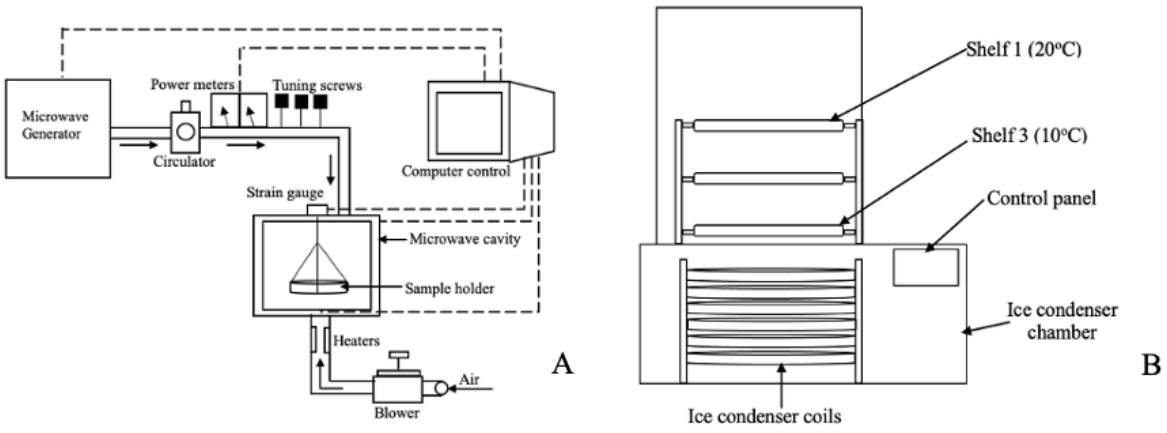

Figure 5.1. Schematic diagram of the (A) microwave-assisted hot air dryer and (B) vacuum freeze dryer.

3.2.3 Freeze drying of samples

Pre-frozen hop bud samples at -80°C in plastic trays were transferred to a laboratory-scale vacuum freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH Gamma 1-16 LSCplus, Osterode, Lower Saxony, Germany) (Figure 3.1B) with a condenser temperature of -55°C. Freeze-drying was carried out at 10°C or 20°C for 24 h at 0.85 mbar. Changes in sample mass during the drying process were recorded every hour. Each experiment was performed in triplicate using three different samples.

3.2.4 Determination of moisture content

The initial moisture content of the hop buds was determined using a hot air oven (Fisher Scientific 6903 Isotemp oven, Waltham, Massachusetts, US). Each sample was dried at 50°C for 24 h. The moisture content of the sample was calculated with Equation (1):

$$\text{Moisture content on wet basis (wb) (\% g water (g wet matter)}^{-1}) = \frac{M_w}{M_w + M_s} \quad (1)$$

where M_w is the mass of water in the sample (g) and M_s is the mass of solids in the sample (g). For analytical purposes, it was preferable to express moisture content as a function of the solid mass. Dry basis (db) moisture content was calculated with Equation (2):

$$\text{Dry basis moisture content (\% g water (g dry matter)}^{-1}) = \frac{M_w}{M_s} \quad (2)$$

3.2.5 Drying kinetics

To investigate the drying kinetics of hop buds under different drying conditions, drying rates and moisture ratios were determined. Drying rates of dried hops samples were calculated using Equation (3):

$$DR = \frac{M_{t_1} - M_{t_2}}{t_1 - t_2} \quad (3)$$

where DR is the drying rate (g water min^{-1}), t_1 and t_2 are different times (min) during drying, and M_{t1} and M_{t2} represent dry basis moisture content at time t_1 and t_2, respectively. Moisture ratio (MR) of samples was calculated by converting the experimental drying data using Equation (4):

$$\text{Moisture ratio (MR)} = \frac{M_t - M_e}{M_i - M_e} \quad (4)$$

where M_t, M_i, and M_e refer to moisture content at time t, initial moisture content, and moisture content at equilibrium, respectively.

3.2.6 Mathematical models

Data collected for hop buds dried under the different conditions were used to analyze the fit of two thin layer drying mathematical models, Page, and Logarithmic models, to the experimental data. Statistical parameters such as the root mean square error (RMSE) [Equation (5)], and the correlation coefficient (R^2) [Equation (6)] were used to estimate the quality of fit of each drying model to the observed values. Specifically:

$$\text{RMSE} = \left[\frac{1}{N}\sum_{i=1}^{N}\left(MR_{pre,i} - MR_{exp,i}\right)^2\right]^{\frac{1}{2}} \quad (5)$$

where $MR_{exp,i}$ is the experimental moisture ratio at time t, $MR_{pre,i}$ is the predicted moisture ratio at time t, and N is the number of observations.

$$R^2 = 1 - \frac{\sum_{i=1}^{N}\left(MR_{pre,i} - MR_{exp,i}\right)^2}{\sum_{i=1}^{N}\left(MR_{pre,i} - \overline{MR_{exp}}\right)^2} \quad (6)$$

where $\overline{MR_{exp}}$ is the mean experimentally measured value of MR.

The reduced sum square error (SSE) was also used as a criterion to analyze the closeness of fit in addition to the above parameters. It was calculated using Equation (7):

$$\text{SSE} = \frac{1}{N}\sum_{i=1}^{N}\left(MR_{pre,i} - MR_{exp,i}\right)^2 \quad (7)$$

3.2.7 Effective moisture diffusion coefficient

Fick's second law of moisture diffusion was used to study the movement of moisture molecules in the sample. The effective moisture diffusion coefficient was determined using Equation (8):

$$\text{MR} = \frac{8}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)}\ exp\left[-(2n+1)^2\ \frac{\pi^2 D_{eff} t_i}{4L^2}\right] \quad (8)$$

where D_{eff} is the effective diffusion coefficient of moisture diffusivity ($m^2\ s^{-1}$) and L is the average half-thickness of the hop bud.

Equation (8) was simplified to a one-term exponential model to determine the effective diffusion coefficient of drying of hop buds (Gekas and Lamberg, 1991) and written in a logarithmic form in Equation (9):

$$\text{In(MR)} = In\frac{8}{\pi^2} - \frac{\pi^2 D_{eff} t_i}{4L^2} \quad (9)$$

The effective diffusion coefficient was determined by plotting the drying data in terms of ln(MR) versus drying time, t_i. The plot gave a straight line with a slope as in Equation (10):

$$\text{Slope} = \frac{\pi^2 D_{eff}}{4L^2} \quad (10)$$

3.2.8 Color measurement

Sample color was measured with a CR-300 Chroma meter (Minolta, Tokyo, Japan) that was calibrated with a standard white plate before measurement. The D65 was used as the light source and the CIE1976 (L*a*b*) color scale was used (McLaren, 1976). The L* color parameter for the lightness from black (0) to white (100), a* from green (−) to red (+), and b* from blue (−) to yellow (+). Total color change (ΔE) was determined with L*, a*, and b* values using the Hunter-Scofield equation [Equation (11)]:

$$\Delta E = \sqrt{\left(L_p - L_i\right)^2 + \left(a_p - a_i\right)^2 + \left(b_p - b_i\right)^2} \quad (11)$$

where suffixes *i* and *p* refer to reference values (fresh undried hops) and dried sample values, respectively. A higher ΔE denotes a greater color change from the reference material.

3.2.9 Scanning electron microscopic analyses

To investigate the effect of pre-freezing on the microstructure of inner and outer surfaces of lupulin glands, scanning electron microscopy was used. Microstructural images were obtained by fixing samples on an adhesive specimen holder, which was then inserted into the scanning electron microscope (Hitachi TM-3000, Hitachi, Japan) at 5 kV acceleration voltage.

3.2.10 Terpene concentrations in hops

3.2.10.1 Sample preparation

Representative samples for each of the drying conditions and fresh samples were immersed in liquid nitrogen before grinding using a coffee grinder (Hamilton Beach, Belleville, ON, Canada). Ground samples were allowed to equilibrate to room temperature before 0.75 g of each representative sample was weighed into a 50 mL Falcon tube and recorded. Each sample was allowed to sit for 10 min on the scale (Mettler AE50 analytical balance, Columbus, Ohio, United States of America) until there was < 1 mg change in mass. This is done to ensure that

most of the liquid nitrogen had evaporated from the sample and the proper sample mass was obtained.

For the extraction of secondary metabolites, 20 mL high-pressure liquid chromatography (HPLC)-grade methanol (Thermo Fisher Scientific, Waltham, Massachusetts, US) was added to each Falcon tube and vortexed (Thermo Scientific vortex, Waltham, Massachusetts, US) for 20 min at 500 rpm. Each sample was filtered using Whatman™ filter paper (Thermo Fisher Scientific, Waltham, Massachusetts, US) and allowed to filter for 20 min. Residual hop biomass was placed into a new 50 mL Falcon tube and subjected to a second extraction process to ensure 99.5% of the terpenes had been extracted. The second extract was added to the corresponding first extract, resulting in a 40x dilution total extract.

3.2.10.2 Terpene analysis

A 1-mL sample of each extract was pipetted into gas chromatography (GC) vials for terpene analysis. Separation of the terpenes was performed with an Agilent 7820A GC coupled to an Agilent 7693 autosampler and a flame ionization detector (FID) (Agilent Technologies, Mississauga, Ontario, Canada). The system was equipped with an injector containing a capillary column (30 m x 250 μm x 0.25 μm nominal Agilent Technologies DB-5 Model) using split injection (ratio 50:1) with a hydrogen carrier gas (40 mL min^{-1}). An injection volume of 5 μL of each sample with a syringe size of 10 μL was used. The oven temperature was initially programmed at 35°C and held for 4 min. The temperature was increased at a rate of 10°C min^{-1} up to 105°C held for 0 min, increased at a rate of 15°C min^{-1} up to 205°C held for 0 min, and lastly increased at a rate of 35°C min^{-1} up to 270°C held for 5 min. The inlet temperature into the FID detector was set at 340°C. Spectra were recorded as three scans from 50 m z^{-1} to 400 m z^{-1}. The ionization mode was used with an electronic impact at 70 eV. Quantification of the terpenes was done using an external calibration of 37 terpenes mostly found in cannabis (LGC standards, Manchester, New Hampshire and Sigma Aldrich, Saint Louis, Missouri, US).

3.2.11 Statistical analysis

Statistical analyses were conducted using JMP software (JMP 4.3 SAS Institute Inc., Cary, NC, US) with a confidence level ($p < 0.05$) of 95%. Mean values of the experimental runs are presented. Drying mathematical models of the hops were fitted and analyzed with JMP

software (JMP 4.3 SAS Institute Inc.). Pairwise comparisons of means were done using the Newman-Keuls test.

3.3 Results and discussion

3.3.1 Drying kinetics

Hops drying curves with hot air, microwave-assisted hot air, and freeze-drying at different drying temperatures for untreated and pre-frozen samples are presented in Figures 3.2 and 3.3. The initial moisture content of the hops was 79% (wb). Freeze-dried hop buds took only 697.5 min at 10°C shelf temperature, to reach a desired final moisture content of 7% (db). The drying time was reduced by 26.8% for hops dried at 20°C when compared to 10°C. Freeze-drying is carried out in three stages, freezing, primary drying, and secondary drying (Addo et al., 2021), and pre-freezing the buds before drying prevents the formation of foams when the vacuum is applied to the system. Primary drying, during freeze-drying, involves the sublimation of ice crystals present in the hops' inflorescence. When sublimation was complete, the sample temperature increased and approached the shelf temperature. Increasing the shelf temperature from 10°C to 20°C provided more energy to the sample and reduced the drying time by improving the drying rate.

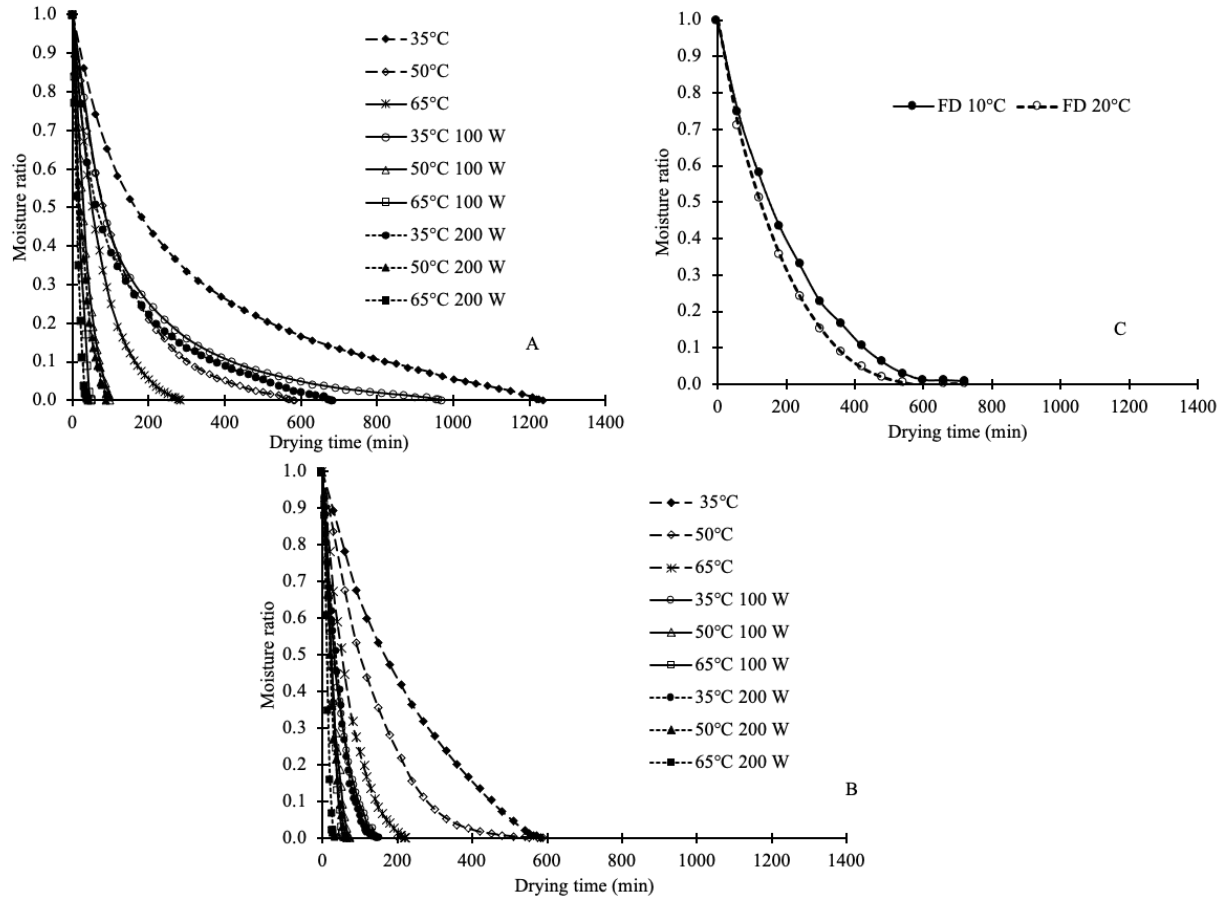

Figure 5.2. Moisture ratio curves of (A) untreated, (B) pre-frozen at -80°C, and (C) freeze dried hops.

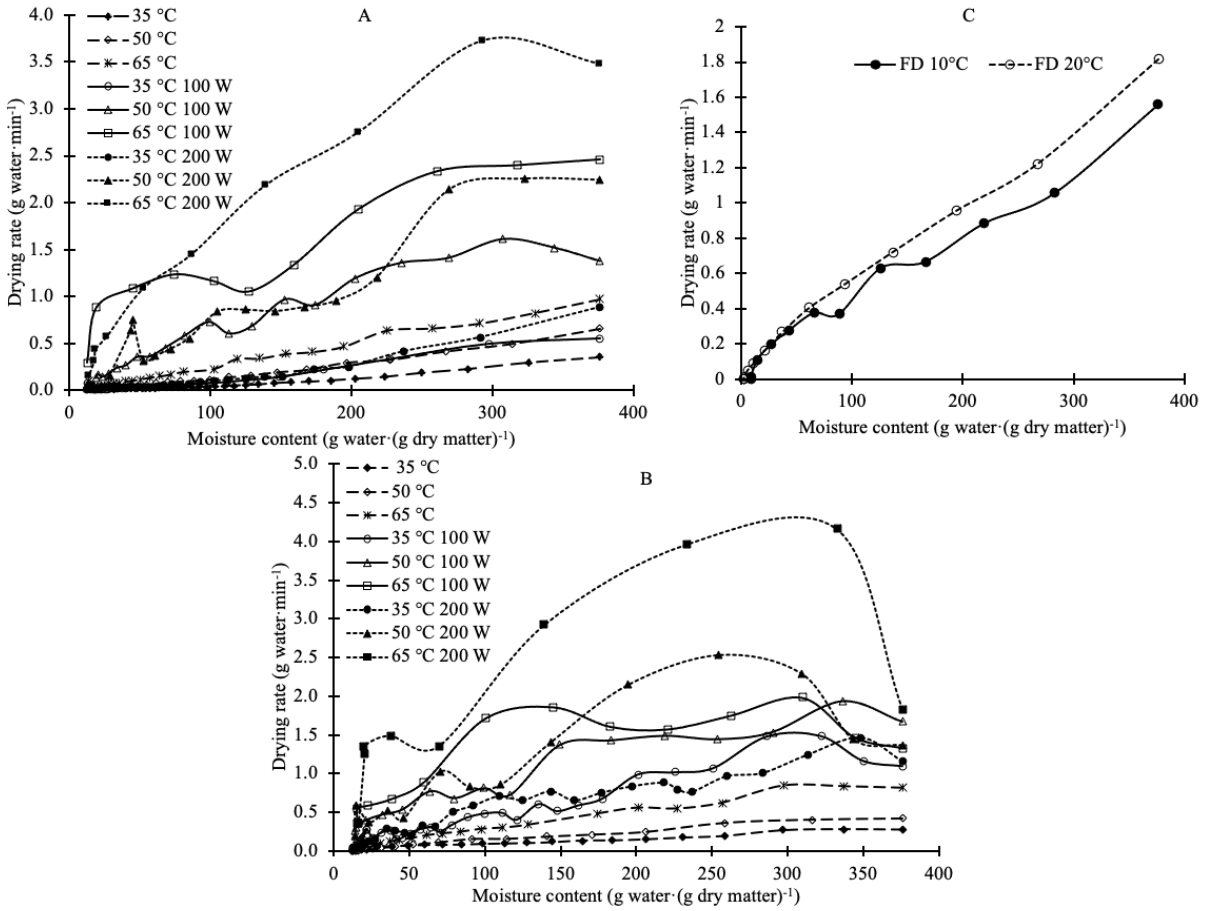

Figure 5.3. Drying rate curves of (A) untreated, (B) pre-frozen at -80°C, and (C) freeze dried hops.

For hot air and microwave-assisted hot air drying, drying time and rate were significantly ($p < 0.05$) affected by the pre-freezing step, drying temperature of the air, and microwave power. Irrespective of the hot air and microwave-assisted hot air drying conditions, pre-freezing samples at -80°C before drying significantly reduced the drying time by at least 0.2% and up to 85.9% (Figure 3.3). This is likely due to the formation of ice crystals leading to cellular breakdown with numerous holes created within the sample matrix making moisture present readily available for diffusion in the presence of a temperature gradient (Dandamrongrak et al., 2003; Searles et al., 2001; Tatemoto et al., 2016).

As expected, the moisture ratio reduced exponentially with time. For untreated samples dried at a microwave power of 200 W, it took only 37 min at 65°C to reach the desired final moisture content of 12% (db) (Figure 3.3). The drying time was reduced by 94.5% and 51.3%,

when compared with untreated hops dried (immediately after harvest) at 35°C and 50°C, respectively. Similar results were obtained for pre-frozen samples dried at a microwave power of 200 W, with the drying time reduced by 80.2% and 52.6% for hops dried at 35°C and 50°C, respectively, when compared to an inlet temperature of 65°C (Figure 3.3). Hot air drying (0 W) of untreated hop buds at 35°C had the longest drying time (20.43 h).

The drying rate decreased with a decrease in the moisture content and increased with an increase in microwave power (Figure 3.3). At the start of the drying period, the drying rate was high, likely resulting from the readily available water on the surface of the material. Along the drying process, surface moisture was reduced, and more energy was required for the transfer of moisture from the inside to the surface of the material, resulting in a reduced drying rate. Similar observations have been reported with moringa (*Moringa oleifera*), pineapple (*Ananas comosus*), ginger (*Zingiber officinale*), and broccoli (*Brassica oleracea*) (Dev et al., 2011; Izli et al., 2018; Izli and Polat, 2019; Md Salim et al., 2016). Increasing the air temperature from 50°C to 70°C (moringa), 60°C to 70°C (pineapple and ginger), and 40°C to 60°C (broccoli) decreased the drying time by 60%, 38%, and 51%, respectively.

High microwave power had a high moisture transfer driving force that resulted in reduced drying time. This is due to the volumetric heating property of microwave drying (Cao et al., 2019). Microwaves excited the water molecules present in the hop bud samples and this energy increased the rate of moisture transfer from the internal matrix of the material to the surface. Increasing the microwave power from 100 W to 200 W, significantly reduced drying time for the untreated hop bud samples at 35°C, 50°C and 60°C. For pre-frozen samples, increasing microwave power did not significantly reduce the drying time at the various drying temperatures.

3.3.2 Mathematical models for predicting drying

The moisture ratio values calculated for hops buds when subjected to different microwave drying conditions were fitted into two thin-layer drying models as displayed in Table 3.1. These include the statistical parameters used to assess the appropriateness of the model fit and drying model coefficients. Both models emerged as best fits due to the high R^2 and low SSE and RMSE values. R^2, SSE and RMSE values obtained for this study ranged between 0.999 to 0.967, 0.035 to 0.002, and 0.149 to 0.001 respectively. These data are comparable to those obtained when applied to drying carrot slices (*Daucus carota*) (Hu et al., 2017) and parboiled

rice (*Oryza sativa*) (Palamanit et al., 2020). Comparing the predicted moisture ratio values with the experimental values under each drying condition validated the established models. The experimental and predicted moisture ratio values laid around a straight line, linear regression. These models can be used to scale up the microwave-assisted hot air and vacuum freeze dryers to a commercial scale.

Table 5.1. Mathematical model parameters and performance of two thin layer drying models for hops under different drying conditions.

Condition	Drying Conditions	Coefficients	R^2	SSE	RMSE
Page model = $MR = \exp(-kt^n)$					
Hot air and microwave-assisted hot air drying					
Untreated (dried immediately after harvest)	35˚C/ 0 W	k = 0.012, n = 0.79	0.996	0.01	0.016
	50˚C/ 0 W	k = 0.012, n = 0.93	0.999	0.002	0.007
	65˚C/ 0 W	k = 0.01, n = 1.06	0.999	0.002	0.009
	35˚C/ 100 W	k = 0.02, n = 0.77	0.997	0.005	0.012
	50˚C/ 100 W	k = 0.01, n = 1.28	0.998	0.004	0.014
	65˚C/ 100 W	k = 0.016, n = 1.34	0.993	0.012	0.035
	35˚C/ 200 W	k = 0.033, n = 0.73	0.998	0.006	0.013
	50˚C/ 200 W	k = 0.03, n = 1.05	0.997	0.006	0.018
	65˚C/ 200 W	k = 0.025, n = 1.39	0.998	0.002	0.149
Pre-freezing step (-80˚C for 24 h)	35˚C/ 0 W	k = 0.002, n = 1.18	0.992	0.018	0.029
	50˚C/ 0 W	k = 0.003, n = 1.17	0.999	0.002	0.009
	65˚C/ 0 W	k = 0.007, n = 1.17	0.998	0.004	0.013
	35˚C/ 100 W	k = 0.014, n = 1.12	0.998	0.005	0.001
	50˚C/ 100 W	k = 0.01, n = 1.36	0.996	0.008	0.023
	65˚C/ 100 W	k = 0.003, n = 1.81	0.994	0.013	0.032
	35˚C/ 200 W	k = 0.008, n = 1.26	0.997	0.01	0.018
	50˚C/ 200 W	k = 0.006, n = 1.58	0.998	0.003	0.016
	65˚C/ 200 W	k = 0.006, n = 1.93	0.999	0.001	0.009
Vacuum freeze-drying					

Drying shelf temperature	10˚C	k = 0.003, n = 1.13	0.967	0.004	0.018
	20˚C	k = 0.003, n = 1.15	0.978	0.003	0.016
Logarithmic model = $MR = a * \exp(-kt) + c$					
Hot air and microwave-assisted hot air drying					
Untreated (dried immediately after harvest)	35˚C/ 0 W	k = 0.003, a = 0.894, c = 0.027	0.988	0.031	0.027
	50˚C/ 0 W	k = 0.008, a = 0.971, c = 0.002	0.998	0.004	0.011
	65˚C/ 0 W	k = 0.013, a = 1.03, c = -0.025	0.999	0.001	0.004
	35˚C/ 100 W	k = 0.007, a = 0.925, c = 0.028	0.989	0.018	0.024
	50˚C/ 100 W	k = 0.023, a = 1.15, c = -0.124	0.998	0.004	0.014
	65˚C/ 100 W	k = 0.028, a = 1.36, c = -0.354	0.999	0.002	0.013
	35˚C/ 200 W	k = 0.008, a = 0.875, c = 0.031	0.982	0.035	0.032
	50˚C/ 200 W	k = 0.032, a = 1.04, c = -0.045	0.998	0.003	0.013
	65˚C/ 200 W	k = 0.059, a = 1.16, c = -0.131	0.995	0.005	0.03
Pre-freezing step (-80˚C for 24 h)	35˚C/ 0 W	k = 0.003, a = 1.17, c = -0.179	0.999	0.002	0.01
	50˚C/ 0 W	k = 0.007, a = 1.06, c = -0.031	0.998	0.004	0.014
	65˚C/ 0 W	k = 0.013, a = 1.09, c = -0.073	0.999	0.001	0.007
	35˚C/ 100 W	k = 0.023, a = 1.27, c = -0.25	0.998	0.003	0.014
	50˚C/ 100 W	k = 0.023, a = 1.27, c = -0.25	0.998	0.003	0.014
	65˚C/ 100 W	k = 0.015, a = 1.88, c = -0.838	0.992	0.015	0.036
	35˚C/ 200 W	k = 0.019, a = 1.12, c = -0.09	0.997	0.011	0.019
	50˚C/ 200 W	k = 0.029, a = 1.3, c = -0.228	0.988	0.022	0.043
	65˚C/ 200 W	k = 0.038, a = 1.58, c = -0.524	0.982	0.024	0.058
Vacuum freeze-dried					
Drying shelf temperature	10˚C	k = 0.004, a = 1.06, c = -0.06	0.986	0.002	0.013
	20˚C	k = 0.006, a = 1.05, c = -0.04	0.988	0.002	0.014

*k is the drying rate constant (min^{-1}).

**n, a, b, and c are the drying coefficients (unit-less) that have different values depending on the model and drying curve; t is drying time (min).

3.3.3 Effective moisture diffusion coefficient

Effective diffusivity is used to describe the rate of moisture movement in a sample over the drying period (Dadmohammadi and Datta, 2019). Sample drying profiles consist of an initial drying stage, a constant-rate period, and a falling-rate period (first and second falling rates). Drying of most agricultural products mostly takes place during the falling rate period (Zhou et al., 2019). This means that moisture transfer during drying is controlled by internal diffusion, and internal diffusion occurring during the falling rate period for most food materials is described by Fick's second law of diffusion (Efremov and Kudra, 2007). The underlying assumption is that moisture migration is driven by a moisture content gradient. Effective diffusivity is affected by pre-freezing, drying temperature, moisture content, and material structure (Chen et al., 2020). Moisture gradient present in hops during drying likely generates stress in cellular structure and cell wall collapse, resulting in physical changes to the structure, dimension, or volume (Chen et al., 2020; Dadmohammadi and Datta, 2019). Such cell wall disruption subsequently affects the diffusing distance of moisture, which moves from inside to the outside.

Effective moisture diffusion coefficients of drying hops with different conditions are shown in Table 3.2. The D_{eff} value increased by 22.7% when the shelf temperature of the freeze-drying system was increased from 10°C to 20°C. The effective moisture diffusion coefficients significantly increased with an increase in drying temperature at the same microwave power. At the same drying temperature, D_{eff} values significantly increased with the increase in microwave power. This shows the effect of microwaves on the movement of water in a material. Pre-freezing hops before drying improved the drying rate because of the significant increase in the D_{eff} values. The lowest D_{eff} value was found at the power of 0 W (hot air drying) with the drying temperature of 35°C. Similar observations were made in the drying of broccoli stalk slices (*Brassica oleracea*) (Md Salim et al., 2016), and carrots (*Daucus carota*) (Hu et al., 2017). Md Salim et al., (2016) showed that D_{eff} values of broccoli stalk slices obtained with microwave-assisted hot air drying were twice higher than hot air drying, ranging between 6.64×10^{-8} and 13.31×10^{-8} m^2 s^{-1}. Hu et al., (2017) showed that the lowest recorded D_{eff} for carrots was at a microwave power density of 0.44 W g^{-1}, with an inlet air temperature of 40°C; this increased by 51.7% when the inlet air temperature was increased to 60°C.

Table 5.2. Effective moisture diffusion coefficients of drying of hops at different drying conditions.

Hot air drying and microwave assisted hot air drying					
Condition	Temperature (°C)	Microwave power (W)	Slope	Moisture diffusivity (m^2 s^{-1})	R^2
Untreated (dried immediately after harvest)	35	0	0.0035	5.91×10^{-10}	0.87
	50	0	0.0101	1.71×10^{-9}	0.87
	65	0	0.0190	3.21×10^{-9}	0.94
	35	100	0.0052	8.78×10^{-10}	0.93
	50	100	0.0444	7.49×10^{-9}	0.94
	65	100	0.1007	1.71×10^{-8}	0.75
	35	200	0.0073	1.23×10^{-9}	0.83
	50	200	0.0424	7.16×10^{-9}	0.96
	65	200	0.1415	2.39×10^{-8}	0.94
Pre-freezing step (-80˚C for 24 h)	35	0	0.0072	1.22×10^{-9}	0.88
	50	0	0.0118	1.99×10^{-9}	0.97
	65	0	0.0228	3.85×10^{-9}	0.91
	35	100	0.0298	5.03×10^{-9}	0.96
	50	100	0.0660	1.11×10^{-8}	0.86
	65	100	0.0835	1.41×10^{-8}	0.86
	35	200	0.0422	7.13×10^{-9}	0.93
	50	200	0.0787	1.33×10^{-8}	0.89
	65	200	0.1519	2.57×10^{-8}	0.92
Vacuum freeze drying					
Shelf temperature (˚C)			Slope	Moisture diffusivity (m^2 s^{-1})	R^2
10			0.0072	2.43×10^{-7}	0.98
20			0.0093	3.14×10^{-7}	0.93

3.3.4 Color changes

Visual perception plays an important role in the selection of agricultural products. It can be used as an indicator of food quality and grade, as various studies have related color changes to the antioxidant properties of plant materials (Cömert et al., 2020; Krawitzky et al., 2014). Color changes in hop buds for all experimental drying conditions were measured with a chroma meter and compared to a reference sample (fresh hops) (Figure 3.4). Visual color degradation (from green to dull green-yellow) was obvious during the drying process. Color parameters, L (A), a (B), b (C), and total color changes (D), of hops subjected to hot air, microwave-assisted hot air (MD) or vacuum freeze-drying (FD) conditions are presented in Figure 3.4.

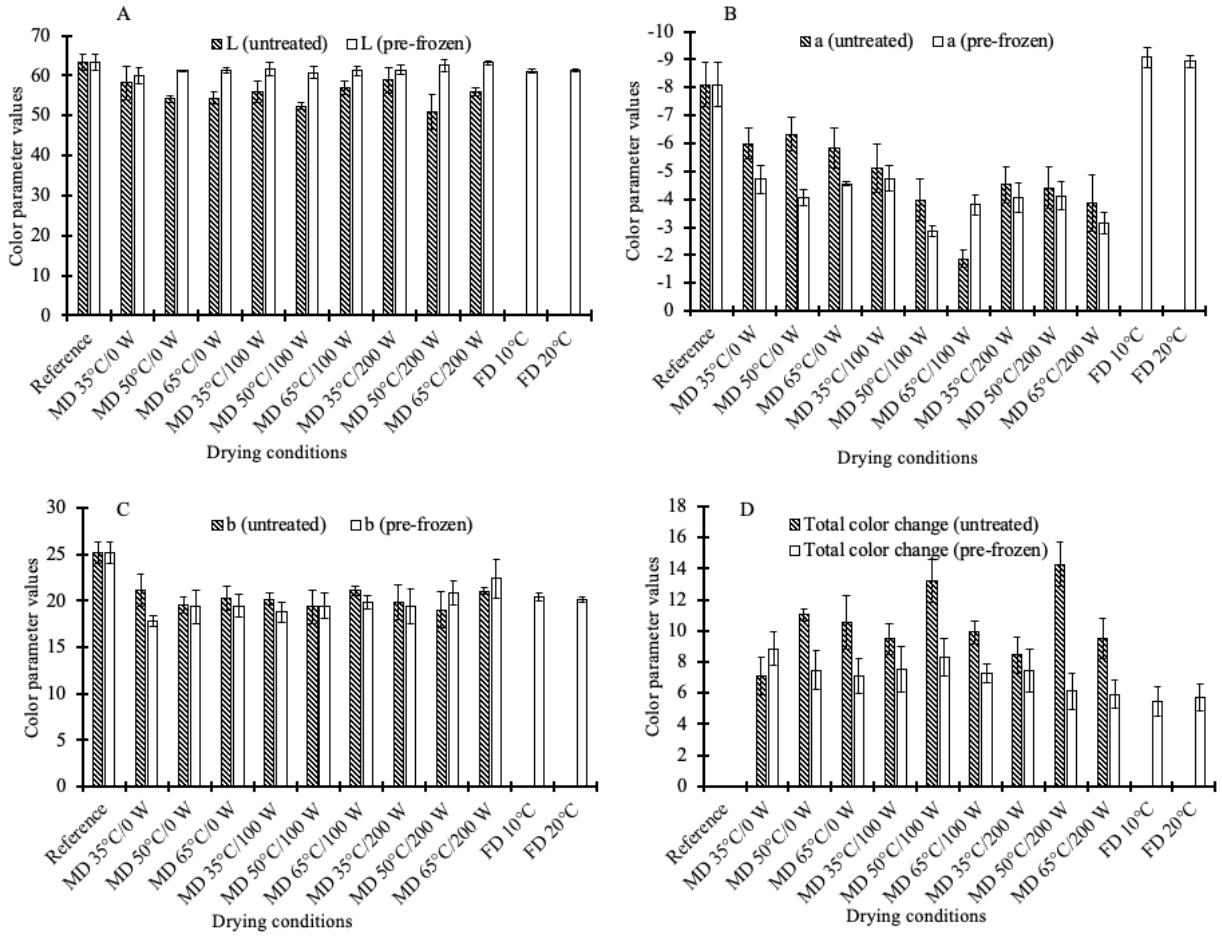

Figure 5.4. Color parameter values of hops subjected to hot air, microwave-assisted hot air (MD) or vacuum freeze-drying (FD).

The L*, a*, and b* values were 63.4, -8.10 and 25.2, respectively, for the reference sample. The fresh sample was the greenest, and this green color was lost during hot air and microwave-assisted hot air drying (a* values increased) and involved gradual development of a dull yellow color (a combined effect of changes in a* and b* values). However, the total color changes of vacuum freeze-dried hop buds when compared to fresh hops were significantly ($p < 0.05$) less than color changes observed for hot air and microwave-assisted hot air dried hops. The color changes observed in the dried samples may be due to less pigment destruction, ascorbic acid browning, and non-enzymatic Maillard browning during vacuum freeze-drying (Dueik et al., 2013; Stępień et al., 2019). It has been reported that chlorophyll degradation results in the formation of pheophytin and pyropheophytin in thermally processed vegetables and herbs (Cui et al., 2004; Hörtensteiner and Kräutler, 2011). Similar results have been reported for pineapples (*Ananas comosus*) dried using freeze-drying and microwave drying (Izli et al., 2018). Vacuum inclusion drying studies using carrots (*Daucus carota*), potatoes (*Solanum tuberosum*), and apples (*Malus domestica*) showed a significant reduction (31%) in the browning formation compared to atmospheric drying (Dueik and Bouchon, 2011; Dueik et al., 2013).

Color values of the hot air and microwave-assisted hot air dried hops were not dependent on the microwave power or temperature; rather, pre-freezing affected this parameter. Similar results have been reported for parsley (*Petroselinum crispum*) (Soysal, 2004), and coriander (*Coriandrum sativum*) leaves (Sarimeseli, 2011). The a*, and b* values of both untreated and pre-frozen hot air and microwave-assisted hot air dried hops decreased significantly when compared to the color of the reference sample. Total color change for pre-frozen samples was significantly lower when compared to untreated hops, indicating that a freezing step before drying can be used to preserve hops color. Pre-freezing samples before drying reduces non-enzymatic Maillard browning thereby reducing the total color change during the drying process.

3.3.5 Scanning electron microscopy analyses

The hop bud is anatomically separated into bracts, bracteoles, strig, and lupulin glands (Mishra et al., 2020). Bracts and bracteoles (small bracts) are leaf-like structures that surround the cone and attach to the string or central axis. Lupulin glands (glandular trichomes) contain secondary metabolites, including α- and β-bitter acids, prenylated flavonoids, and essential oils composed mainly of myrcene, limonene, α-humulene and β-caryophyllene, which are

responsible for a particular beer flavor and bitterness (Patzak et al., 2015; Raut et al., 2020b). To determine the effect of pre-freezing on lupulin glands, SEM analyses were performed on pre-frozen samples before drying. When evaluating the structure of the hops (Figure 3.5), it can be concluded that pre-freezing caused significant structural damage to the hops lupulin glands. Therefore, pre-freezing can be an important additional step in the extraction of essential oils. It is possible that structural damage caused by freezing can make oils readily available for extraction, thereby reducing extracting time and increasing yield. In the reference sample (fresh), lupulin glands appeared firm and intact, with a mushroom-like shape (Figure 3.5). Pre-freezing caused shrinkage and disruption to cell structure within the lupulin glands. This can be attributed to the formation of ice crystals during freezing (Ando et al., 2019; Nowak et al., 2016; Vallespir et al., 2019).

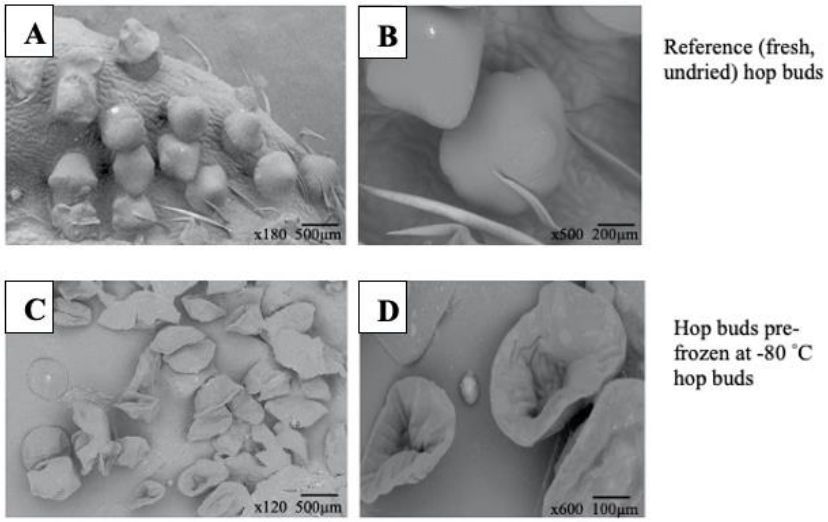

Figure 5.5. Representative scanning electron microscopy images of reference hop buds and -80°C pre-frozen buds.

3.3.6 Terpenes in hops

Hops are mostly used in the brewing industries due to their unique chemical compounds that contribute greatly to the bitterness, flavor, and aroma of beers. The secondary metabolites and essential oils are produced in the glandular trichomes known as the lupulin glands developed in the female inflorescences (Patzak et al., 2015). A total of 18 terpenes were identified, while the four major terpenes in Brewer's gold variety, namely myrcene, limonene, humulene, and

caryophyllene, were analyzed for this study (Figure 3.6). These molecules provide the hops with a peppery, citrus, and hoppy mixed aroma (Surendran et al., 2021; Vieira et al., 2018). Myrcene (A), D-limonene (B), humulene (C), and caryophyllene (D) concentrations in hops subjected to hot air, microwave-assisted hot air (MD) and vacuum freeze-drying (FD) conditions are presented in Figure 3.6.

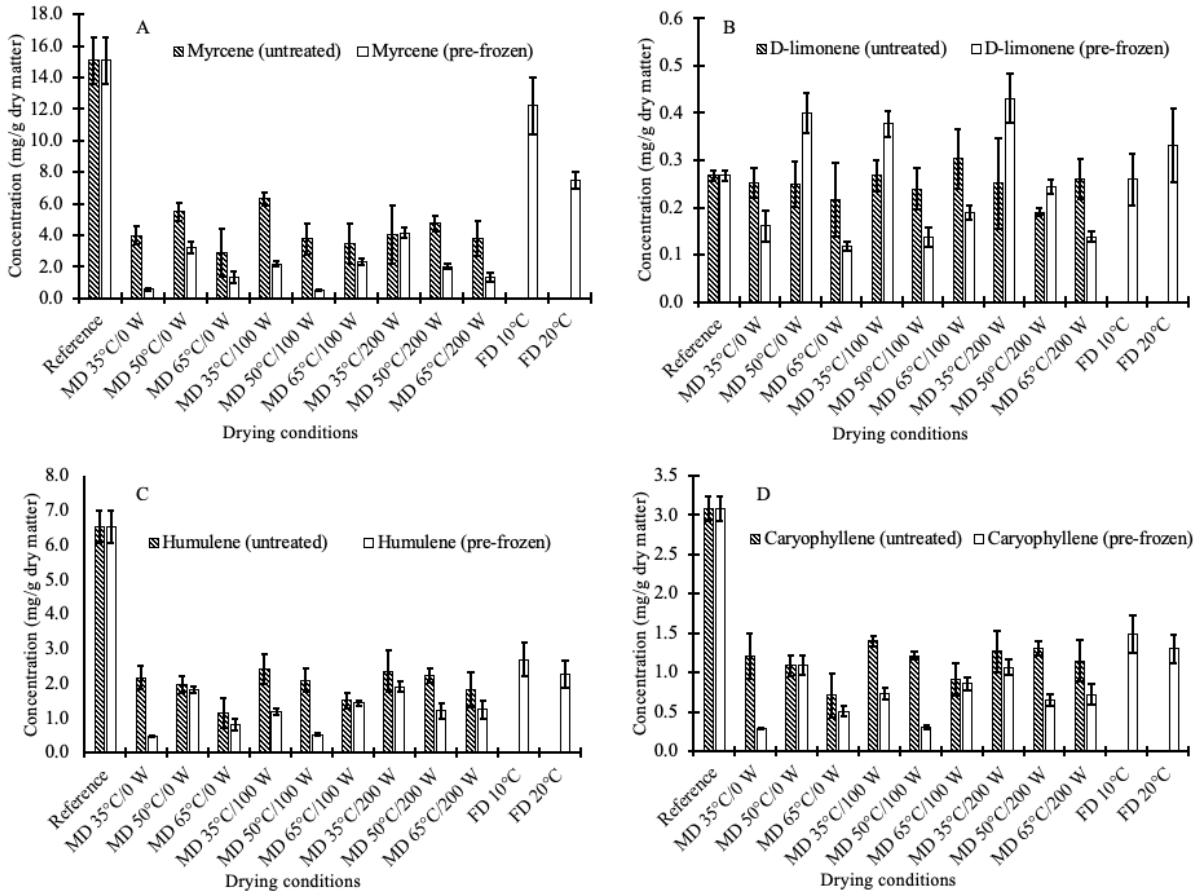

Figure 5.6. Terpene concentrations in hops subjected to hot air, microwave-assisted hot air (MD) or vacuum freeze-drying (FD).

The average concentration of myrcene, limonene, caryophyllene, and humulene for fresh, undried hops were 15.1 mg g^{-1}, 0.3 mg g^{-1}, 3.1 mg g^{-1}, and 6.5 mg g^{-1} respectively. For the dried samples under the various process conditions, the concentration ranged from 12.2 mg g^{-1} to 0.5 mg g^{-1} (myrcene), 0.3 mg g^{-1} to 0.1 mg g^{-1} (limonene), 1.5 mg g^{-1} to 0.3 mg g^{-1} (caryophyllene), and 2.7 mg g^{-1} to 0.5 mg g^{-1} (humulene). Rybka et al., (2018) showed that compared to fresh

hops, drying at 40°C and 55°C significantly ($p < 0.05$) reduced the yield of essential oil by 10% and 36% respectively thereby reduces the aroma of the dried hops. ANOVA analyses of hot air and microwave-assisted hot air dried samples showed a significant ($p < 0.05$) pre-freezing effect on the concentration of myrcene, caryophyllene, humulene, and drying temperature effect on the concentration of limonene. Pre-freezing reduced the concentration of terpenes by 28.2% - 20.7%. However, the results did not show a microwave effect as the microwaves only helped reduce the drying time by providing more energy for the evaporation of the water molecules.

Based on the volatile characteristics of secondary compounds and essential oils of hops, it can be hypothesized that there is a positive correlation between drying temperature and the loss of secondary compounds and reduced concentration of essential oils in dried hops (Kwaśnica et al., 2020; Raut et al., 2020b; Rybka et al., 2018). Similar results were observed for the freeze-dried samples when the shelf temperature was increased from 10°C to 20°C. However, the low temperature used during freeze-drying preserved 68.3% –16.6% of the major terpenes present in hops compared to hot air and microwave-assisted hot air drying systems. Hence, freeze-drying can be used to preserve terpenes present in hops. Compared to the other three terpenes, the concentration of limonene was not significantly affected ($p < 0.05$) by freeze-drying.

3.4 Conclusion

The aim of this study was to evaluate the effects of pre-freezing on hops that were freeze-dried or dried with microwave-assisted hot air at different drying temperatures and microwave power. Moisture was removed from the hops samples during the falling rate period, and pre-freezing hops prior to drying improved the drying rate. This was largely due to a significant increase in the effective moisture diffusion coefficient, which increased when drying temperature and microwave power density were increased. Pre-freezing can be used to preserve hops color; however, it can significantly ($p > 0.05$) reduce terpene concentrations by possible over-release of cellular content sensitive to temperature degradation. In contrast, pre-freezing may be beneficial to oil extraction, as SEM analyses show evidence of structural damage incurred by lupulin glands. When comparing the drying techniques investigated in this study, low temperatures used during freeze-drying successfully preserved hops quality and its flavoring molecules. This study could be used to improve postharvest processing for other industrial crops such as cannabis since hops and cannabis belong to the same Cannabaceae family and have similar physiological traits.

3.5 References

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Connecting text

Chapter 3 highlighted the importance of controlled postharvest processing to ensure industrial crop quality. Results of the study affirmed that pre-freezing plant material before drying can shorten postharvest processing times, and this method can potentially be applied to other industrial crops. Chapter 4 investigated the relationship between cannabis mass reduction and relative humidity during freeze-drying and the effects of pre-freezing and freeze-drying temperature on cannabis trichome structure, drying kinetics, color, and cannabinoids and terpenes concentrations. Chapter 4 showed the potential use of relative humidity sensors for real-time determination of the end of a drying process.

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Chapter 4: Freeze-drying *Cannabis sativa* using real-time relative humidity monitoring and mathematical modeling for the cannabis industry

Abstract

Pre-freezing has an impact on the drying time and physicochemical properties of cannabis. This study investigated the relationship between sample mass reduction and relative humidity during freeze-drying and the effects of pre-freezing and freeze-drying temperature on cannabis drying kinetics, trichome structure, and color, in addition to cannabinoid and terpene concentrations. Three cannabis accessions, Qrazy Train, Qrazy Apple, and Qrazy Angel, were dried at 10˚C, and 20˚C, with different pre-freezing conditions (-20˚C and -40˚C). Pre-freezing rates of 0.13˚C min^{-1} for both Qrazy Train and Qrazy Angel and 0.15˚C min^{-1} for Qrazy Apple were recorded for pre-freezing at -20˚C and significantly ($p < 0.05$) increased by 73.5%, 71.2%, and 72.9% for Qrazy Train, Qrazy Apple, and Qrazy Angel, respectively, when inflorescence was pre-frozen at -40˚C. Rational regression model best explains the relationship between mass reduction and relative humidity during drying, while drying kinetics can be described using the Page and Logarithmic models. Total color changes ranged from 1.16 to 9.69 total color changes measured for all dried samples compared to respective fresh, undried samples were not significantly ($p < 0.05$) different. THCA concentrations for fresh, undried *Cannabis sativa* accessions ranged from 214.4 mg g^{-1} to 257.5 mg g^{-1}; this was higher than their CBDA concentrations, ranging from 0.03 mg g^{-1} to 0.1 mg g^{-1}. Freeze-drying increased [CBDA], [CBGA], and [CBG] in dried samples ranging from 0.45 mg g^{-1} to 0.38 mg g^{-1}, 2.87 mg g^{-1} to 4.91 mg g^{-1}, and 0.57 mg g^{-1} to 1.33 mg g^{-1}, respectively. Mean terpene concentration ranged from 17.7 mg g^{-1} to 40.3 mg g^{-1}. Irrespective of the pre-freezing condition or cannabis accession, drying at 20˚C reduced drying time by 10.4% to 31.9%. Findings could be of industrial relevance for improving post-harvest processes while maintaining quality of this regulated crop.

4.1 Introduction

Drying agricultural products is a complex operation that involves heat and mass transfer phenomena. Drying can result in physical, chemical, and/or biochemical changes, some of which are desirable in final products. Generally, drying is defined as a unit operation that transforms liquid, solid, or semi-solid material into a solid product by evaporation of water, and is influenced by a temperature gradient (Kwaśnica et al., 2020). Freeze-drying, one of the best methods for water removal from agricultural products, is the exception to this definition (Adams et al., 2015; Jangam, 2011). Compared to traditional drying methods such as conventional hot-air drying, freeze-drying produces high-quality dried products that retain nutrients and color through dehydration by sublimation and surface desorption of the frozen product (Cao et al., 2018; Ratti, 2001).

Two main parameters are commonly used to evaluate the degree of drying: moisture content and water activity (Li et al., 2018). Moisture content refers to the amount of free and bound water present in the material. Water activity refers to the amount of water available for microbial activity and as such, is an indicator of food stability. To maintain food quality and safety, moisture content and water activity must be lower than 15% and 0.3, respectively (de Bruijn et al., 2016; Pou and Raghavan, 2020). Hence, real-time accurate prediction of the end of the drying process is important when optimizing a drying system that prevents insufficient drying or ashing plant material.

Various studies have investigated the use of novel sensing methods to accurately predict of the end of a drying process (Kiani et al., 2018; Li et al., 2014; Wang et al., 2022; Wu et al., 2012; Yadollahinia et al., 2009). Kiani et al. (2018) developed a prototypic hot-air drying system for drying mint (*Mentha spicata*) leaves that was equipped with an electronic nose. This study showed that the changes in the aroma patterns during drying were highly correlated to changes in moisture content, and this could be used as an indicator for the end of the drying process. However, immersing the sensors in the headspace without periodically cleaning saturated the sensors and the response signals were not accurate. One computer vision-based drying system showed that morphological features of food decreased with an increase in drying time (Yadollahinia et al., 2009). The disadvantage of using computer vision in a dryer is that it can only acquire external image information within a visible range, rather than internal compositional attributes. In an enclosed dryer, relative humidity can be used as an indicator for

the end of a drying process. Relative humidity is defined as the ratio of the partial pressure of water vapour in an air-water mixture to the saturated vapour pressure of water at a given temperature. Thus, relative humidity is a function of both water content and temperature (Bradford et al., 2016; Phitakwinai et al., 2019).

Slow drying and screen drying are commonly used industrial practices for postharvest processing of the *Cannabis sativa* plant. Some challenges include long drying time and microbial contamination, all affecting the quality and safety of cannabis products, and resulting in considerable financial loss (Addo et al., 2021; Challa et al., 2021). The main objective of this study was to optimize freeze-drying methodology for this crop using relative humidity sensors. Importantly, the relationship between relative humidity and sample mass reduction during freeze-drying was explored, and mathematical models considered for application in scaled up operations of this valuable crop. Pre-freezing rates for different pre-treatment conditions were determined, and the effects of pre-freezing and freeze-drying temperature on drying kinetics, cannabis trichome structure, color, and the retention of cannabinoids and terpenes were investigated.

4.2 Materials and methods

4.2.1 Sample preparation (pre-freezing)

Harvested inflorescence from three cannabis accessions, Qrazy Train, Qrazy Apple, and Qrazy Angel that were cultivated indoors using the same growing conditions obtained from EXKA Inc. for this study. Inflorescences were divided into two groups and subjected to pre-freezing at either -20˚C or -40˚C. Specifically, 100 grams cannabis inflorescence was placed in a single layer on plastic trays at -20˚C and -40˚C in chest freezers for 24 h to obtain various freezing rates. Temperature data were collected every minute using an Onset 12-bit temperature/relative humidity smart sensor (Onset Computer Corporation, Bourne, MA, US) connected to a Hobo U30 USB weather station data logger (Onset Computer Corporation, Bourne, MA, US). The sensor was placed next to a representative cannabis bud in the center of the tray prior to pre-freezing. Each experiment was performed in triplicate using three different cannabis inflorescence samples.

4.2.2 Scanning electron microscopy analyses

Microstructural images were obtained by fixing cannabis samples on an adhesive specimen holder that was inserted into the scanning electron microscope (Hitachi TM-3000, Hitachi, Japan) at 5 kV acceleration voltage.

4.2.3 Freeze-drying of samples

Pre-frozen cannabis inflorescence samples in plastic trays were transferred to a laboratory-scale vacuum freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH Gamma 1-16 LSCplus, Osterode, Lower Saxony, Germany) with a condenser temperature of -55˚C. Freeze-drying was carried out at 10˚C or 20˚C for 24 h at 0.85 mbar. Temperature and humidity data during freeze-drying were collected every minute using a Python-coded automatic data logger that was connected to three BME680 temperature/pressure/humidity sensors (sensors A, B, and C; Figure 4.1). Sensor A was placed in the headspace to measure the relative humidity in the freeze dryer during the drying process. Sensor B was placed 0.05 m away from the tray on the same drying shelf to measure the relative humidity conditions around the tray. Sensor C was placed next to a representative cannabis bud in the centre of the tray (Figure 4.1). Sensor location was used to determine the accuracy of the relative humidity values by virtue of the sensor location. The sensors have a relative humidity range of 0% to 100% (+/-3%) and a temperature range of -40˚C to 85˚C (+/-1˚C). Changes in sample mass during the drying process were recorded every hour. Each experiment was performed in triplicate using three different samples.

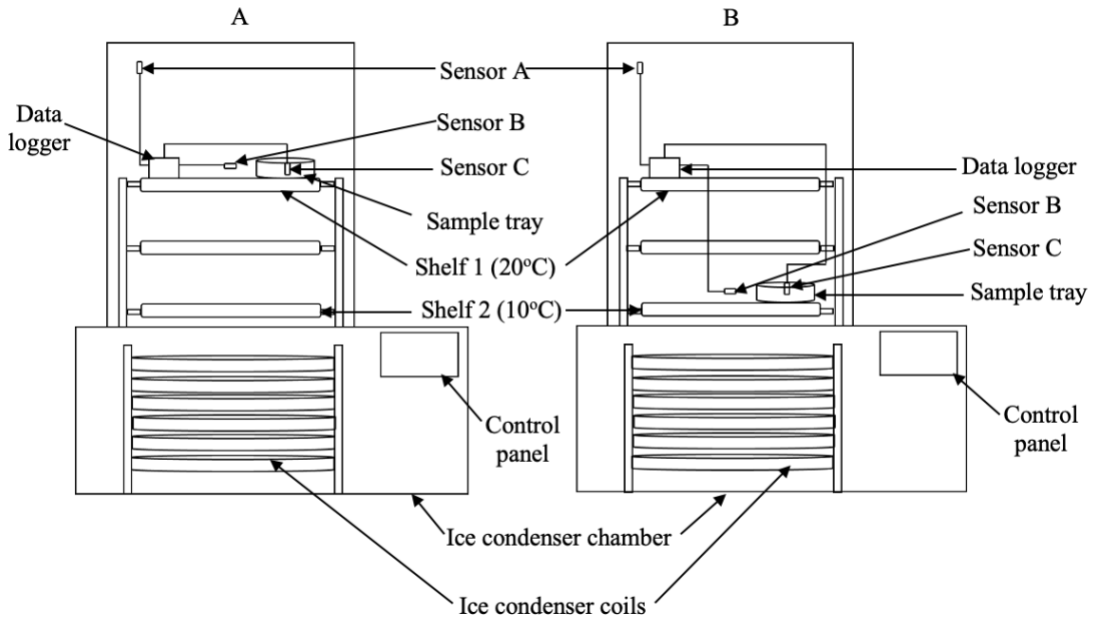

Figure 6.1. Sensor location and pre-frozen cannabis samples placed on (A) shelf 1 (top) and (B) shelf 2 (bottom) for the freeze-drying experiment.

4.2.4 Relationship between changes in sample mass and relative humidity

Data collected for cannabis inflorescence dried under different conditions were used to analyze the relationship between sample mass and relative humidity using Curve Expert Professional software ver. 2.6.5 (Hyams Development, US). Statistical parameters and correlation coefficient (R^2) were used to select the best model and to verify the accuracy of the prediction model.

4.2.5 Drying curves and kinetics

The initial moisture content of the cannabis inflorescence was determined using a hot air oven (Fisher Scientific 6903 Isotemp, Waltham, MA, US). Each sample was dried at 50˚C for 24 h. The moisture content of each sample was calculated with Equation (1):

Moisture content on wet basis (wb) (% g water·(g wet matter)$^{-1}$) = $Mw / (Mw + Ms)$ (1)

where Mw is the mass of water in the sample (g) and Ms is the mass of solids in the sample (g).

For analytical purposes, moisture content was expressed as a function of the solid mass. Dry basis (db) moisture content was calculated with Equation (2):

Dry basis moisture content (% g water·(g dry matter)$^{-1}$) = Mw / Ms (2)

To investigate the drying kinetics of cannabis inflorescences under different drying conditions, moisture ratios (MR) and effective diffusion coefficients were determined. MR of the samples was calculated by converting the experimental drying data using Equation (3):

Moisture ratio (MR) = *(Mt – Me) / (Mi – Me)* (3)

where Mt, Mi, and Me refer to moisture content at time t, initial moisture content, and moisture content at equilibrium, respectively. As Me is relatively small compared with Mt, and Mi the moisture ratio was simplified to Equation (4) as described previously (Raut et al., 2020b):

Moisture ratio (MR) =*Mt / Mi* (4)

4.2.6 Mathematical models

Data collected for cannabis inflorescence dried under different conditions were used to analyze the fit of two thin layer drying mathematical models (Page and logarithmic models) to the experimental data. Statistical parameters were used to estimate the quality of fit of each drying model to the observed values.

4.2.7 Color measurement

Sample color was measured with a CR-300 Chroma meter (Minolta, Tokyo, Japan) calibrated with a standard white plate before measurements were taken. A D65 light source and the CIE1976 (L*a*b*) color scale were used (McLaren, 1976). L* represents lightness from black (0) to white (100), a* from green (−) to red (+), and b* from blue (−) to yellow (+). Total color change (ΔE) was determined with L*, a*, and b* values using the Hunter-Scofield equation [Equation (5)]:

$$\Delta E = \sqrt{\left(L_p - L_i\right)^2 + \left(a_p - a_i\right)^2 + \left(b_p - b_i\right)^2} \quad (5)$$

where suffixes *i* and *p* refer to reference values (fresh undried cannabis) and dried sample values, respectively. A higher ΔE denotes a greater color change from the reference material.

4.2.8 Cannabinoid and terpene analyses

4.2.8.1 Sample preparation

Representative cannabis inflorescence samples from each of the experimental drying conditions (and fresh cannabis inflorescence samples) were immersed in liquid nitrogen before

grinding using a coffee grinder (Hamilton Beach, Belleville, Ontario, Canada). Ground samples were allowed to equilibrate to room temperature before 0.75 g of each representative sample was weighed into a 50 mL Falcon tube and recorded. Each sample was allowed to sit for 10 min on the scale (Mettler AE50 analytical balance, Columbus, Ohio, US) until there was < 1 mg change in mass. This is done to ensure that most of the liquid nitrogen had evaporated from the sample and an accurate sample mass was recorded. For secondary metabolite extraction, 20 mL high-pressure liquid chromatography (HPLC) grade methanol (Thermo Fisher Scientific, Waltham, Massachusetts, US) was added to each Falcon tube and vortexed (Thermo Scientific vortex, Waltham, Massachusetts, US) for 20 min at 500 rpm. Each sample was filtered using Whatman™ filter paper (Thermo Fisher Scientific, Waltham, Massachusetts, US) and allowed to filter for 20 min. Residual cannabis biomass was placed into a new 50 mL Falcon tube and subjected to a second extraction process to ensure 99.5% of the secondary metabolites had been extracted. The second extract was added to the corresponding first extract, resulting in 40 ml total extract.

4.2.8.2 Cannabinoid analyses

Each extract was further diluted 50x (for analysis of major cannabinoids) or 4x (for analysis of minor cannabinoids and terpenes) using HPLC grade methanol (Thermo Fisher Scientific, Waltham, Massachusetts, US). One-milliliter samples of each extract were pipetted into HPLC vials for cannabinoid analysis. The Waters Acquity Ultra High-Performance Liquid Chromatography (UPLC) with a tunable ultraviolet (TUV) detector (Waters™, Mississauga, Ontario, Canada) was used for cannabinoid analyses. The Waters Cortex column was used to separate cannabinoids with a sample injection volume of 2 µL and a column temperature of 30˚C, equipped with an isocratic gradient pump. Mobile phase A consisted of 22% reverse osmosis water and 0.1% formic acid (Sigma-Aldrich, Saint Louis, Missouri, US). Mobile phase B was 78% HPLC grade acetonitrile (Thermo Fisher Scientific, Waltham, Massachusetts, US). Quantification of the cannabinoids was done using an external calibration curve developed using 7 standard cannabinoids (LGC standards, Manchester, New Hampshire, US and Sigma Aldrich, Saint Louis, Missouri, US).

4.2.8.3 Terpene analysis

One-milliliter samples of each extract were pipetted into gas chromatograph (GC) vials for terpene analysis. Separation of the terpenes was performed with an Agilent 7820A GC coupled to an Agilent 7693 autosampler and a flame ionization detector (FID) (Agilent Technologies, Mississauga, Ontario, Canada). The system was equipped with an injector containing a capillary column (30 m x 250 μm x 0.25 μm nominal Agilent Technologies DB-5 Model) using split injection (ratio 50:1) with a hydrogen carrier gas (40 mL min^{-1}). An injection volume of 5 μL of each sample with a syringe size of 10 μL was used. The gas chromatography oven temperature was initially programmed at 35˚C and held for 4 min. The temperature of the oven was increased at a rate of 10˚C min^{-1} up to 105˚C held for 0 min, increased at a rate of 15˚C min^{-1} up to 205˚C held for 0 min, and lastly increased at a rate of 35˚C min^{-1} up to 270˚C held for 5 min. The inlet temperature into the FID detector was set at 340˚C. Spectra were recorded at three scans from 50 to 400 m z^{-1}. The ionization mode was used with an electronic impact at 70 eV. Quantification of the terpenes was done using an external calibration of 37 terpenes mostly found in cannabis (LGC standards, Manchester, New Hampshire, US and Sigma Aldrich, Saint Louis, Missouri, US).

4.2.9 Statistical analysis

Statistical analyses were conducted using the JMP software (JMP 4.3 SAS Institute Inc., Cary, North Carolina, US) with a confidence level ($p < 0.05$) of 95%. Drying mathematical models of the cannabis were fitted and analyzed with Curve Expert Professional software ver. 2.6.5 (Hyams Development, US) and JMP software (JMP 4.3 SAS Institute Inc., Cary, North Carolina, US). Pairwise comparisons of means were done using the Student's t statistical test.

4.3 Results and discussion

4.3.1 Freezing rate curves

Pre-freezing is required for efficient freeze-drying, as it influences the number and size of ice crystals that affect drying performance and residual moisture content (Kasper and Friess, 2011). To determine the freezing rate curves of cannabis inflorescences subjected to different pre-freezing temperatures, -20˚C and -40˚C, temperature changes were plotted against freezing time (Figure 4.2). The total freezing time was defined as the time taken for the temperature at the

centre of a representative bud to reach -20˚C, as commercially frozen foods are kept at -18˚C to -20˚C (Skåra et al., 2019). Total freezing time required for the three cannabis accessions to reach -20˚C when pre-frozen at -20˚C was 190.2 ± 11.1 min, 189.1 ± 13.1 min, and 196.4 ± 3.58 min for Qrazy Train, Qrazy Apple, and Qrazy Angel, respectively. Freezing times for pre-freezing at -40˚C were 51.4 ± 9.26 min, 59.3 ± 1.21 min, and 63.2 ± 2.08 min for Qrazy Train, Qrazy Apple, and Qrazy Angel, respectively. Factor effect analyses and Student's t pairwise comparisons of the freeze times showed significant differences ($p < 0.05$) between the two freezing temperatures for all accessions. At the same freezing temperature, no significant differences ($p < 0.05$) were observed between the three accessions.

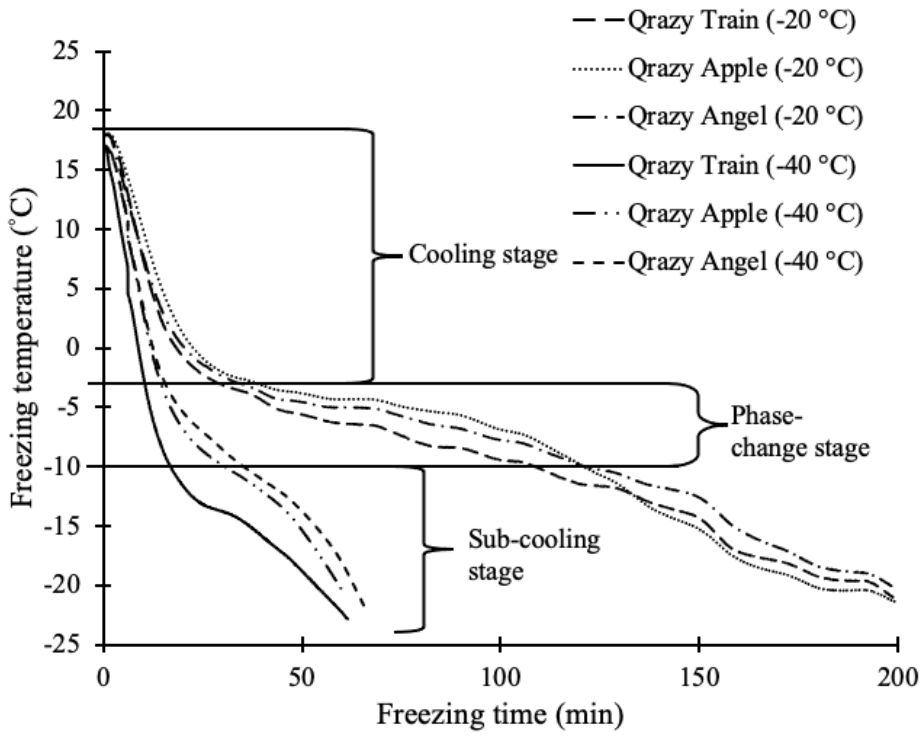

Figure 6.2. Pre-freezing curves for cannabis accessions submitted to different pre-freezing temperatures.

When determining the freezing kinetics of agricultural products, freezing curves can be divided into three stages: the cooling stage, the phase-change stage (or maximum ice-crystal formation zone), and the sub-cooling stage (Figure 4.2) (Cao et al., 2018; Gao et al., 2019). During the cooling stage, cannabis inflorescence was cooled down from atmospheric temperature to -3˚C, releasing sensible heat due to a rapid temperature change. First phase

durations were 29.9 ± 10.4 min, 41.3 ± 13.6 min, and 32.5 ± 10.1 min for Qrazy Train, Qrazy Apple, and Qrazy Angel respectively during pre-freezing at -20˚C. First phase durations significantly ($p < 0.05$) reduced by 65.1%, 65.2%, and 52.4% for Qrazy Train, Qrazy Apple, and Qrazy Angel, respectively, when samples were pre-frozen at -40˚C.

During the phase-change stage, most of the water in the cannabis inflorescence was transformed into ice crystals as the temperature decreased slowly and latent heat was released. Duration of the phase-change stage determines ice crystal size, and this is critical to the quality of frozen food products. Less time spent in the ice-crystal formation zone is preferred, as small and evenly distributed ice crystals are formed, causing minimal or no damage to cellular structures. Duration times recorded were 77.1 ± 12.3 min, 78.6 ± 12.4 min, and 95.5 ± 18.9 min for Qrazy Train, Qrazy Apple, and Qrazy Angel, respectively, during pre-freezing at -20˚C. Phase-change durations were significantly ($p < 0.05$) reduced to 8.51 ± 6.43 min, 16.4 ± 1.57 min, and 19.7 ± 1.66 min for Qrazy Train, Qrazy Apple, and Qrazy Angel, respectively when the freezing temperature decreased to -40˚C. These data are comparable to reported freezing curves for blueberries (*Vaccinium corymbosum*) (Cao et al., 2018).

During the sub-cooling stage, residual water continues to freeze and ice crystals continually form. The temperature of the cannabis inflorescence samples dropped because the thermal conductivity of ice was higher than that of water. Overall, the total freezing rates recorded for cannabis inflorescences pre-frozen at -20˚C were 0.13˚C min^{-1} for both Qrazy Train and Qrazy Angel, and 0.15˚C min^{-1} for Qrazy Apple. Total freezing rates significantly ($p < 0.05$) increased by 73.5%, 71.2%, and 72.9% for Qrazy Train, Qrazy Apple, and Qrazy Angel, respectively, when inflorescences were pre-frozen at -40˚C. Similar results were reported in studies using beetroots (*Beta vulgaris*), apples (*Malus domestica*), eggplants (*Solanum melongena*), and celeries (*Apium graveolens*) (Nowak et al., 2016; Vallespir et al., 2019). Nowak et al., (2016) showed that decreasing the freezing temperature for celery from -20˚C to -40˚C increased the average cooling rate from 0.013˚C min^{-1} to 0.252˚C min^{-1}. Based on the total freezing rates, freezing at -20˚C can be considered a slow freezing process with larger ice crystal production if compared to freezing at -40˚C.

4.3.2 Scanning electron microscopy analyses

Scanning electron microscopy was used to investigate the effect of different pre-freezing and freeze-drying temperatures on inner and outer trichome surface microstructure. In the reference cannabis inflorescence samples (fresh, untreated), trichomes appeared firm and intact, with a large globular head on a long stalk (Figure 4.3). These trichomes were classified as stalked trichomes based on structure, as stalked trichomes have trichome heads elevated above the epidermis on a multicellular stalk and produce the greatest concentration of cannabinoids (Livingston et al., 2020; Tanney et al., 2021). Irrespective of cannabis accession, pre-freezing temperature and drying condition, trichomes incurred marked structural damage. The cold temperature used during pre-freezing and ice crystal formation resulted in trichome stalk wrinkling and trichome heads fell off. Freeze-drying caused noticeable damage to the trichome heads and sugar leaf surfaces were covered with epidermis debris. Although not yet explored for cannabis, structural damage caused by freezing could make moisture and oils more readily available for drying and extraction, respectively, which might translate to reduced drying and extraction time with improved oil yield. Preliminary studies using hops (*Humulus lupulus*) showed that pre-freezing hop buds at -80˚C increased the effective moisture diffusion coefficient, and it increased with higher drying temperature and microwave power, ranging between 5.91 x 10^{-10} m^2 s^{-1} and 2.43 x 10^{-7} m^2 s^{-1} (Addo et al., 2022c).

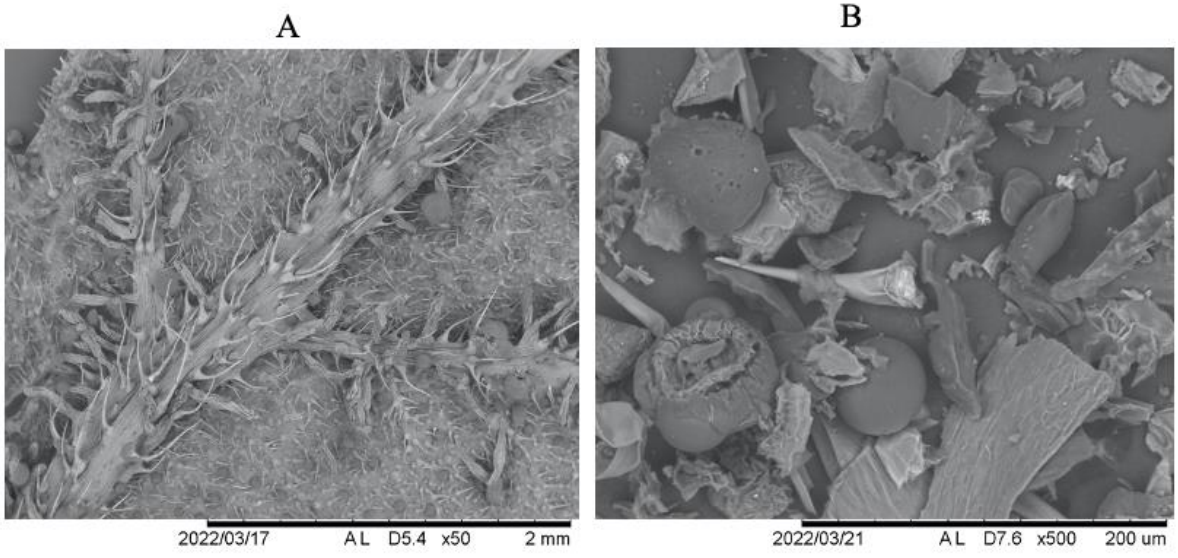

Figure 6.3. Representative SEM images of cannabis (Qrazy Train) trichomes after (A) pre-freezing and (B) freeze-drying at 20°C.

4.3.3 Relationship model between changes in mass and relative humidity

Relative humidity sensors in a drying system make it convenient for determining when plant material has been sufficiently dried. In this study, Sensor A and B were placed in the air stream of the dryer. Specifically, sensor A was placed in the headspace and Sensor B was placed 0.05 m away from the tray. Senor C was placed in the centre of the drying tray next to a representative cannabis inflorescence. Drying is influenced by relative humidity and temperature. The thermal gradient inside and outside a material increases with an increase in temperature. This causes more moisture displacement, thereby increasing the relative humidity in the system. Applying vacuum during drying additionally results in the expansion of air and steam inside the material which forms a puffy structure. This structure of foodstuff leads to the easier escape of water molecules (Dueik and Bouchon, 2011; Dueik et al., 2013). In this study, relative humidity was monitored during freeze-drying at two drying shelf temperatures (10˚C and 20˚C), to determine optimal drying time. Changes in relative humidity within the freeze dryer were recorded when cannabis samples pre-frozen at different temperatures (-20˚C and -40˚C) were freeze-dried. Figures 4.4, 4.5, and 4.6 show exponential decrease in relative humidity during the drying process for the different cannabis accessions used in the study.

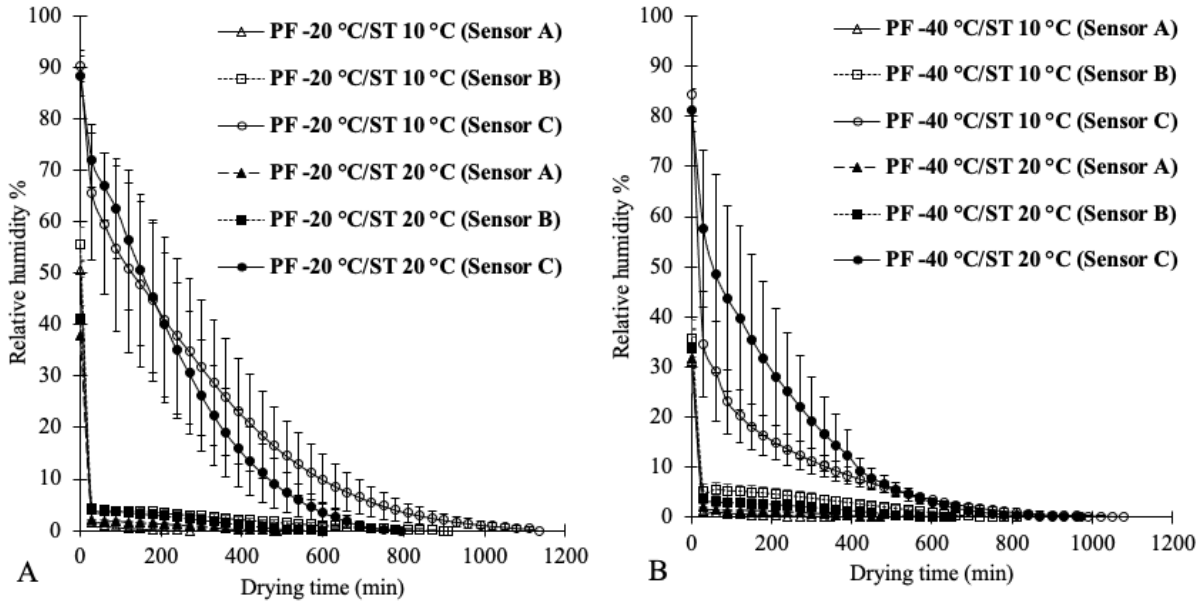

Figure 6.4. Relative humidity for Qrazy Train subjected to different pre-freezing temperatures (PF) and freeze-drying shelf temperatures (ST).

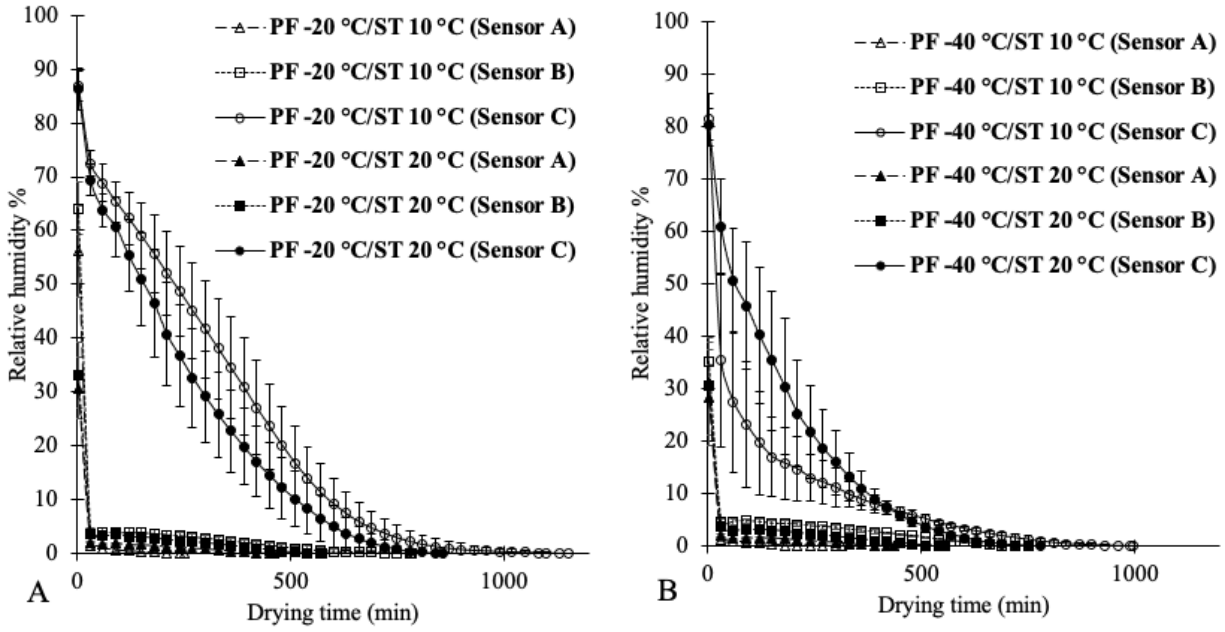

Figure 6.5. Relative humidity for Qrazy Apple subjected to different pre-freezing temperatures (PF) and freeze-drying shelf temperatures (ST).

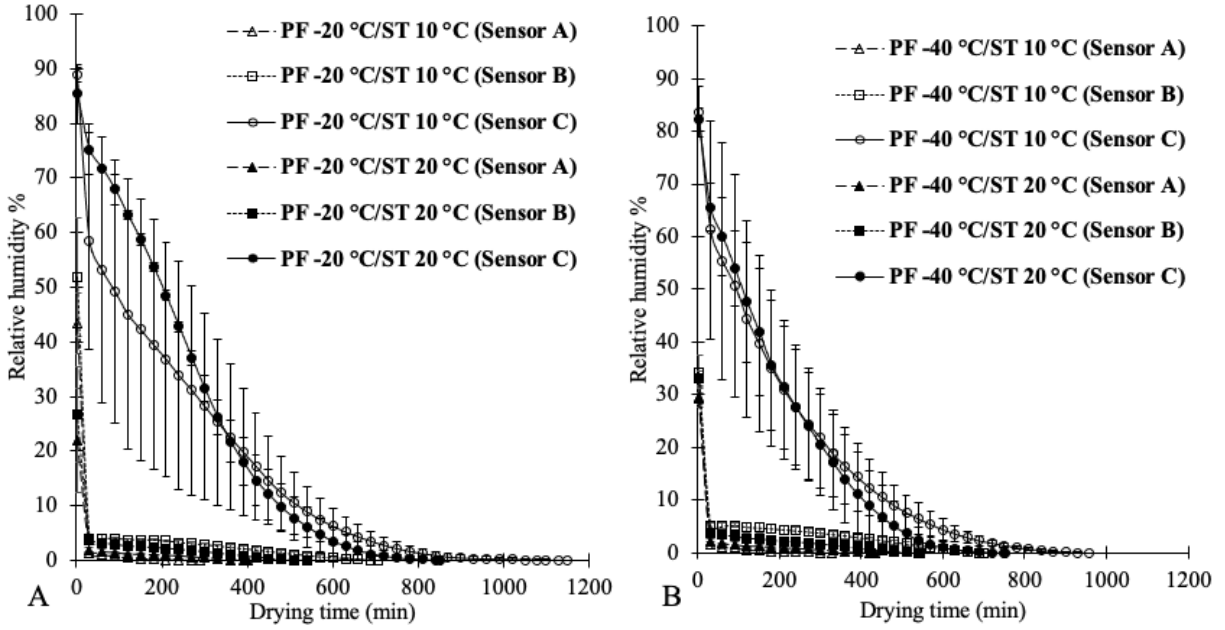

Figure 6.6. Relative humidity values for Qrazy Angel subjected to different pre-freezing temperatures (PF) and freeze-drying shelf temperatures (ST).

Preliminary tests showed that relative humidity quickly reduced to 0% when there was no biomass sample in the freeze-dryer, indicating that the recorded relative humidity values for the different drying conditions of cannabis were due to the evaporation of water molecules from the samples as described previously (Bradford et al., 2016; Phitakwinai et al., 2019). Data logged by the three sensors (A, B, and C) emphasise the importance of sensor location within the dryer. Irrespective of the drying condition, shelf temperature, and accession, only sensor C recorded the closest to the actual changes in relative humidity during the entire drying process. This can be attributed to placing the sensor near a representative bud in the center of the drying tray (Figure 4.1). At the start of the drying process, high standard deviation values were recorded for the relative humidity values measured by sensor C. This can be attributed to differences in moisture content of the plant biomass used for each drying condition. Standard deviation values reduced during the drying process and towards the end of the process, there were no significant differences ($p < 0.05$) between the measured relative humidity values for the triplicates for each drying condition. Sensors A and B reduced to 0% when the vacuum pump was engaged and remained at 0% during the drying process. Sensor A recorded the relative humidity in the headspace of the dryer and sensor B recorded the relative humidity around the drying tray containing the cannabis bud samples.

Drying times and relative humidity rates were significantly ($p < 0.05$) affected by shelf temperature of the freeze-dryer, but not by pre-freezing temperature (-20˚C and -40˚C). Results indicate a strong relationship between changes in sample mass and relative humidity during drying and shows that relative humidity can be used as indicator to determine the end of a drying process of a freeze-dryer. Model analyses showed that the data best fit the rational regression model with high R^2 (Table 4.1). The rational regression model is simply a generalization of a polynomial model as a ratio of two polynomial functions. To verify these findings, ANOVA analyses were performed using the predicted sample mass changes with different drying conditions, using the rational model as a function of the measured mass changes. High correlation coefficients (0.97 - 0.99) and R^2 values were recorded (0.96 - 0.99).

Table 6.1. Rational model parameters for drying three cannabis accessions.

Rational model

$$M = \frac{a + (b * RH)}{1 + (c * RH) + (d * RH^2)}$$

Cannabis accession	Pre-freezing temperature	Freeze-drying shelf temperature	a	b	c	d	R^2
Qrazy Train	-20˚C	10˚C	21.95	1.95	0.01	0	0.96
		20˚C	19.70	0.27	-0.01	0	0.99
	-40˚C	10˚C	19.42	2.47	-0.01	0	0.97
		20˚C	22.55	1.03	-0.01	0	0.99
Qrazy Apple	-20˚C	10˚C	20.95	3.21	0.04	0	0.98
		20˚C	20.87	0.10	-0.02	0	0.99
	-40˚C	10˚C	18.95	2.60	-0.01	0	0.96
		20˚C	19.97	0.92	-0.01	0	0.99
Qrazy Angel	-20˚C	10˚C	22.81	0.91	-0.01	0	0.98
		20˚C	21.30	5.76	0.09	0	0.99
	-40˚C	10˚C	21.52	2.76	0.02	0	0.96
		20˚C	21.17	0.64	-0.01	0	0.99

a, b, c, and d are the model coefficients (unit-less) that have different values depending on the equation. M is the mass of the sample (grams) and RH is the relative humidity (%).

4.3.4 Drying curves and kinetics

Freeze-drying is carried out in three stages (Figure 4.7); freezing, primary drying, and secondary drying (Addo et al., 2021). Freezing cannabis inflorescence prevents water foams from forming when the vacuum is applied to the system. Primary drying involves the sublimation of ice crystals present in the inflorescences. When sublimation is complete, the sample temperature will increase and approach the shelf temperature. Increasing the shelf temperature from 10˚C to 20˚C provided more energy to the sample and reduced the drying time by improving the drying rate. More energy is required to evaporate the bound moisture present in a sample during the secondary drying stage irrespective of the pre-freezing temperature. A significant ($p < 0.05$) decrease in drying time was observed when the drying shelf temperature

was increased form 10˚C to 20˚C. Similar observations were made for all three cannabis accessions used for the study. Kwaśnica et al., (2020) showed that when using a convectional air dryer for cannabis, drying time can be reduced from 840 min to 510 min by increasing air temperature from 50˚C to 70˚C.

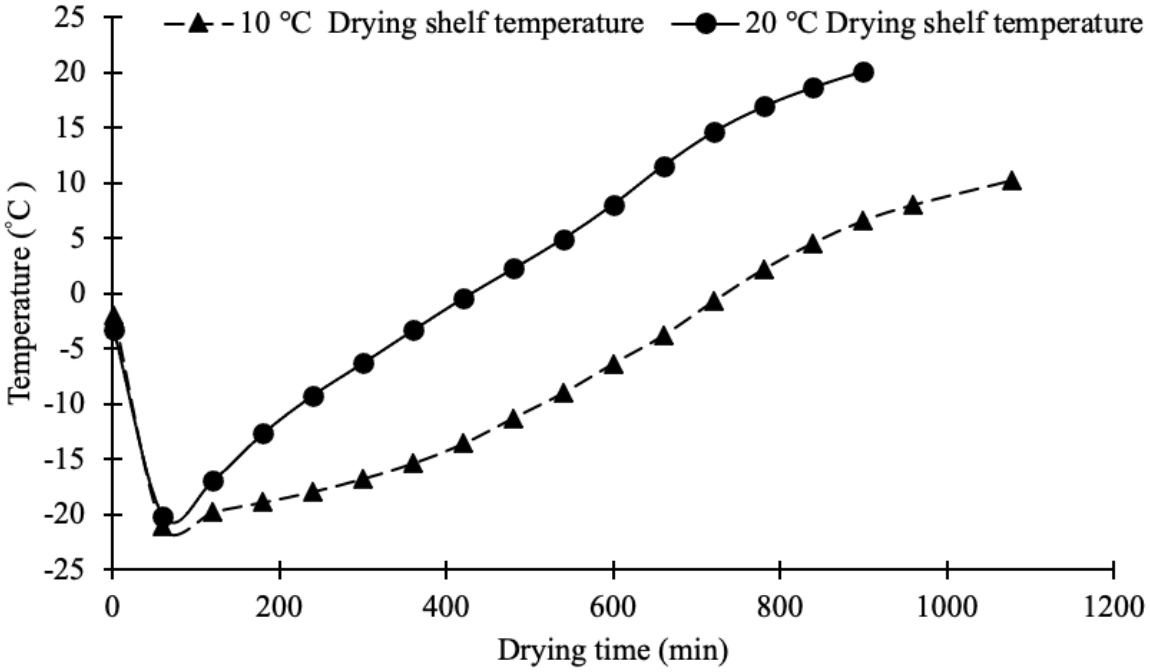

Figure 6.7. Comparison of freeze-drying curves of Qrazy Train pre-frozen at -20°C at different shelf temperatures.

Moisture ratios as a function of drying time were plotted in semi-log graphs for cannabis inflorescence dried at the different drying conditions. As expected, moisture ratios reduced exponentially with time (Figure 4.8). Initial moisture content of the three accessions ranged between 77% to 79% (wb). Drying curves were significantly ($p < 0.05$) affected by shelf drying temperature but not pre-freezing temperature. For -20˚C pre-frozen cannabis inflorecence samples freeze-dried at a shelf temperature of 10˚C, drying times were 1115 min (Qrazy Train), 957 min (Qrazy Apple), and 988 min (Qrazy Angel). Freeze-drying time was significantly ($p < 0.05$) reduced by 31.9% (Qrazy Train), 20.1% (Qrazy Apple), and 22.6% (Qrazy Angel) when the shelf temperature was increased to 20˚C. This can be attributed to the increased product temperature during the secondary drying stage of the freeze-drying process (Figure 4.7). Similar

observations were made for samples pre-frozen at -40˚C; drying time was reduced was by 10.5% (Qrazy Train), 18.9% (Qrazy Apple), and 26.0% (Qrazy Angel). In another Cannabaceae crop, Addo et al., (2022) showed that the drying time for hops decreased by 94.5% and 51.3% if compared to untreated hops dried immediately after harvest at 35˚C and 50˚C, respectively, using a microwave drying unit.

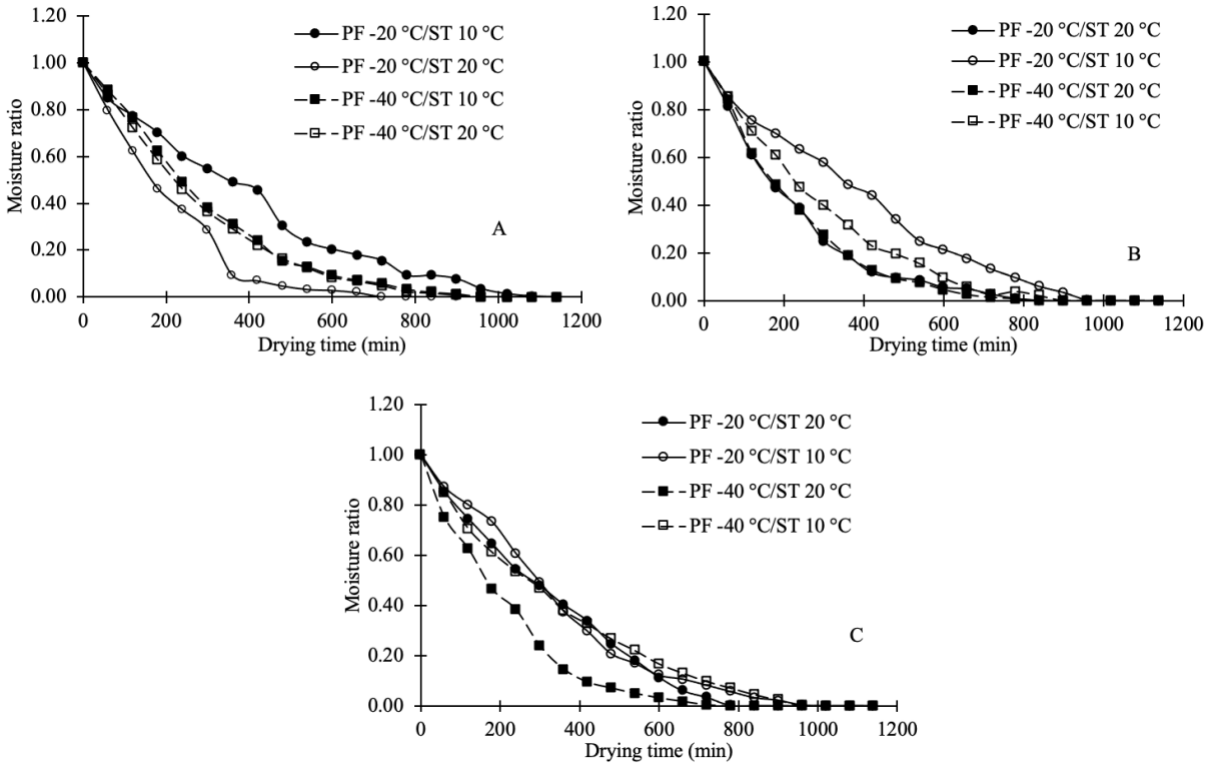

Figure 6.8. Moisture ratio curves of for Qrazy Train (A), Qrazy Apple (B), and Qrazy Angel (C) submitted to different pre-freezing (PF*)* and freeze-drying shelf temperatures (ST).

4.3.5 Mathematical models

Drying models are important when designing industrial energy-efficient dryers. The moisture ratio values calculated for cannabis inflorescence when subjected to different freeze-drying shelf temperatures were fitted into two thin-layer drying models (Page and Logarithmic models) that include R^2 values to assess the appropriateness of the model fit and drying model coefficients (Table 4.2). The Page model is an empirical modification of the Lewis Newton model that comprises a dimensionless empirical constant (n). It has been used to determine the drying kinetics of shelled corn (*Zea mays*) and other agricultural products (Page, 1949). Chandra

and Singh proposed a new model, the logarithmic form of the Henderson and Pabis model, with an empirical term addition (Chandra and Singh, 1994). This model is based on Fick's second law of diffusion, and it has been used to describe the drying of laurel leaves (*Laurus nobilis*) (Yagcioglu, 1999). Various studies have shown that both models are the best drying models (Hu et al., 2017; Palamanit et al., 2020; Raut et al., 2020b). All the models emerged as best fits due to the high R^2 ranging from 0.93 to 0.99.

Table 6.2. Thin layer model parameters for cannabis accessions submitted to different drying conditions.

Logarithmic model = Moisture ratio = $a\ exp\ (-kt) + c$						
Sample	Pre-freezing temperature	Drying temperature	a	k	c	R^2
Qrazy Train	-20˚C	10˚C	1.20	0.10	-0.20	0.94
		20˚C	1.07	0.28	-0.03	0.98
	-40˚C	10˚C	1.12	0.18	-0.06	0.96
		20˚C	1.09	0.19	-0.05	0.96
Qrazy Apple	-20˚C	10˚C	1.27	0.09	-0.27	0.99
		20˚C	1.04	0.26	-0.02	0.95
	-40˚C	10˚C	1.09	0.18	-0.07	0.93
		20˚C	1.07	0.25	-0.03	0.96
Qrazy Angel	-20˚C	10˚C	1.18	0.14	-0.13	0.98
		20˚C	1.16	0.14	-0.13	0.98
	-40˚C	10˚C	1.10	0.14	-0.11	0.94
		20˚C	1.05	0.27	-0.03	0.99
Page model = Moisture ratio = $exp\ (-kt^n)$						
Sample	Pre-freezing temperature	Drying temperature	k	n	R^2	
Qrazy Train	-20˚C	10˚C	0.08	1.27	0.98	
		20˚C	0.19	1.30	0.96	
	-40˚C	10˚C	0.11	1.34	0.93	
		20˚C	0.14	1.25	0.99	
Qrazy Apple	-20˚C	10˚C	0.07	1.35	0.98	
		20˚C	0.21	1.14	0.94	

	-40˚C	10˚C	0.13	1.23	0.98
		20˚C	0.19	1.22	0.99
Qrazy Angel	-20˚C	10˚C	0.07	1.46	0.96
		20˚C	0.09	1.35	0.99
	-40˚C	10˚C	0.13	1.16	0.98
		20˚C	0.21	1.19	0.95

k is the drying rate constant ($hour^{-1}$), a, b, and c are the drying coefficients (unit-less) that have different values depending on the equation and the drying curve.

4.3.6 Color measurement

The color of agricultural products can be used as an indicator of food quality and grade, as various studies relate color changes to nutritional properties of plant material (Cömert et al., 2020; Dueik and Bouchon, 2011; Krawitzky et al., 2014). Color additionally plays an important role in the selection of agricultural products. Color changes in cannabis inflorescence for all drying conditions investigated in this work were measured with a chroma meter and compared to their respective reference sample (fresh, undried cannabis inflorescence) (Figure 4.9). No visual color degradation was obvious during the drying process. ANOVA and Student's t pairwise comparisons analyses showed that the total color changes measured for all cannabis accessions compared to the respective fresh, undried samples were not significantly ($p < 0.05$) different. Total color changes ranged from 1.16 to 9.69 and similar results were reported for freeze-dried orange (*Citrus x sinensis*) puree using different drying temperatures and chamber pressures (Silva-Espinoza et al., 2020). No significant differences were recorded for the total color changes for all drying conditions, except for a pre-freezing significant difference for both pre-freezing conditions for Qrazy Train inflorescence dried at 10˚C. Significant total color changes were recorded between Qrazy Apple inflorescence pre-frozen at -40˚C and freeze-dried at a shelf temperature of 20˚C, and between Qrazy Apple inflorescence pre-frozen at -20˚C and freeze-dried at a shelf temperature of 10˚C.

L* values determine the lightness of the sample from black (0) to white (100). L* values were not affected by pre-freezing or freeze-drying shelf temperature and ranged between 41.71 – 48.87 for Qrazy Train, 41.72 – 50.32 for Qrazy Apple, and 41.84 – 48.77 for Qrazy Angel. The a*, and b* values are used to measure the sample position on the green (−) to red (+) and blue (−)

to yellow (+) spectrum, respectively. Both the a* and b* values for all drying conditions, except Qrazy Train and Qrazy Apple samples pre-frozen at -20˚C and dried at 10˚C, were not significantly ($p < 0.05$) different when compared to the reference sample.

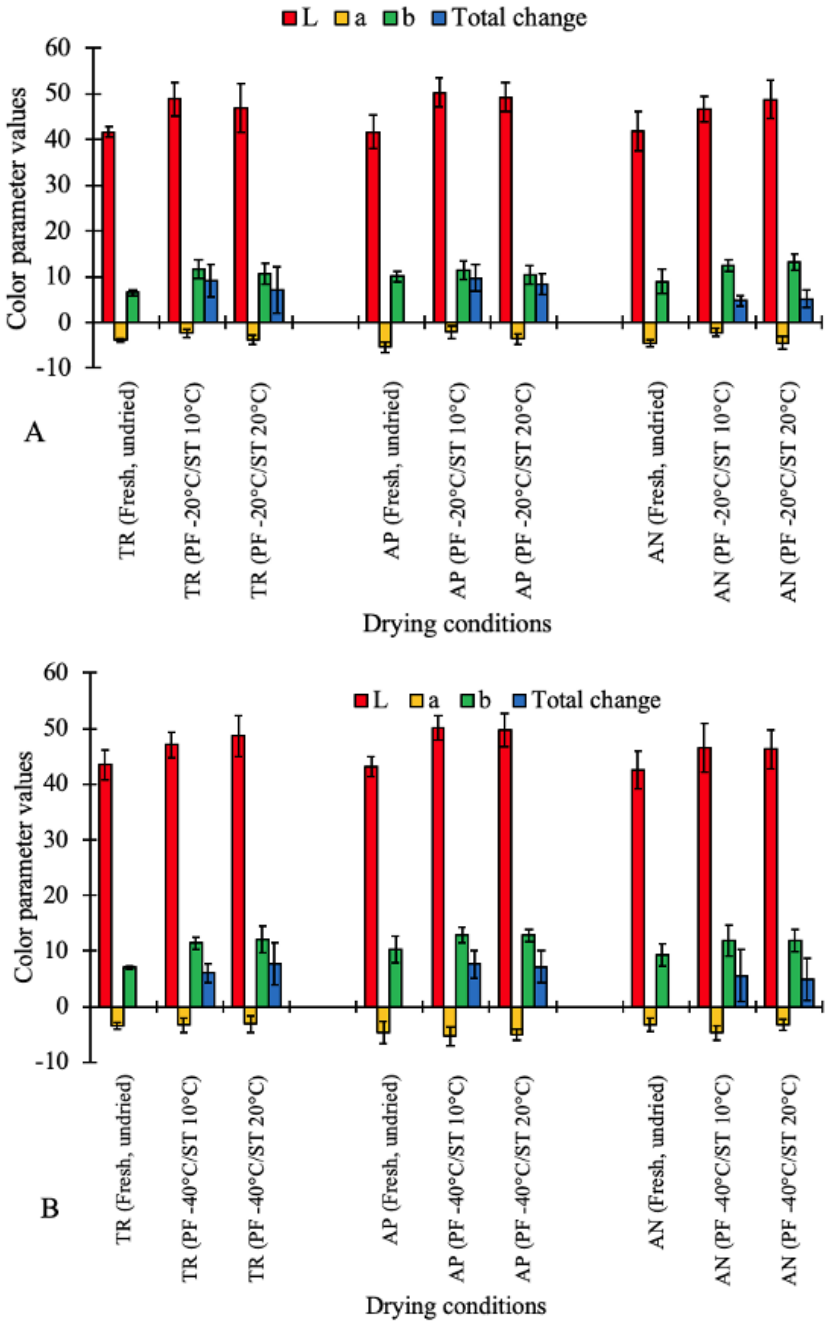

Figure 6.9. Color changes of Qrazy Train (TR), Qrazy Apple (AP), and Qrazy Angel (AN) submitted to different pre-freezing (PF) and freeze-drying shelf temperatures (ST).

4.3.7 Cannabinoid and terpene analyses

Plant metabolites can be classified as primary or secondary relative to their involvement in plant development and growth. Although secondary metabolites are not directly involved in development and growth, they help protect plants against biotic and abiotic stress caused by unfavourable environmental conditions (Addo et al., 2021; Ashraf et al., 2018; Desaulniers Brousseau et al., 2021). Secondary metabolite concentration in plants of the same species can vary under environmental stresses such as temperature, light intensity, drought, etc. Vacuum freeze-drying process is as a form of cryopreservation that may be considered a low temperature-induced stress that causes wounding and drought to cannabis inflorescence (Figure 4.3). The effect of these postharvest activities on the commercially relevant secondary plant metabolites in cannabis, namely cannabinoids and terpenes, were examined using ultra high-performance liquid chromatography with a tunable ultraviolet detector, and gas chromatography with a flame ionization detector.

Total THC content and major cannabinoid concentrations (cannabidiolic acid [CBDA], cannabigerolic acid [CBGA], cannabigerol [CBG], tetrahydrocannabinol [Δ9-THC], and tetrahydrocannabinolic acid [THCA]) in three cannabis accessions subjected to different postharvest pre-freezing and freeze-drying conditions were determined (Figures 4.10 and 4.11). CBD and total CBD content were not presented as the concentration of CBD was below the limit of detection of the instrumentation and methodology. Fresh, undried cannabis inflorescence from Qrazy Train, Qrazy Apple, and Qrazy Angel, had THCA concentrations of 25.8 g 100 g dry matter^{-1}, 21.4 g 100 g dry matter^{-1}, and 23.1 g 100 g dry matter^{-1}, respectively, which were significantly ($p < 0.05$) higher than their CBDA concentration of 0.01 g g dry matter^{-1}, 0.004 g 100 g dry matter^{-1}, and 0.003 g g dry matter^{-1}, respectively. They may be considered Type I chemovars according to the classification set by Lewis et. al. (2018). Pre-freezing and freeze-drying significantly increased THCA concentration, except for Qrazy Train pre-frozen at -20˚C and freeze-dried at 20˚C (27.1 g 100 g dry matter^{-1}), Qrazy Apple pre-frozen at -40˚C and freeze-dried at 10˚C for (22.4 g 100 g dry matter^{-1}), and all drying conditions for Qrazy Angel.

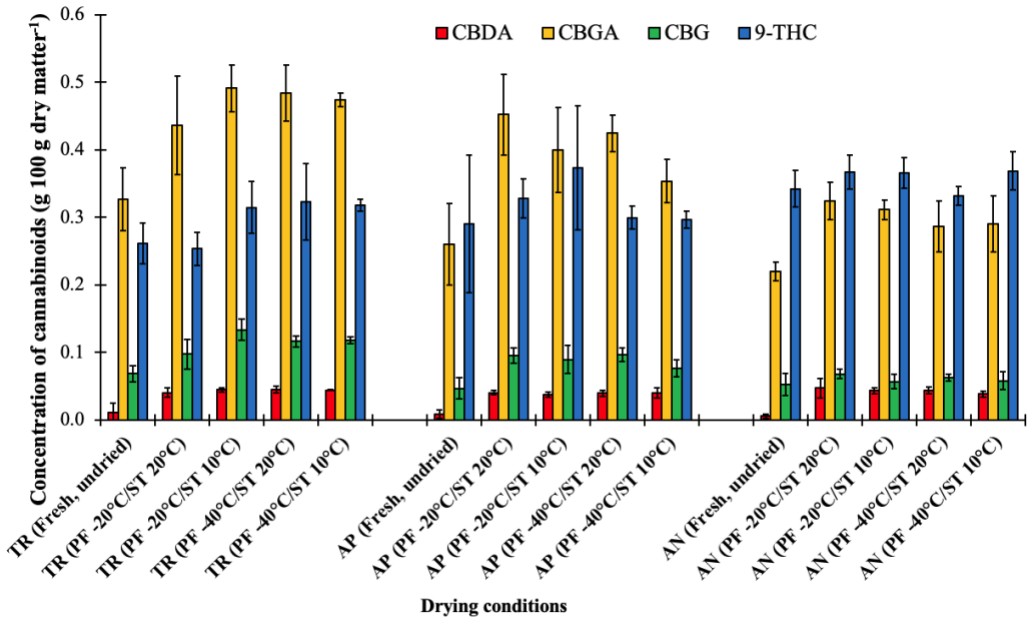

Figure 6.10. Concentration of CBDA, CBGA, and CBG in Qrazy Train (TR), Qrazy Apple (AP), and Qrazy Angel (AN) submitted to different pre-freezing (PF*)* and freeze-drying shelf temperatures (ST).

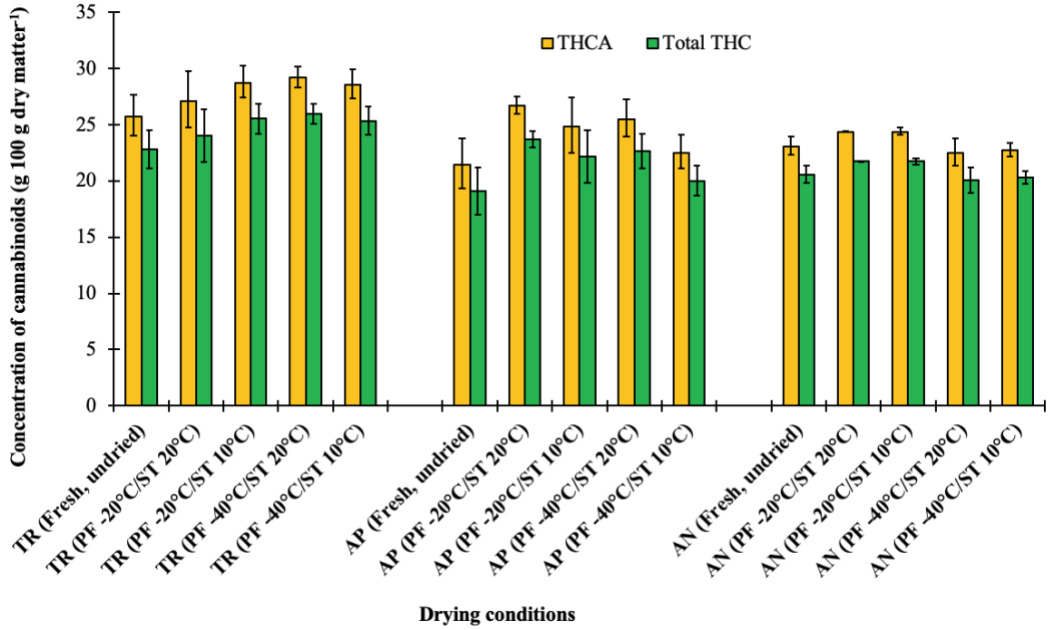

Figure 6.11. Concentration of total THC and THC in Qrazy Train (TR), Qrazy Apple (AP), and Qrazy Angel (AN) submitted to different pre-freezing (PF*)* and freeze-drying shelf temperatures (ST).

ANOVA analyses showed that for each dried cannabis accessions, inflorescence subjected to different pre-freezing and freeze-drying temperatures did not have a significant effect ($p < 0.05$) on the mean concentration of the major cannabinoids. However, when comparing freeze-dried samples to their respective fresh, undried inflorescence, significant ($p < 0.05$) increases were observed for CBDA concentrations for all three accessions. CBG was only significantly ($p < 0.05$) increased for Qrazy Train and Qrazy Apple. For CBGA, significant ($p < 0.05$) increases in concentrations were observed for Qrazy Train and Qrazy Apple (all conditions), and pre-freezing at -20˚C at both drying temperatures for Qrazy Angel. CBDA, CBGA, and CBG concentrations in all dried samples increased by 73.3% to 87.7%, 23.4% to 42.4%, and 6.9% to 51.9% respectively.

Various studies have documented increases in secondary metabolites in response to drought and wounding stress in medical plants (Caplan et al., 2019; Lewis et al., 2018; Savatin et al., 2014; Toth et al., 2021). Caplan et al. (2019) showed that drought stress applied to the cannabis plants through gradual growing substrate drying in a controlled environment can increase THCA and CBDA concentrations by 12% and 13% respectively, compared to the control upon harvest. In other species such as the *Phillyrea angustifolia* plant, loss of moisture during the drying process can lead to the formation of reactive oxygen species in chloroplasts that increase the production of antioxidants such as carotenoids to prevent cellular damage (Peñuelas et al., 2004). Wounding inflicted by ice crystals formed during pre-freezing induced the production of plant hormones such as jasmonic acid and abscisic acid, which have been linked to changes in cannabinoid concentrations (Peč et al., 2010; Savatin et al., 2014). Plant stress parameters that increase the terpenoid synthesis and accumulation may do the same for cannabinoids because of their related biochemical origins. Although the lower temperature during the freezing and primary stages of the freeze-drying inactivates enzymes present in cannabis plant material, the increased product temperature during the secondary drying provides a favourable environment for enzymatic activity which can lead to an increase in cannabinoid and terpene concentrations.

For fresh, undried cannabis inflorescence, Qrazy Angel had the highest concentration (0.34 g 100 g dry matter^{-1}) of the decarboxylated form of THCA, Δ9-THC, followed by Qrazy Apple (0.29 g 100 g dry matter^{-1}) and Qrazy Train (0.26 g 100 g dry matter^{-1}). In the dried samples, the highest concentration of Δ9-THC for Qrazy Train (0.32 g 100 g dry matter^{-1})

obtained was with pre-freezing at -40˚C and drying at 20˚C, pre-freezing at -20˚C and drying at 10˚C for Qrazy Apple (0.37 g 100 g dry matter^{-1}), and pre-freezing at -40˚C and drying at 10˚C for Qrazy Angel (0.37 g 100 g dry matter^{-1}). Factor effect analyses and Student's t pairwise comparisons showed that Δ9-THC concentrations were not affected by pre-freezing and drying temperature, and they were not significantly ($p < 0.05$) different compared to the reference samples for all accessions. Although significant decarboxylation of THCA to Δ9-THC was not observed in this study, likely due to the low freeze drying temperature used, storage studies have shown that postharvest processing of cannabis inflorescence or extracts at 25˚C or temperatures higher even in a dark room can cause cannabinoid decarboxylation (Meija et al., 2021; Milay et al., 2020).

Major terpene content was similarly determined and compared for inflorescence from the three cannabis accessions subjected to the same postharvest drying conditions (Figure 4.12 and 4.13). A total of 18 terpene compounds were identified from the tested samples. The average total terpene content for fresh, undried inflorescence from accessions Qrazy Train, Qrazy Apple, and Qrazy Angel was 3.1 g 100 g dry matter^{-1}, 2.7 g 100 g dry matter^{-1}, and 4.5 g 100 g dry matter^{-1}, respectively. For the dried samples under the various conditions, the mean terpene concentration ranged from 2 g 100 g dry matter^{-1} to 2.4 g 100 g dry matter^{-1} (Qrazy Train), 1.8 g 100 g dry matter^{-1} to 2.3 g 100 g dry matter^{-1} (Qrazy Apple), and 3.3 g 100 g dry matter^{-1} to 4 g 100 g dry matter^{-1} (Qrazy Angel). The main terpenes for Qrazy Train and Qrazy Apple were myrcene, limonene, caryophyllene, and humulene. These provide accessions with a peppery, citrus, and hoppy mixed aroma (Surendran et al., 2021; Vieira et al., 2018). The racemic mixture of the two major terpenes in Qrazy Angel, α-and β-pinene, maybe responsible for providing this accession with a woody pine, and turpentine-like aroma (Vespermann et al., 2017). ANOVA and Student's t mean pairwise comparison analyses showed that compared to the fresh, undried samples, the different drying conditions did not significantly ($p < 0.05$) influence the concentrations of 78% of the identified terpenes. Compared to the fresh, undried samples, the concentration of camphor was significantly ($p < 0.05$) reduced by 72% in dried Qrazy Train, 77% in dried Qrazy Apple, and 78% in dried Qrazy Angel. Similar observations were made for camphene, fenchol, and caryophyllene.

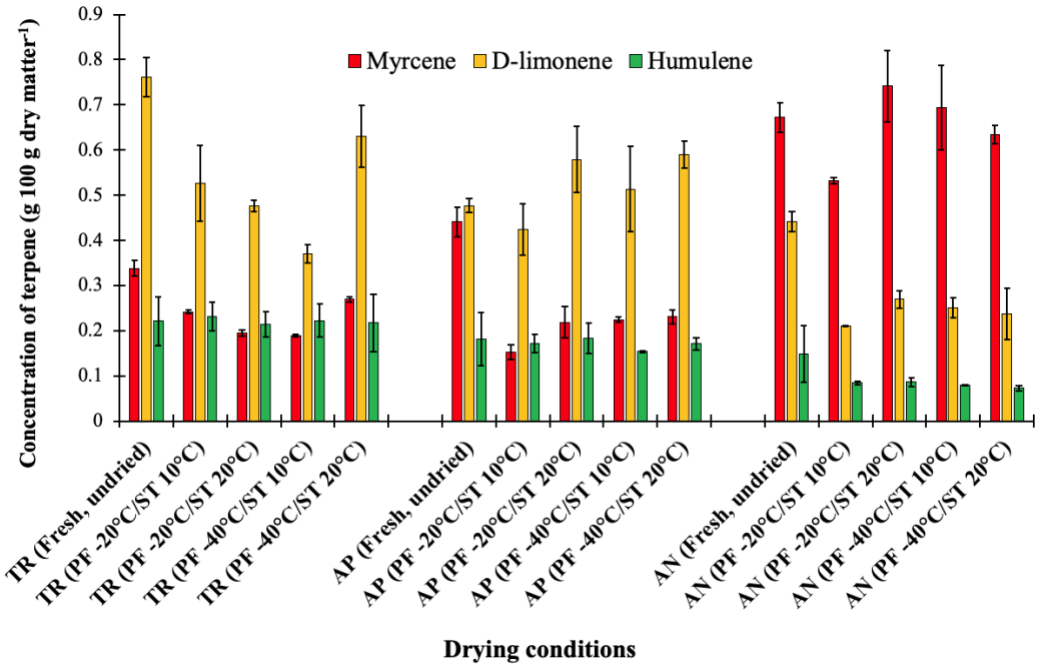

Figure 6.12. Concentration of myrcene, D-limonene, and humulene in Qrazy Train (TR), Qrazy Apple (AP), and Qrazy Angel (AN) submitted to different pre-freezing (PF) and freeze-drying shelf temperatures (ST).

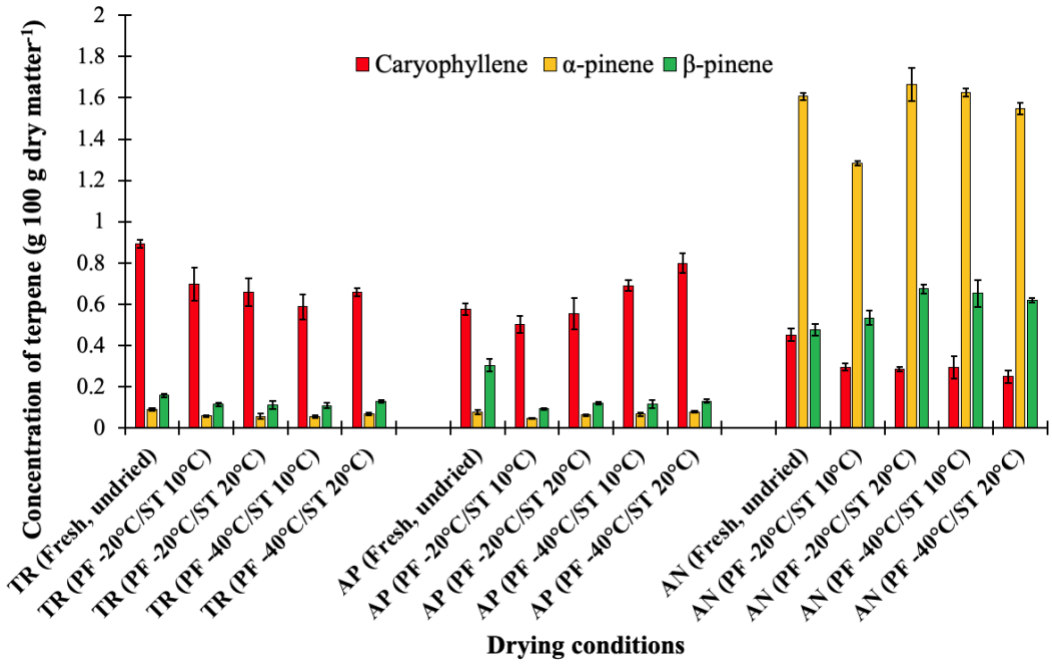

Figure 6.13. Concentration of caryophyllene, and α-and β-pinene in Qrazy Train (TR), Qrazy Apple (AP), and Qrazy Angel (AN) submitted to different pre-freezing (PF) and freeze-drying shelf temperatures (ST).

4.4 Conclusion

This study investigated the relationship between relative humidity and sample mass reduction during freeze-drying and the effects of pre-freezing and freeze-drying temperature on cannabis trichome structure, drying kinetics, color, and the retention of cannabinoids and terpenes. Freezing times for pre-freezing at -40˚C were 51.4 min (Qrazy Train), 59.3 min (Qrazy Apple), and 63.2 min (Qrazy Angel). Total freezing time required for the three cannabis accessions to reach -20˚C when pre-frozen at -20˚C were 190.2 min, 189.1 min, and 196.4 min for Qrazy Train, Qrazy Apple, and Qrazy Angel, respectively. Statistical analyses of the freeze times showed significant differences ($p < 0.05$) between the two freezing temperatures for all accessions. Results showed a strong correlation (0.97 - 0.99) between changes in sample mass and relative humidity during drying and can be explained using the rational regression model. The study showed that relative humidity can be used as an indicator to determine the end of the drying process using a freeze-dryer. However, the relative humidity sensor must be placed near a representative inflorescence in the center of the sample tray. Drying kinetics for scale-up studies can be described using the predictive Page and Logarithmic mathematical models due to the high R^2 ranging from 0.93 to 0.99. ANOVA and Student's t pairwise comparisons showed that the total color changes measured for all cannabis accessions compared to the respective fresh, undried samples were not significantly ($p < 0.05$) different. Terpene analyses showed that mean terpene concentration ranged from 1.8 g 100 g dry matter^{-1} to 4 g 100 g dry matter^{-1}. Fresh, undried *C. sativa* accessions had THCA concentrations ranging from 21.4 g 100 g dry matter^{-1} to 25.8 g 100 g dry matter^{-1}. Freeze drying increased the concentration of CBDA, CBGA, and CBG in all dried samples increased by 73.3% to 87.7%, 23.4% to 42.4%, and 6.9% to 51.9% respectively compared to their respective fresh, undried samples. These findings add new and important industry-relevant knowledge to the growing body of evidence that can support and optimize postharvest processes for this regulated crop by reducing drying time. More research examining the effect of freeze-drying on secondary metabolites should be conducted to further explore cannabinoid and terpene biosynthesis at the molecular level postharvest.

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Connecting text

Based on the findings in Chapters 3 and 4, there was a need in Chapter 5 to compare the efficiency of 2,2-diphenyl-1-picrylhydrazine (DPPH) and ferric reducing ability of power (FRAP) assays to estimate total antioxidant activity (TAC) in hops and cannabis. Chapter 4 showed that pre-freezing and freezing drying increased the concentration of cannabinoids in dried samples. Chapter 5 explored the effects of pre-freezing before drying and drying conditions on antioxidants and antioxidant activity.

Chapter 5 was submitted for publication consideration and is cited as the following:

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Software – Philip Wiredu Addo and Michelle Shearer
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Chapter 5: Correlation between total antioxidant capacity, cannabinoids and terpenoids in hops and cannabis

Abstract

Efficient determination of antioxidant activity in medicinal plants may provide added value to medical extracts. The effects of postharvest pre-freezing and drying [microwave-assisted hot air (MAHD) and freeze drying] on hops and cannabis were evaluated to determine the relationship between antioxidant activity and secondary metabolites. The 2,2-diphenyl-1-picrylhydrazine (DPPH) reduction and ferric reducing ability of power (FRAP) assays were assessed for suitability in estimating antioxidant activity of extracted hops and cannabis inflorescence and correlation with cannabinoid and terpene content. Antioxidant activity in extracts obtained from fresh, undried samples amounted to 3.6 Trolox equivalent antioxidant activity (TEAC) (M) dry matter^{-1} and 2.32 FRAP (M) dry matter^{-1} for hops, in addition to 2.29 TEAC (M) dry matter^{-1} and 0.25 FRAP (M) dry matter^{-1} for cannabis. Pre-freezing significantly increased antioxidant values by 13% (DPPH) and 29.9% (FRAP) for hops, and by 7.7% (DPPH) and 19.4% (FRAP) for cannabis. ANOVA analyses showed a significant ($p < 0.05$) increase in total THC (24.2) and THCA (27.2) concentrations (g 100 g dry matter^{-1}) in pre-frozen, undried samples compared to fresh, undried samples. Freeze-drying and MAHD significantly ($p < 0.05$) reduced antioxidant activity in hops by 79% and 80.2% [DPPH], respectively and 70.1% and 70.4% [FRAP], respectively, when compared to antioxidant activity in extracts obtained from pre-frozen, undried hops. DPPH assay showed that both freeze-drying and MAHD significantly ($p < 0.05$) reduced the antioxidant activity of cannabis by 60.5% compared to the pre-frozen samples although, there was no significant ($p < 0.05$) reduction in the antioxidant activity using the FRAP method. Greater THC content was measured in MAHD-samples when compared to fresh, undried (64.7%) and pre-frozen, undried (57%), likely because of decarboxylation. Both drying systems showed a significant loss in the total terpene concentration yet freeze-drying has a higher metabolite retention compared to MAHD. These results may prove useful for future experiments investigating antioxidant activity and added value to cannabis biomass.

5.1 Introduction

Hops (*Humulus lupulus*) possess unique chemical compounds that contribute greatly to the bitterness, flavour, and aroma of beer. Cannabis (*Cannabis sativa*), is a close relative of hops and is predominately cultivated for its medicinal and psychotropic properties (Ren et al., 2019). Hops and cannabis both belong to the taxonomy family Cannabaceae and thus have related physiological traits (Figure 5.1) and contain similar secondary metabolites, some of which exhibit antioxidant capacity. Plant antioxidants play important roles in acclimation or adaptation of plants to a variety of environmental stressors and are beneficial for human health (Gabriel et al., 2020). As part of a balanced nutritional diet, these antioxidants provide protection against damage caused by free radicals involved in the development of many chronic illnesses such as cancer and cardiovascular diseases (Cuma and Beyza, 2019).

Figure 8.1. Cannabis (A) and hop (B) inflorescences.

Hops contain α-acids (cohumulene, humulone, and adhumulone), β-acids (colupulene, n-lupulone and adlupulone), and xanthohumol, which are the precursors of bittering agents in beer (De Keukeleire et al., 2003). Xanthohumol is the major prenylated flavonoid in hops and it is synthesized in glandular trichomes of hop inflorescence (Liu et al., 2015a). Bitter acids in hops are formed from the acylation of one molecule of acyl-CoA and three molecules of malonyl-CoA to form phlorisovalerophenone (Clark et al., 2013; Tsurumaru et al., 2012). Hops can be used as natural antioxidants as the α-acids, β-acids, and xanthohumol present in hops have significant

hydroxyl radical scavenging and antioxidant activities (Kontek et al., 2021; Liu et al., 2015a; Liu et al., 2018).

Major active secondary compounds found in the cannabis plant are the cannabinoids, a group of chemical compounds that alter neurotransmission activity of the brain by acting on the cannabinoid receptors (Grafström et al., 2019; Howlett, 2002; Luo et al., 2020; Moreno et al., 2020a; Ranganathan and D'Souza, 2006). Research studies have shown that cannabinoids exhibit antioxidant properties (Atalay et al., 2019; Dawidowicz et al., 2021; Kopustinskiene et al., 2022). Cannabinoids, like other antioxidants, interrupt free radical chain reactions, chelating free radicals by donating their electrons or hydrogen atom and transforming them into less active forms (Haida and Hakiman, 2019). Dawidowicz et. al. (2021) showed that the degree of antioxidant activity by acidic and neutral cannabinoids can be attributed to the number of phenolic hydroxyl groups in individual cannabinoids. Cannabinolic acid (CBDA) (13.3%) and cannabidiol (CBD) (53.3%) showed significantly ($p < 0.05$) greater scavenging power compared to tetrahydrocannabinolic acid (THCA) and tetrahydrocannabinol (THC), respectively. Hops cannot synthesize cannabinoids as they lack the oxidocyclase enzymes to convert cannabigerolic acid (CBGA) to the various cannabinoids (Tahir et al., 2021).

Other antioxidant compounds of interest produced by hops and cannabis are terpenes and phenols (André et al., 2020). Terpenes, or isoprenoids, are one of the largest and most diverse group in plants (Surendran et al., 2021). Although terpenes and volatile phenols are mostly responsible for its characteristic aroma, they possess beneficial health benefits such as anticancer, antimicrobial, antifungal, antiviral, analgesic, anti-inflammatory, and antiparasitic activities (Ojeda-Sana et al., 2013; Rufino et al., 2015; Schieber and Wüst, 2020). *In vitro* studies by Rufino et al. (2015) showed that myrcene, one of the most abundant terpenes in hops and cannabis, has significant anti-inflammatory and anti-catabolic properties, is useful for halting or slowing down cartilage destruction and osteoarthritis progression. Phenolic compounds including terpenes are reportedly powerful antioxidants with high scavenging properties (Pandey and Rizvi, 2009).

Plant secondary metabolite biosynthesis and antioxidant activity can be disrupted and altered during postharvest storage and drying (ElSohly et al., 2017; Taschwer and Schmid, 2015). Storage studies by Grafström et al. (2019) over four years showed that CBD is not prone to oxidative degradation and is stable over time, while decarboxylation of THCA to THC which

occurs in stored plant material is increased by the presence of oxygen and higher temperatures (Das et al., 2022a; Wang et al., 2016). Specifically, THC concentrations markedly increase from 1.5% to 2.1%, 12.3% and 12.8% when stored at 50°C, 100°C, and 150°C, respectively, due to THCA decarboxylation (Taschwer and Schmid, 2015). Storing hop buds at 20°C in a dark room showed decreased α-acid concentrations from 186.9 μmol g^{-1} to 37.0 μmol g^{-1} and β-acids from 107.7 μmol g^{-1} to 50.9 μmol g^{-1}. Both α-acids and β-acids are oxidized rapidly during hop storage (Taniguchi et al., 2013). Decreases in α-acids and β-acids can decrease the antioxidant capacity of hops.

The effects of pre-freezing and drying on hops terpene content has been reported and the optimal conditions for freeze-drying and microwave assisted hot air drying (MAHD) were explored (Addo et al., 2022c). This previous study showed that the low temperature used during freeze-drying preserved 16.6% to 68.3% of the major terpenes present in hops compared to hot air and MAHD systems, respectively. Pre-freezing caused significant structural damages to hops and this was similarly observed for cannabis in a related trial (Addo et al., 2022c). In this follow-up study, the effects of pre-freezing, prior to drying hops and cannabis, on antioxidant capacity were examined using optimal drying conditions. The suitability and efficiency of 2,2-diphenyl-1-picrylhydrazine (DPPH) and ferric reducing ability of power (FRAP) assays to estimate total antioxidant activity (TAC) in hops and cannabis extracts from biomass subjected to these postharvest methods were compared. Given the legislative focus on documenting scientific literature that scrutinizes the therapeutical potential of cannabis for medical use, the relationship between antioxidant capacity and valued secondary metabolites in these two crops was examined.

5.2 Materials and methods

5.2.1 Sample preparation

Hops (Brewer's gold) were cultivated outdoors at McGill University's Macdonald Campus farm in Sainte-Anne-de-Bellevue, QC, Canada. Hops were planted on May 3, 2022 and harvested from mid-September to the end of October 2022. Preliminary tests were conducted using a split plot design to limit the differences between the hops harvested from the different plots. Cannabis inflorescence was harvested from an indoor-grown accession (Qrazy Train). Harvested hops and cannabis biomass was pre-frozen at -20°C for a minimum of 24 h prior to

drying and analysis as described previously (Addo et al., 2022c). The initial moisture content of the hops and cannabis inflorescence was determined using a hot air oven (Fisher Scientific 6903 Isotemp oven, Waltham, Massachusetts, US). Each sample was dried at 50°C for 24 h.

5.2.2 Freeze drying of hops and cannabis

Optimal freeze-drying conditions for cannabis and hops biomass identified previously were applied to this experiment (Addo et al., 2022c). For each condition, approximately 100 g pre-frozen cannabis and hops inflorescence samples were placed in plastic trays and transferred to a laboratory-scale vacuum freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH Gamma 1-16 LSCplus, Osterode, Lower Saxony, Germany) with a condenser temperature of -55°C. Freeze-drying was carried out at 20°C for 24 h at 0.85 mbar until the sample reached a dry basis moisture content of 12%. Dried samples were transferred into a food-grade plastic bag and stored in a refrigerator at 5°C before analyses. Each experiment was performed in triplicate using three different biomass samples.

5.2.3 Microwave-assisted hot air drying of hops and cannabis (MAHD)

Optimal MAHD conditions for cannabis and hops biomass identified previously were applied to this experiment (Addo et al., 2022c). MAHD was conducted in an automated laboratory-scale microwave oven with several modifications. Briefly, the main components were a 2,450-MHz microwave generator (Gold Star 2M214, Seoul, South Korea) with adjustable power (0 to 750 W), waveguides, a three-port circulator, a manual three-stub tuner to match the load impedance, microwave couplers to measure forward and reflected power, a carbon load to absorb reflected power, and a microwave cavity made of brass (0.47 × 0.47 × 0.27 m) in which the samples were processed. In each experiment, approximately 100 g pre-frozen hops and cannabis inflorescence were placed in a nylon mesh sample holder tray (diameter = 0.21 m). The plant material was spread in one layer and placed inside the microwave cavity. Drying was performed until the sample reached a dry basis moisture content of 12%. Dried samples were transferred into a plastic bag and stored in a refrigerator at 5°C before analyses. Drying was performed in triplicate under each condition.

5.2.4 Extraction of secondary metabolites

Representative samples for each of the drying conditions and fresh samples were immersed in liquid nitrogen before grinding with a coffee grinder (Hamilton Beach, Belleville, ON, Canada). Ground samples were allowed to equilibrate to room temperature before 0.75 g was weighed in a 50 mL Falcon tube and recorded. Each sample was allowed to sit for 10 min on the scale (Mettler AE50 analytical balance, Columbus, Ohio, United States of America) until there was < 1 mg change in mass. This is done to ensure that most of the liquid nitrogen had evaporated from the sample and the proper sample mass was obtained. For the extraction of secondary metabolites, 20 mL high-pressure liquid chromatography (HPLC)-grade methanol (Thermo Fisher Scientific, Waltham, Massachusetts, US) was added to each Falcon tube and vortexed (Thermo Scientific vortex, Waltham, Massachusetts, US) for 20 min at 500 rpm. Each sample was filtered using Whatman™ filter paper (Thermo Fisher Scientific, Waltham, Massachusetts, US) and allowed to filter for 20 min. Residual cannabis biomass was placed into a new 50 mL Falcon tubes and subjected to a second extraction process to ensure 99.5% of the secondary metabolites were extracted. The second extract was added to the corresponding first extract, resulting in a 40x dilution total extract.

5.2.5 Measuring of antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

Antioxidant activities of hops and cannabis were determined using the DPPH assay introduced by Brand-Williams et al. (1995) and used by Dawidowicz et al. (2021) for cannabis, with some modifications. A calibration curve was generated using different serial dilutions of a 10 mM Trolox® standard (Sigma-Aldrich, Saint Louis, Missouri, US) in HPLC-grade methanol (Thermo Fisher Scientific, Waltham, Massachusetts, US). A stock solution of 0.1 mM DPPH ion (Sigma-Aldrich, Saint Louis, Missouri, US) in HPLC-grade methanol was prepared fresh daily. Aliquots (100 µL) of extracted samples or standards were placed in 15-mL Falcon tubes and 2900 µL of DPPH ion stock solution was added. The mixture was subjected to vigorous vortexing (Thermo Scientific vortex, Waltham, Massachusetts, US) for 30 sec then incubated for 30 min at room temperature in the dark. Absorbances were measured at 517 nm using the Ultropec 2100 pro ultraviolet/visible spectrophotometer (Biochrom Limited, Cambridge, England). A DPPH ion solution was used as a control and HPLC-grade methanol was used to zero the spectrophotometer. The average radical scavenging activity of the samples was

calculated and the DPPH inhibition (%) was calculated using Equation 1. Concentration (M) of Trolox equivalent antioxidant activity (TEAC) using the calibration curve was calculated using Equation 2. Results are reported as the concentration (M) of Trolox equivalent antioxidant activity (TEAC) per gram dry matter sample using Equation 3.

$$\%\ DPPH\ inhibition = \frac{Absorbance_{control} - Absorbance_{sample}}{Absorbance_{control}} \quad (1)$$

$$TEAC\ (M) = \frac{\left(\frac{\%\ DPPH\ inhibition - 8.7009}{36.361}\right)}{1000} \quad (2)$$

$$TEAC\ (M)\ dry\ matter\ (g)^{-1} = \frac{Extraction\ volume\ (0.04\ L) * TEAC\ (M)}{Analysis\ volume\ (0.0001\ L) * (sample\ mass - (\%\ mc * sample\ mass))} \quad (3)$$

The experiment was carried out in triplicate.

5.2.6 Measuring of antioxidant activity using ferric reducing antioxidant power (FRAP) assay

The antioxidant capacity of hops and cannabis was additionally determined using the ferric reducing antioxidant power (FRAP) assay based on methods developed by Benzie and Strain (1996) and Dawidowicz et al. (2021) for cannabis, with some modifications. The standard curve was prepared using different serial dilution concentrations (10 – 0.004 mM) of Trolox (Sigma-Aldrich, Saint Louis, Missouri, US). The FRAP reagent was prepared from 300 mM sodium acetate buffer (pH 3.6), 20 mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) (Sigma-Aldrich, Saint Louis, Missouri, US) solution in 40 M hydrochloric acid (Thermo Fisher Scientific, Waltham, Massachusetts, US) and 20 mM ferric chloride ($FeCl_3$) (Sigma-Aldrich, Saint Louis, Missouri, US) solution in proportions of 10:1:1 (v/v), respectively. The FRAP solution was prepared fresh daily and warmed to 37°C in a water bath for 10 min prior to use. Aliquot (100 µL) of extracted samples or standards was placed in 15-mL Falcon tubes and 2900 µL FRAP stock solution was added. The mixture after vigorous vortex (Thermo Scientific vortex, Waltham, Massachusetts, US) for 30 sec was incubated for 60 min at room temperature and in darkness. Absorbances were measured at 593 nm using the Ultropec 2100 pro ultraviolet/visible spectrophotometer (Biochrom Limited, Cambridge, England). The FRAP solution was used as a control and HPLC-grade methanol was used to zero the spectrophotometer. The experiment was carried out in triplicate. FRAP inhibition was calculated using Equation 4. The FRAP value (antioxidant activity) was calculated using the calibration curve and Equation 5. Results are reported as FRAP value (M) per gram dry matter sample using Equation 6.

$$FRAP\ inhibition\ (AU) = Absorbance_{sample} - Absorbance_{control} \quad (4)$$

$$FRAP\ value\ (M) = \frac{\left(\frac{FRAP\ inhibition - 0.1263}{1.2228}\right)}{1000} \quad (5)$$

$$FRAP\ value\ (M)\ dry\ matter\ (g)^{-1} = \frac{Extraction\ volume\ (0.04\ L) * FRAP\ value\ (M)}{Analysis\ volume\ (0.0001\ L) * (sample\ mass - (\%\ mc * sample\ mass))} \quad (6)$$

5.2.7 Cannabinoid analyses

Waters Acquity Ultra High-Performance Liquid Chromatography (UPLC) with a tunable ultraviolet (TUV) detector (Waters™, Mississauga, Ontario, Canada) was used for cannabinoid analyses. Each extract was further diluted 50x (for analysis of major cannabinoids) and 4x (for analysis of minor cannabinoids and terpenes) using HPLC-grade methanol (Thermo Fisher Scientific, Waltham, Massachusetts, US). One-milliliter samples of each extract were pipetted into HPLC vials for cannabinoid analysis. The Waters cortex column was used to separate cannabinoids with a sample injection volume of 2 µL and a column temperature of 30°C, equipped with an isocratic gradient pump. Mobile phase A consisted of 22% reverse osmosis water and 0.1% formic acid (Sigma-Aldrich, Saint Louis, Missouri, US). HPLC-grade acetonitrile (78%) (Thermo Fisher Scientific, Waltham, Massachusetts, US) was used for mobile phase B. Quantification of the cannabinoids was done using an external calibration curve developed using 7 standard cannabinoids (LGC standards, Manchester, New Hampshire, US and Sigma Aldrich, Saint Louis, Missouri, US).

5.2.8 Terpene analysis

Terpene analysis assay previously described by Addo et al. (2022) was used for this study. Gas chromatography-tandem mass spectrometer was used for terpene analyses. One-milliliter samples of each extract were pipetted into gas chromatograph (GC) vials for terpene analysis. Separation of the terpenes was performed with an Agilent 7820A GC coupled to an Agilent 7693 autosampler and a flame ionization detector (FID) (Agilent Technologies, Mississauga, Ontario, Canada). The system was equipped with an injector containing a capillary column (30 m x 250 µm x 0.25 µm nominal Agilent Technologies DB-5 Model) using split injection (ratio 50:1) with a hydrogen carrier gas (40 mL min^{-1}). An injection volume of 5 µL each sample with a 10-µL syringe size was used. The oven temperature of the mass spectrometer

was initially programmed at 35°C and held for 4 min. The temperature was increased at a rate of 10°C min^{-1} up to 105°C held for 0 min, increased at a rate of 15°C min^{-1} up to 205°C held for 0 min, and lastly increased at a rate of 35°C min^{-1} up to 270°C held for 5 min. The inlet temperature into the FID detector was set at 340°C. Spectra were recorded at three scans from 50 m z^{-1} to 400 m z^{-1}. The ionization mode was used with an electronic impact at 70 eV. Quantification of the terpenes was done using an external calibration of 37 terpenes mostly found in cannabis (LGC standards, Manchester, New Hampshire, US and Sigma Aldrich, Saint Louis, Missouri, US).

5.2.9 Statistical analysis

Statistical analyses were conducted using the JMP software (JMP 4.3 SAS Institute Inc., Cary, North Carolina, US) with a confidence level ($p < 0.05$) of 95%. Pairwise comparisons of means were done using the Student's t statistical test.

5.3 Results and discussion

5.3.1 Ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) calibration curves

This study aimed to compare the suitability and efficiency of the DPPH and FRAP assays when measuring total antioxidant activity in hops and cannabis extracts procured from differently processed biomass, including a pre-freezing step followed by freeze-drying or MAHD. The DPPH and FRAP colorimetric assays are universal tools that are currently used for assessing nonenzymatic antioxidants present in plants (Pisoschi et al., 2009; Sochor et al., 2010). The DPPH assay measures the radical scavenging activity of most phenolic compounds such as flavonoids and tannins (Brand-Williams et al., 1995; Dawidowicz et al., 2021). The FRAP assay is a measure of the transition metal ion chelating activity of antioxidants such as ascorbic acid, uric acid and polyphenolic compounds such as catechins under acidic conditions (Benzie and Strain, 1996; Dawidowicz et al., 2021). Bleaching of the DPPH solution from violet to pale yellow increases with an increase of antioxidant activity in each sample (Figure 5.2A). This assay is based on the reduction of the free radical DPPH to DPPH-H. The FRAP assay uses the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) as the signal and measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe^{2+}-tripyridyltriazine compound

from the colorless oxidized Fe^{3+} form by the action of electron-donating antioxidants (Figure 5.2B).

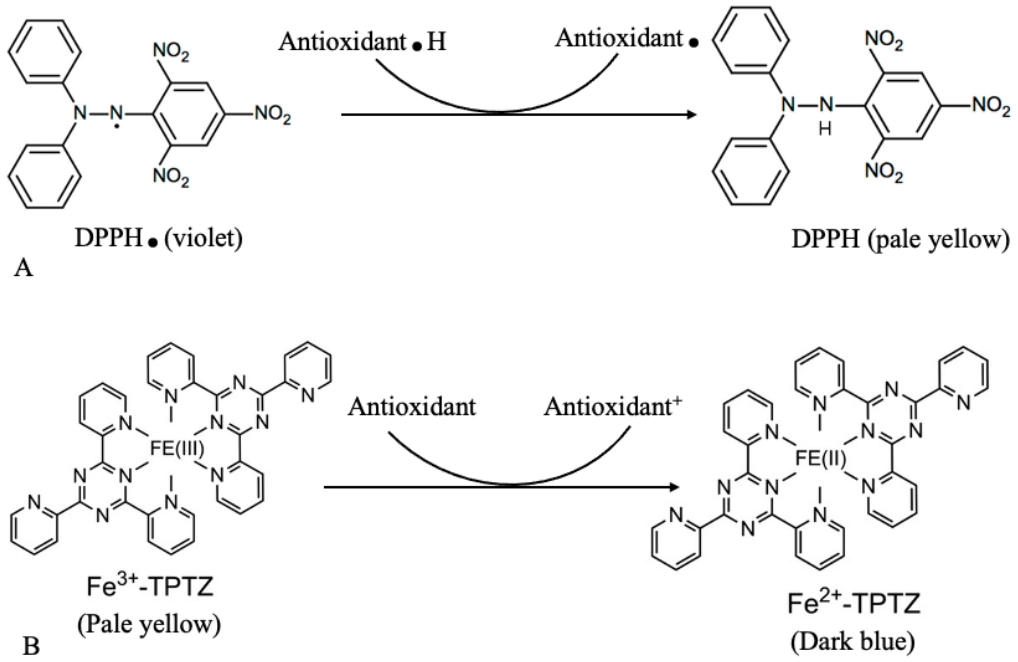

Figure 8.2. Reaction underlying the (A) DPPH and (B) FRAP antioxidant assays.

The percentage of radical scavenging capacity for different Trolox concentrations used for DPPH and FRAP assay calibration curves is shown in Figure 5.3. The results showed inhibition values using 0.005 mM to 10 mM and 0.005 to 2.5 mM Trolox concentrations, respectively. Data exhibit a flattening of the graph between 2.5 to 10 mM Trolox concentration. This can be attributed to the almost complete quenching of DPPH and FRAP by Trolox, which does not affect the absorbance values. As such, sample dilution is necessary to dilute samples to within the measurable range. A similar curve flattening observation was made by Sochor et al. (2010) and Pisoschi et al. (2009) where the absorbance of Trolox did not change at concentrations of 200 – 1000 μmol L^{-1} and 0.15 – 0.2 mM, respectively. Calibration graphs (Figure 5.3) used to quantify the antioxidant capacities of hops and cannabis in this study are linear, in the range 0.005 to 2.5 mM for Trolox, with strong correlation coefficients (R^2) of 0.996 and 0.982 for DPPH and FRAP, respectively.

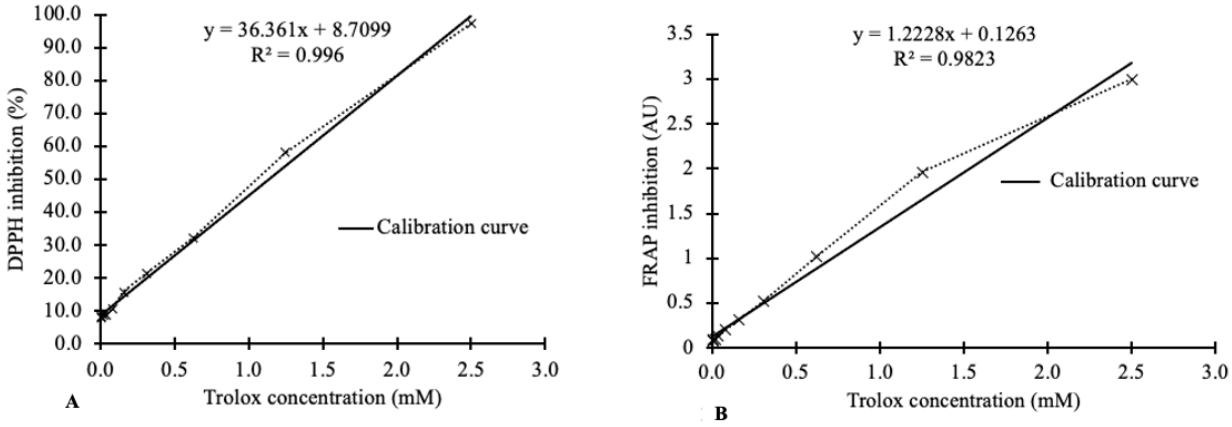

Figure 8.3. Calibration curves used for DPPH and FRAP assays in the presence of different Trolox concentrations.

5.3.2 Antioxidant activity of hops and cannabis

Antioxidants may be hydrophobic (lipid-soluble) and hydrophilic (water-soluble) substances; however, plant-based antioxidants are mostly hydrophilic (Haida and Hakiman, 2019; Neri et al., 2020). The observed TEAC and FRAP values determined in hops and cannabis are presented in Figure 5.4. Bars with the same letter in the graph are not significantly ($p < 0.05$) different (Figure 5.4). The antioxidant activity of extracts derived from fresh, untreated hops was 3.6 TEAC (M) dry matter^{-1} and 2.32 FRAP (M) dry matter^{-1}. Extracts from fresh, untreated cannabis samples had 2.29 TEAC (M) dry matter^{-1} and 0.25 FRAP (M) dry matter^{-1} antioxidant values. The lower antioxidant activity observed in cannabis relative to hops can be attributed to the presence of α-acids and β-acids in hops (De Keukeleire et al., 2003). Analysis of variance tests showed that pre-freezing, freeze-drying and MAHD significantly affected ($p < 0.05$) the antioxidant activity of hops and cannabis when evaluated with the DPPH and FRAP assays.

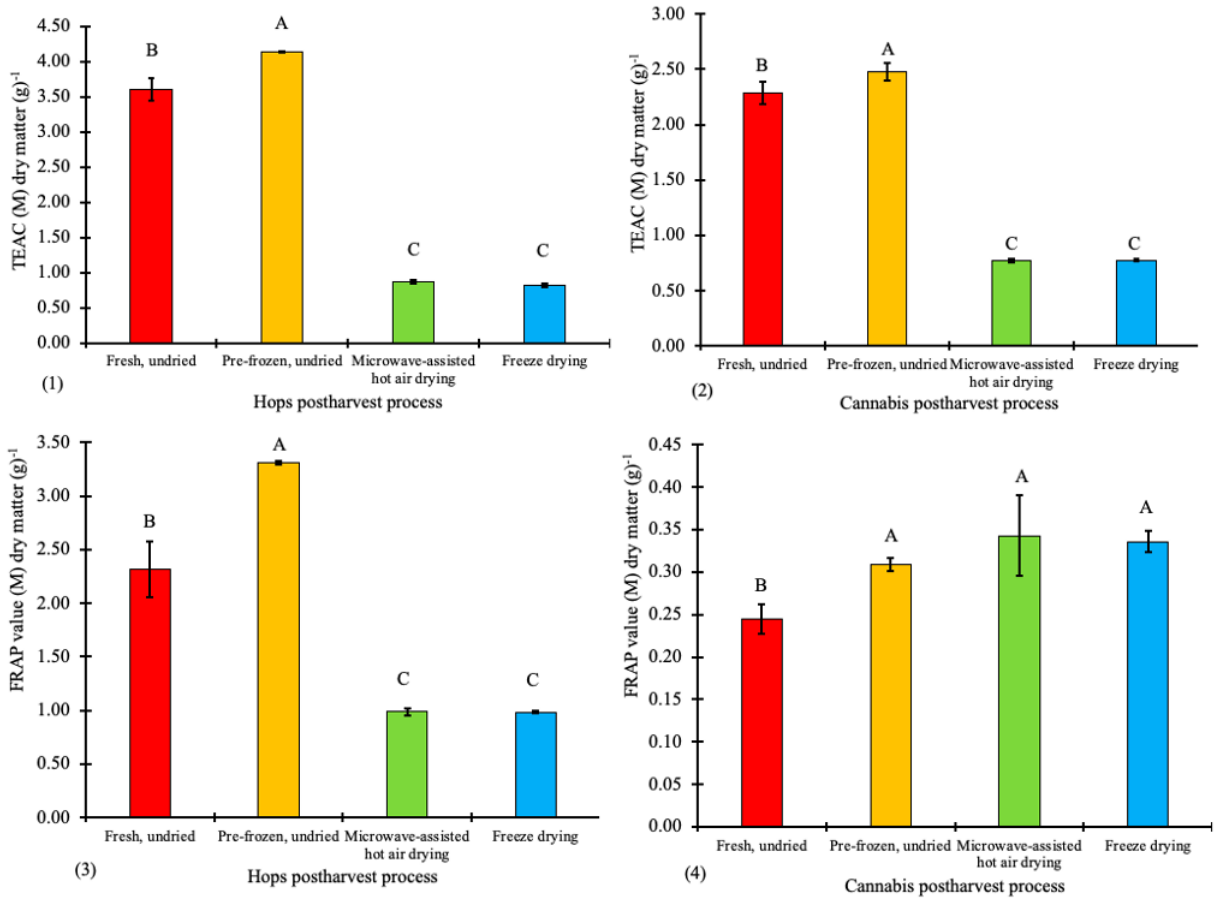

Figure 8.4. Total antioxidant activity in extracts from hops and cannabis using the DPPH (1 and 2) and FRAP (3 and 4) assays.

Pre-freezing the hops and cannabis samples before drying increased the antioxidant values by 13% (DPPH assay) and 29.9% (FRAP assay) for hops, and by 7.7% (DPPH assay) and 19.4% (FRAP assay) for cannabis (Figure 5.4). Increased antioxidant activity in the pre-frozen samples can be attributed to the structural damage caused by the ice crystal formation (Addo et al., 2022c). In a preceding study, scanning electron microscopy analyses of cannabis samples showed that the cold temperature used during pre-freezing and the consequent ice crystal formation caused wrinkling of cannabis trichome stalks and cannabis trichome heads to fall off. Other research has shown that pre-freezing exerts positive effects on the organoleptic quality and functional properties of plant material since the frozen state allows the release of bioactive compounds as bound phenolic acids and anthocyanins, resulting in increased antioxidant activity (Leong and Oey, 2012; Mullen et al., 2002). Leong and Oey (2012) showed that pre-freezing

apricots (*Prunus armeniaca*) at -20°C increased the concentration of vitamin C and β-carotene by 55.5% and 10.7%, respectively.

DPPH assays used for this study show that freeze-drying and MAHD significantly ($p < 0.05$) reduced the antioxidant activity in hops by 79% and 80.2%, respectively, compared to pre-frozen, undried samples. A similar observation was made for hops using the FRAP assay, as antioxidant activity was reduced by 70.1% and 70.4% under freeze-drying and microwave-assisted hot air drying, respectively, when compared to pre-frozen, undried hops. Both freeze-drying and MAHD significantly ($p < 0.05$) reduced antioxidant activity of cannabis by 60.5% using the DPPH assay. However, there was no significant ($p < 0.05$) difference between the antioxidant activity values for pre-frozen, freeze-dried and microwave-assisted hot air dried cannabis samples using the FRAP method. This difference can be attributed to the presence of iron-chelating compounds such as cannabinoids in the cannabis extract samples. Cannabinoids can interfere with the FRAP assay by chelating the Fe^{3+} irons in the FRAP reagent mixture. Dawidowicz et al. (2021) showed that cannabinoids are antioxidant agents as they can scavenge free radicals, and the antioxidant activity of THC was greater by 35.3% with the FRAP method when compared to DPPH assay. Given these data, the FRAP assay is recommended for determining antioxidant activity in cannabis and hop inflorescence. Further studies using other antioxidant activity assays such as oxygen radical absorbance capacity (ORAC) and determining presence of antioxidants in different cannabis and hop plant organs could be explored.

5.3.3 Cannabinoid and terpenes in hops and cannabis

For a comparison of different postharvest treatments and valued phytochemicals in extracted hops and cannabis extra inflorescence, total THC content and major cannabinoid concentrations (tetrahydrocannabinolic acid [THCA], tetrahydrocannabinol [Δ9-THC], tetrahydrocannabivarin [THCV], cannabigerolic acid [CBGA], and cannabigerol [CBG]) in *C. sativa* were determined (Figure 5.5). Bars with the same letter in the graph are not significantly ($p < 0.05$) different (Figure 5.5). In the same figure, cannabinoid and terpene content in extracts obtained from fresh, undried cannabis samples were compared to cannabinoids and terpene content in extracts obtained in this study. CBDA, CBD, and total CBD content are not presented as the concentration of CBDA and CBD was below the limit of detection of the instrumentation and methodology. Extracts from fresh, undried *Cannabis sativa* had total THC, THCA, THC,

and CBG concentrations of 20.5 g 100 g dry matter^{-1}, 23.1 g 100 g dry matter^{-1}, 0.27 g 100 g dry matter^{-1}, and 0.16 g 100 g dry matter^{-1}, respectively. ANOVA analyses showed a significant ($p < 0.05$) increase in the total THC (24.2 g 100 g dry matter^{-1}) and THCA (27.2 g 100 g dry matter^{-1}) concentrations in extracts obtained from pre-frozen, undried samples compared to fresh, undried samples. However, there was no significant ($p < 0.05$) increase in THC (0.32 g 100 g dry matter^{-1}) and CBG (0.22 g 100 g dry matter^{-1}) concentrations in extracts from the pre-frozen, undried samples compared to the fresh, undried samples. The increase in total THC and THCA concentrations can be attributed to the pre-freezing step. Pre-freezing causes structural damage to trichome structures and can be considered as an abiotic stressor (Addo et al., 2022c; Ahmed et al., 2013). Ahmed et al., (2013) reported that abiotic stresses increased total phenolic compounds (TPC) by 62.5% in barley (*Hordeum vulgare*) compared to the control upon harvest. It is plausible that the structural damage incurred by trichomes during pre-freezing step helps release trapped secondary metabolites.

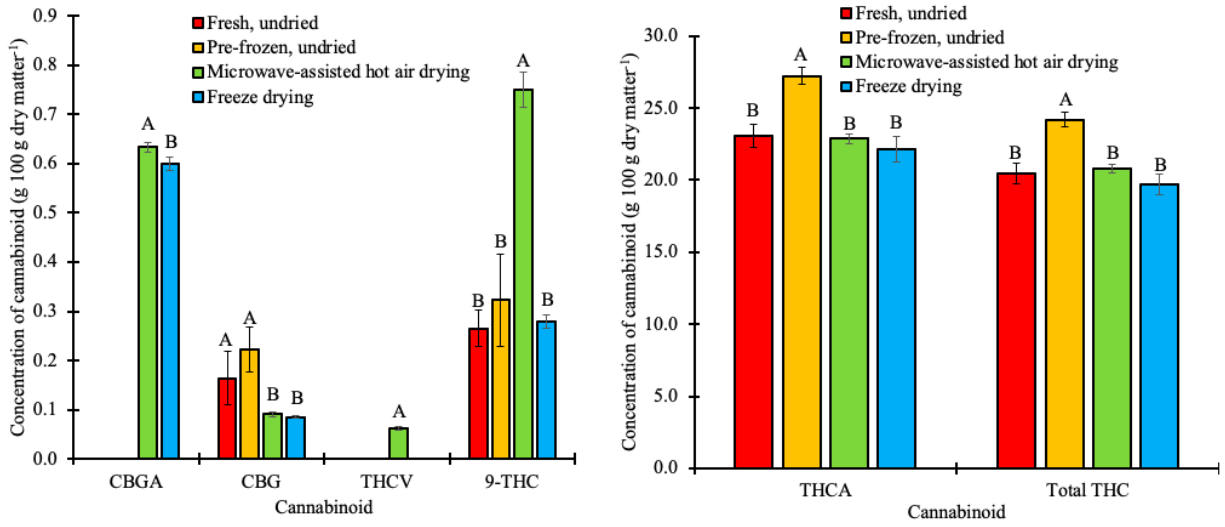

Figure 8.5. Concentration of cannabinoids in pre-frozen, undried, microwave-assisted hot air dried, and freeze dried cannabis.

The concentration of CBGA measured herein was below the limit of detection of the instrumentation and methodology in the extracts of fresh and pre-frozen, undried samples, likely because CBGA serves as the precursory molecule to the other cannabinoids (Nachnani et al., 2021). Various bioengineering studies have demonstrated that the prenylation of olivetolic acid

(OA) by geranyl diphosphate (GPP) to form a cannabigerolic acid is an anabolic process (Blatt-Janmaat and Qu, 2021; Luo et al., 2020; Thomas et al., 2020). Hence, the observed increase in the average concentration of CBGA to 0.63 g 100 g dry matter^{-1} (MAHD-dried samples) and 0.6 g 100 g dry matter^{-1} (freeze-dried samples) can be attributed to the high drying temperatures used.

Recent published reviews of the cannabis post-harvest processing literature (Addo et al., 2021; Das et al., 2022a) indicate that with the application of heat, THCA and THCVA change into their active forms of THC and THCV, respectively. Compared to the fresh and pre-frozen, undried samples, extracts from MAHD biomass had significantly ($p < 0.05$) greater THC content by 64.7% and 57%, respectively. ANOVA analyses show that the change in THCA and THC in freeze-dried samples compared to the fresh and pre-frozen, undried samples was not significant ($p < 0.05$). Hence, freeze-drying can be used to preserve the secondary metabolites present in cannabis and these data support previous findings (Addo et al., 2022c). These findings are comparable to other crops preserved in this manner (Ferenezi et al., 2018; Silva-Espinoza et al., 2020). Significant ($p < 0.05$) increase in the THC in MHAD-dried samples compared to fresh, undried (64.7%) and pre-frozen, undried (57%) can be explained by the non-enzymatic decarboxylation process. Moreno et al. (2020) showed that the non-enzymatic decarboxylation of acidic cannabinoids to neutral cannabinoids increase with the increase in temperature. Using a decarboxylation time of 60 min, the concentration of THC increased from 0.02 g 100 g dry matter^{-1} (80°C) to 0.03 mg g dry matter^{-1} (120°C).

A total of 16 and 7 terpene compounds were identified in the cannabis and hops samples, respectively (Figures 5.6 and 5.7). Bars with the same letter in the graph are not significantly ($p < 0.05$) different (Figure 5.6 and 5.7). All 7 terpene compounds identified in hops were present in cannabis at different concentrations. Despite the major differences in secondary compounds in cannabis and hops used for the study, the main terpenes were myrcene, caryophyllene, and humulene. These provide inflorescence with a peppery, citrus, and hoppy mixed aroma (Surendran et al., 2021; Vieira et al., 2018). The caryophyllene concentration in cannabis was 71.2% greater than that of hops. However, humulene had a higher concentration (54.8%) in hops compared to cannabis. Data represented in Figures 5.6 and 5.7 indicate that the concentration of myrcene in fresh, undried hops was reduced from 1.9 to 0.3 g 100 g dry matter^{-1} (MAHD) and to 0.7g 100 g dry matter^{-1} (freeze-dried) and for fresh, undried cannabis, the concentration reduced

from 0.3 to 0.1 g 100 g dry matter^{-1} (MAHD) and to 0.2 g 100 g dry matter^{-1} (freeze-dried). Rajkumar et al. (2017) showed that compared to fresh, undried carrots, myrcene was reduced from 2.3 to 0.4 g 100 g dry matter^{-1} (MAHD) and to 1.6 g 100 g dry matter^{-1} (FD). This shows that freeze-drying has a higher terpene retention compared to MAHD for these crops.

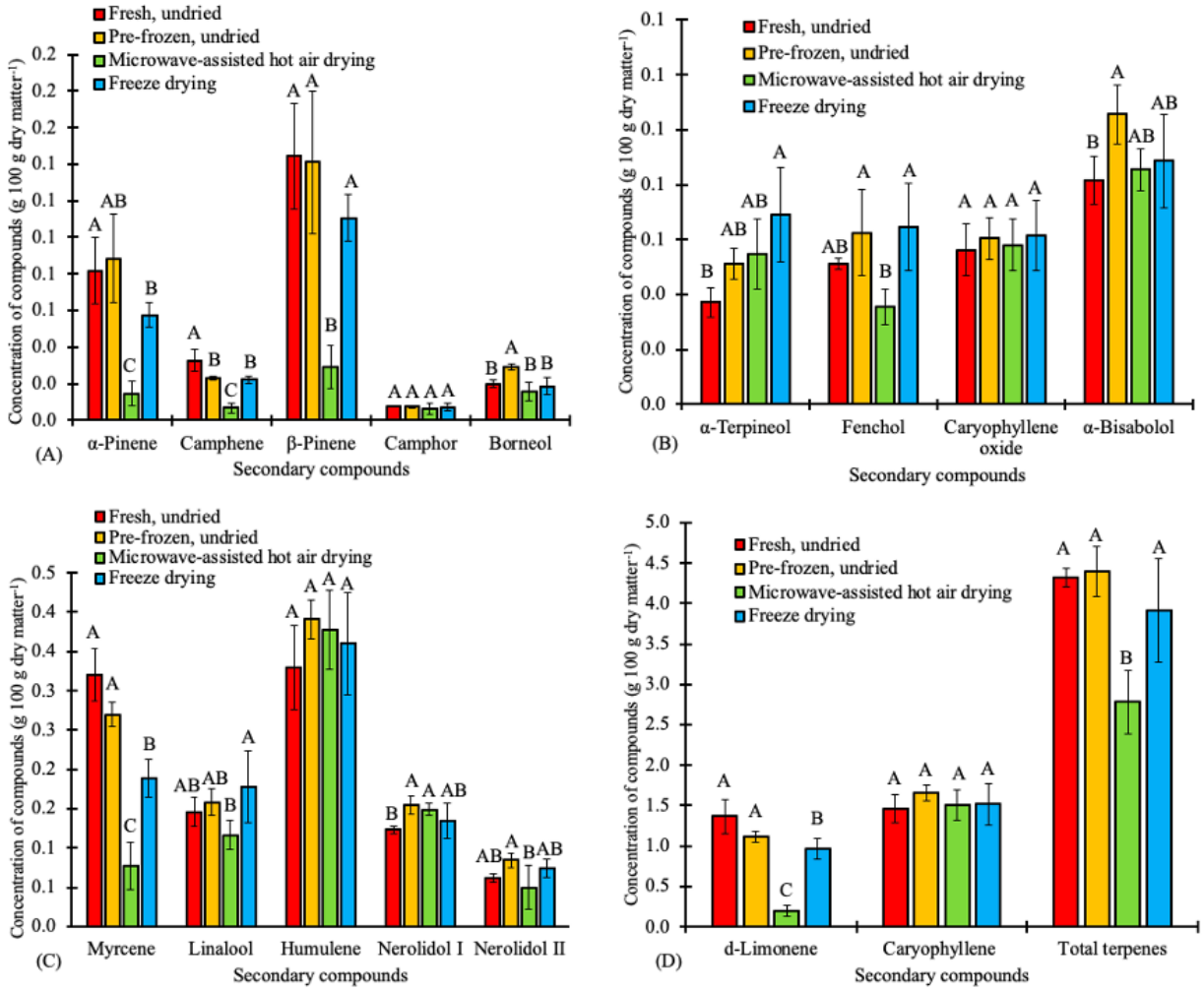

Figure 8.6. Concentration of terpenes in cannabis in fresh, undried, pre-frozen, undried, microwave-assisted hot air dried, and freeze-dried cannabis.

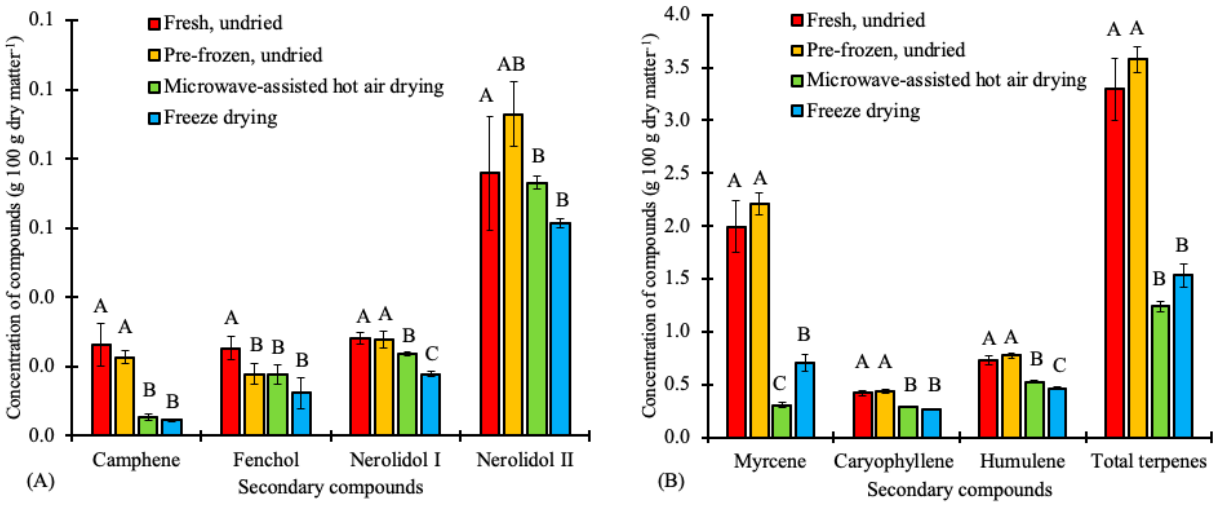

Figure 8.7. Concentration of terpenes in hops in fresh, undried, pre-frozen, undried, microwave-assisted hot air dried, and freeze-dried hops.

Major terpene content was similarly determined and compared for cannabis and hops subjected to the same postharvest drying conditions (Figures 5.6 and 5.7). The average total terpene content from fresh, undried cannabis and hop samples was 4.3 g 100 g dry matter^{-1} and 3.3 g 100 g dry matter^{-1}, respectively. ANOVA analyses showed that the increase in the total terpene content to 4.4 g 100 g dry matter^{-1} and 3.6 g 100 g dry matter^{-1} for cannabis and hops, respectively by pre-freezing was not significant ($p < 0.05$). For freeze-dried and microwave-assisted hot air dried hop samples (Figure 5.7), the average total terpene significantly ($p < 0.05$) reduced to 1.5 g 100 g dry matter^{-1} and 1.2 g 100 g dry matter^{-1} respectively. However, freeze drying preserved the total terpenes (3.9 g 100 g dry matter^{-1}) in cannabis samples compared to microwave-assisted hot air drying (2.8 g 100 g dry matter^{-1}) (Figure 5.6). The high temperature used during MHAD significantly ($p < 0.05$) reduced total terpene content in the fresh, undried samples from 4.3 to 2.8 g 100 g dry matter^{-1}. Terpenes evaporate easily in MAHD since the cannabis and hop structures and dimensions permit its evaporation even at 35°C, while freeze-drying uses a relatively very low temperature which limits the evaporation of terpenes (Rajkumar et al., 2017). Hence, freeze-drying, rather than hot-air drying, is recommended to help preserve terpenes in hops and cannabis during postharvest processing.

5.4 Conclusion

The effects of postharvest processing on hops and cannabis were evaluated to determine the relationship between antioxidant capacity and secondary metabolites. The study compared the efficiency of DPPH and FRAP assays to estimate total antioxidant activity in hops and cannabis extracts. The antioxidant activity of extracts derived from fresh, untreated samples were 3.6 TEAC (M) dry matter^{-1} and 2.32 FRAP (M) dry matter^{-1} for hops and 2.29 TEAC (M) dry matter^{-1} and 0.25 FRAP (M) dry matter^{-1} for cannabis. The results showed that although freezing of inflorescences is a preservation technique, pre-freezing the hops and cannabis samples before drying increased the antioxidant values by 13% (DPPH assay) and 29.9% (FRAP assay) for hops, and by 7.7% (DPPH assay) and 19.4% (FRAP assay) for cannabis. Data showed that freeze-drying and MAHD significantly ($p < 0.05$) reduced the antioxidant activity in hops by 79% and 80.2% [DPPH], respectively and 70.1% and 70.4% [FRAP], respectively, compared to pre-frozen, undried hops. For cannabis, the DPPH assay showed that both freeze-drying and MAHD significantly ($p < 0.05$) reduced the antioxidant activity of cannabis. However, there was no significant ($p < 0.05$) difference between the antioxidant activity values for pre-frozen, freeze-dried, and MAHD cannabis samples using the FRAP method because of the presence of iron-chelating cannabinoids in the cannabis. Results showed that the FRAP assay accurately determines the antioxidant activities of cannabinoids compared to the DPPH assay and is a valuable assay for the cannabis industry. ANOVA analyses showed a significant ($p < 0.05$) increase in the total THC (24.2 g 100 g dry matter^{-1}) and THCA (27.2 g 100 g dry matter^{-1}) concentrations in pre-frozen, undried samples compared to fresh, undried samples. Non-enzymatic decarboxylation was observed by the significant ($p < 0.05$) increase in the THC in MAHD-dried samples compared to fresh, undried (64.7%) and pre-frozen, undried (57%). Although both drying systems showed a significant loss in the total terpene concentration, freeze-drying has higher terpene retention compared to MAHD. Freeze drying should be used as the drying system for medicinal plants to reduce the postharvest losses of secondary metabolites and decarboxylation of cannabinoids.

5.5 References

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Connecting text

Chapters 3, 4, and 5 explained the effects of pre-freezing before drying and drying conditions on the secondary metabolites and antioxidant activity present in medicinal plants. The chapters showed that pre-freezing caused structural damages and improved the drying rate hence pre-freezing can also improve the extraction efficiency. Chapter 6 explored the use of different cold extraction temperatures to maximize cannabis extraction yield and concentrations of cannabinoids and terpenes.

Chapter 6 has been published and is cited as the following:

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Chapter 6: Cold ethanol extraction of cannabinoids and terpenes from cannabis using response surface methodology: optimization and comparative study

Abstract

Efficient cannabis biomass extraction can increase yield while reducing costs and minimizing waste. Cold ethanol extraction was evaluated to maximize yield and concentrations of cannabinoids and terpenes at different temperatures. Central composite rotatable design was used to optimize two independent factors: sample-to-solvent ratio (1:2.9 to 1:17.1) and extraction time (5.7 min–34.1 min). With response surface methodology, predicted optimal conditions at different extraction temperatures were a cannabis-to-ethanol ratio of 1:15 and a 10 min extraction time. With these conditions, yields (g 100 g dry matter^{-1}) were 18.2, 19.7, and 18.5 for −20°C, −40°C and room temperature, respectively. Compared to the reference ground sample, tetrahydrocannabinolic acid changed from 17.9 (g 100 g dry matter^{-1}) to 15, 17.5, and 18.3 with an extraction efficiency of 83.6%, 97.7%, 102.1% for −20°C, −40°C, and room temperature, respectively. Terpene content decreased by 54.1% and 32.2% for extraction at −20°C and room temperature, respectively, compared to extraction at −40°C. Principal component analysis showed that principal component 1 and principal component 2 account for 88% and 7.31% of total variance, respectively, although no significant differences in cold ethanol extraction at different temperatures were observed.

6.1 Introduction

Plant metabolites may be classified as primary or secondary based on their involvement in plant development and growth (Akula and Ravishankar, 2011). Although secondary metabolites are not directly involved in development and growth, they protect plants against biotic (insects, viruses, and bacteria) and abiotic stress (unfavourable environmental conditions) (Akula and Ravishankar, 2011; Khare et al., 2020), add colour and odour, as well as attracting insects for pollination (Schreiner et al., 2012). They are divided into three chemically distinct groups based on their synthesis: phenolics, terpenes, and nitrogen-containing compounds (Kabera et al., 2014).

Major active secondary compounds in the cannabis (*Cannabis sativa*) plant include the terpenophenolic phytocannabinoids, a group of chemical compounds that alter neurotransmission activity in the brain by acting on cannabinoid receptors (Grafström et al., 2019; Howlett, 2002; Luo et al., 2020; Ranganathan and D'Souza, 2006). Phytocannabinoids were considered exclusive to cannabis for many years, until they were discovered in some liverwort and fungi species (Gülck and Møller, 2020). Luo et al. (2020) genetically modified brewer's yeast (*Saccharomyces cerevisiae*) to produce two of the most common cannabinoids, tetrahydrocannabinol (THC) and cannabidiol (CBD).

Extraction of secondary metabolites from plant biomass is the first step for any medicinal plant study (Pattnaik et al., 2022; Rodriguez Garcia and Raghavan, 2022; Rodríguez García and Raghavan, 2022; Szalata et al., 2022). Understanding the genetic composition, plant metabolite biosynthesis, and the prevention of metabolite degradation during postharvest, formulation of cannabis oil, and consumer consumption are important when selecting an extraction technique (Darvishzadeh and Orsat, 2022; Krishnaswamy et al., 2013; Lapornik et al., 2005; Namdar et al., 2018). Commonly used extraction methods in the medicinal industry include the mechanical press and conventional Soxhlet systems. Low extraction rate and poor oil quality are the major disadvantages of the mechanical press system (Qi et al., 2019). The Soxhlet extractor dates to 1879 and is based on the principle that the desired compounds are highly soluble in the solvent used, while impurities are insoluble (Azwanida, 2015; De Castro and Priego-Capote, 2010; Redfern et al., 2014). Soxhlet extraction is normally performed at the boiling point of the solvent for an extended period, which can lead to thermal decomposition of the metabolites.

Addo et al. (2021) and Ubeed et al. (2022) reviewed studies on the cannabis industry showed that modern extraction techniques such as cold ethanol, supercritical CO_2 extraction, ultrasound extraction, and microwave systems have been developed for medicinal plants to improve extraction efficiency and extract quality. Compared to supercritical CO_2 extraction and other traditional extraction systems, cold ethanol extraction limits the extraction of chlorophylls and waxes; hence, it does not require an extract purification or winterization step (Addo et al., 2021; Baldino et al., 2020; Ubeed et al., 2022). Food-grade ethanol is commonly used as an extraction solvent as it is considered a "green" and GRAS (generally recognized as safe) solvent, although other solvents such as hexane and butanol reportedly improve extraction yields (Chang et al., 2017).

Central composite rotatable design (CCRD) was used for the study. Like central composite designs, Box–Behnken designs are response surface designs that require three levels for each independent variable and can only fit second-order quadratic models (Peng et al., 2020). Central composite designs can be classified into three groups namely, circumscribed (CCC), inscribed (CCI), and face centered (CCF) central composite designs (Rakić et al., 2014; Zin and Kim, 2019). Classification of central composite designs are based on the position of the axial points. The axial (α) points of the CCC are placed outside the set experimental parameter limits. This allows for the determination of the effect of values beyond or below the chosen levels of factors on the experimental dependent values/responses. Inscribed central composite design is used when it is not possible to leave the limits of the independent variables and gives a poor prediction compared to CCC. The CCI design uses the factor settings as the axial points and creates a factorial or fractional factorial design within those limits (Zin and Kim, 2019). Five levels are required for each independent variable for CCC and CCI and both designs are rotatable. For CCF designs, the axial points are at the center of each face of the factorial space, so $\alpha = \pm 1$. CCF requires three levels.

This study aimed to optimize cold ethanol for cannabis biomass extraction. Effects of independent variables, including sample-to-solvent ratios, extraction temperatures, and extraction times, on the crude oil yield and concentration of cannabinoids and terpenes were investigated. Response surface methodology was used to optimize the conditions and compare the effects of the dependent variables using quantitative results.

6.2 Materials and methods

6.2.1 Sample preparation

Harvested cannabis inflorescences from three cannabis accessions, Qrazy Train, Qrazy Apple, and Qrazy Angel, cultivated indoors using the same growing conditions were obtained from EXKA Inc. (Mirabel, QC, Canada). Harvested inflorescences were pre-frozen at −20°C for 24 h before transferring to a laboratory-scale vacuum freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH Gamma 1−16 LSCplus, Osterode, Lower Saxony, Germany) with a condenser temperature of −55°C. Freeze-drying was carried out at plate temperatures of 10°C for 24 h at 0.85 mbar. The initial moisture content of the inflorescence ranged from 78.5% (wb) to 80.5% (wb). Freeze-dried inflorescence from the different cannabis accessions were mixed and cryo-ground to uniform particle size (0.25–0.5 mm) using liquid nitrogen and a mortar and pestle. Ground samples were kept in zip-locked plastic bags, manually homogenized, then stored at either −20°C, −40°C, or room temperature before extraction and analysis.

6.2.2 Reagents

Food-grade ethanol was purchased from Commercial Alcohols (Brampton, Ontario, Canada). Reference standards of cannabinoids and isotopically labeled cannabinoids were purchased from Cerilliant (Round Rock, TX, USA). All neutral cannabinoids including Δ9-THC (tetrahydrocannabinol), Δ8-THC, CBD (cannabidiol), CBG (cannabigerol), CBN (cannabinol), CBC (cannabichromene), THCV (tetrahydrocannabivarin), CBDV (cannabidivarin), CBGV (cannabigerivarin), and CBV (cannabivarin) were provided at 1.0 mg mL^{-1} in methanol. CBL (cannabicyclol) was provided at 1.0 mg mL^{-1} in acetonitrile. The acidic cannabinoids, including Δ9-THCA (tetrahydrocannabinolic acid), CBDA (cannabidiolic acid), CBGA (cannabigerolic acid), CBNA (cannabinolic acid), CBCA (cannabichromenic acid), THCVA (tetrahydrocannabivarin acid), CBDVA (cannabidivarinic acid), and CBGVA (cannabigerovarinic acid), were provided at 1.0 mg mL^{-1} in acetonitrile. CBLA (cannabicyclolic acid) was provided at 0.5 mg mL^{-1} in acetonitrile.

Isotopically labeled cannabinoids, including Δ9-THC-d_3, CBD-d_3, CBN-d_3, and CBG-d_3, were provided at 0.1 mg mL^{-1} in methanol while Δ9-THCA-d_3, CBGA-d_3, and CBCA-d_3 were provided at 0.1 mg mL^{-1} in acetonitrile. THC-d_3 was used as internal standard for Δ9-THC, Δ8-THC, THCV, CBC, and CBL. THCA-d_3 was used for THCA, CBNA, and THCVA. CBD-d_3 was

used for CBD, CBDA, CBDV, and CBDVA. CBN-d_3 was used for CBN and CBV. CBG-d_3 was used for CBG and CBGV. CBGA-d_3 was used for CBGA and CBGVA and CBCA-d_3 was used for CBCA and CBLA. Ultrapure water was collected from a Millipore Milli-Q Advantage A10 mixed bed ion exchange system fed with reverse osmosis domestic water (Jaffrey, NH, USA). Optima® grade acetonitrile, methanol, and formic acid were procured from Fisher Scientific (Fair Lawn, NJ, USA).

Terpene reference standards were purchased from Restek (Bellefonte, PA, USA) and provided at 2.5 mg mL^{-1} in isopropanol. Isotopically labeled terpene (±)-linalool-d3 (vinyl-d3) was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada) and used as an internal standard. Hexane (HPLC Plus, ≥95%) was purchased from Millipore-Sigma (Oakville, ON, Canada).

6.2.3 Cold ethanol extraction

The effect of ethanol temperature on the extraction efficiency for cannabis was determined by varying the temperature of the cold ethanol (−20°C, −40°C, and room temperature) during extraction. To emulate the industrial reflux cold ethanol extraction (CEE) system, 40 mL ethanol in 50-mL Falcon tubes were stored at either −20°C and −40°C for 24 h. Required cryo-ground cannabis biomass to achieve desired sample-to-solvent ratios were added to the cold ethanol and placed on a Corning LSE variable speed vortex mixer (Corning, Glendale, AZ, USA). Cold ethanol extraction was done by placing the vortex mixer with the sample soaked in ethanol in a freezer at the required temperature. Extractions were carried out with different sample-to-solvent ratios, extraction temperatures, and extraction times. The sample-to-solvent ratios used for this study were calculated by varying cannabis biomass (g) within 40 mL of ethanol with Equation (1).

$$\text{Cannabis biomass in grams} = 40\ \text{mL} \times \frac{\text{Density of ethanol } (0.789 \frac{\text{g}}{\text{mL}})}{\text{mass of ethanol (g)}} \quad (1)$$

6.2.4 Calculation of extraction yield and efficiency

After extraction, each extract containing the solvent and cannabis biomass mixture was subjected to vacuum filtration using Whatman 4 filter paper (Sigma Aldrich, St. Louis, MO, USA) to remove any residual biomass. Vacuum rotary evaporator operating at 35 rpm and 50°C

was used to evaporate the ethanol present in the extract to determine the yield of crude cannabis oil. Extraction yield of the crude cannabis oil was calculated using Equation (2). Extraction efficiency at the optimal condition was calculated based on THCA concentration using Equation (3).

$$\text{Yield (g 100 g dry matter}^{-1}) = \frac{\text{mass of extracted crude cannabis oil (g)}}{\text{mass of dried sample (100 g)}} \quad (2)$$

$$\text{Efficiency (\%)} = \frac{\text{Concentration of THCA in extract } \left(\frac{\text{g}}{\text{100 g dry matter}}\right)}{\text{Concentration of THCA in cryo-ground sample } \left(\frac{\text{g}}{\text{100 g dry matter}}\right)} \times 100\% \quad (3)$$

6.2.5 Cannabinoid analyses by liquid chromatography-tandem mass spectrometer (LC-MS/MS)

A cannabinoid analysis method developed and described previously by the National Research Council of Canada was modified and used for this study (McRae and Melanson, 2020; Meija et al., 2021). Extracted crude cannabis oil samples were centrifuged at 5000 rpm for 5 min. An aliquot of the supernatant was diluted in methanol based on the initial sample biomass (Table 6.1) used for the extraction (this sample is referred to as the diluted cannabis extract). Samples, standards, and quality control (QC) samples (100 μL) were transferred to high-pressure liquid chromatography (HPLC) vials containing glass inserts. The internal standard (50 μL, 500 ng mL^{-1} in methanol) was added prior to injection onto the liquid chromatography tandem mass spectrometer (LC-MS/MS) system. The LC-MS/MS system consisted of a HPLC (Ultimate3000; Thermo Fisher Scientific, Waltham, MA, USA) coupled to a triple quadrupole mass spectrometer (TSQ Quantiva; Thermo Fisher Scientific, MA, USA). Chromatographic separation was carried out on C_{18} bonded phase column (Accucore C_{18}, 150 mm × 2.1 mm i.d. with 2.6 μm particle size; Thermo Fisher Scientific, MA, USA) maintained at 40°C and the mobile phases consisted of water/formic acid and acetonitrile/formic acid both mixed in a 1000:1 volume ratio. An injection volume of 1 μL was used for the study.

Table 10.1. Dilution factors used for cannabinoid and terpene analyses for the extracted sample biomass.

Dilution Factor	Approximate Initial Mass of Biomass (g)
Cannabinoid analysis	
5000-fold	10
3000-fold	6
1500-fold	3
1000-fold	2
Terpene analysis	
1000-fold	10
500-fold	6
200-fold	3
100-fold	2

The MS/MS detection of cannabinoids was performed via electrospray ionization in positive ion mode using quasi-molecular ion to product ion transitions (McRae and Melanson, 2020). The LC-MS/MS method includes both acidic and neutral forms of the cannabinoids. The neutral forms ionize only in positive mode while the acidic forms ionize equally well in both positive and negative mode. Using positive ionization mode for both neutral and acidic cannabinoids produced more consistent and more similar signal responses for all cannabinoids and resulted in a simplified method, relative to a polarity-switching method. External calibration standard solutions containing 20 cannabinoids were prepared in methanol at concentrations of 10, 20, 100, 1000, 6000, 9000 and 10,000 ng mL^{-1} with quality control samples prepared at 30, 1500 and 8 000 ng mL^{-1}. Linear regression, weighted $1/x^2$, was used for calibration with peak area ratio of cannabinoid and internal standard as the response variable.

6.2.6 Terpene analysis by gas chromatography-tandem mass spectrometer (GC-MS/MS)

For terpene analysis, extracted crude cannabis oil samples were centrifuged at 5000 rpm for 5 min. An aliquot of the supernatant was diluted in hexane based on the initial sample biomass (Table 6.1) used for the extraction (referred to as the diluted cannabis extract). Samples, standards, and QC samples (150 μL) were transferred to HPLC vials containing glass inserts and

the internal standard (50 µL, 1 µg mL^{-1} of linalool-d_3 in hexane) was added before injection onto the gas chromatography-tandem mass spectrometer (GC-MS/MS) system (Trace 1310 GC coupled to a TSQ 9000 Triple Quadrupole MS/MS; Thermo Fisher Scientific, MA, USA). An injection volume of 1 µL was used for the study.

Chromatographic separation of the analytes was obtained using the TraceGOLD TG-5SilMS column (30 m x 0.25 mm i.d. with 0.25 µm film thickness; Thermo Fisher Scientific, MA, USA) and helium as the carrier gas. The inlet temperature was held at 250°C with a deactivated splitless quartz wool single taper liner (78.5 mm × 4 mm i.d. × 6.3 mm o.d.; Thermo Fisher Scientific, MA, USA). A constant inlet flow of 1.5 mL min^{-1} with a split flow of 15 mL min^{-1} and a split ratio of 10 was used. Selected reaction monitoring (SRM) scan type with electron impact ionization mode was used for the tandem mass spectrometer, while the ion source temperature and MS transfer line temperature were held at 300°C and 250°C, respectively. The temperature program for the GC oven can be found in Table 6.2.

Table 10.2. Gas chromatography oven temperature program.

Retention Time (min)	Rate (°C min^{-1})	Target Value (°C)	Hold Time (min)
2.000	0.00	65.0	2.00
8.000	10.00	125.0	0.00
18.333	15.00	250.0	2.00
25.000	30.00	300.0	5.00
25.000	Stop Time		

Calibration curves (0.005–2.5 µg mL^{-1}) were generated using weighted linear regression (1/x) of the peak area ratios (analyte/internal standard) versus the concentration of the calibration standards. The concentration of individual terpenes in extracts was determined using the appropriate calibration curve for the metabolite using the resulting peak area ratios. Monitored ions, ion transitions, and mass spectrometer voltage parameters are listed in Table 6.3.

Table 10.3. Gas chromatography-tandem mass spectrometer acquisition parameters for terpenes.

Name	Q1 (*m*/*z*)	Q3 (*m*/*z*)	CE (eV)	Q1 (*m*/*z*)	Q3 (*m*/*z*)	CE (eV)	RT (min)
α-pinene	*93.1*	*77.1*	*10*	93.1	91.1	6	4.1
camphene	*93.1*	*77.1*	*12*	121.1	93.1	8	4.4
β-pinene	*93.1*	*77.1*	*10*	93.1	91.1	6	4.8
β-myrcene	*93.1*	*77.1*	*10*	93.1	91.1	6	4.9
Δ-3-carene	*93.1*	*77.1*	*10*	105.1	79.1	7	5.3
α-terpinene	*136.1*	*121.1*	*10*	121.1	93.1	8	5.4
p-isopropyl toluene	*134.1*	*119.1*	*6*	119.1	117.1	8	5.5
d-limonene	*121.1*	*93.1*	*8*	93.1	77.1	12	5.6
eucalyptol	*108.1*	*93.1*	*5*	108.1	77.1	20	5.7
ocimene	*93.1*	*77.1*	*10*	121.1	93.1	5	5.8
γ-terpinene	*136.1*	*121.1*	*7*	136.1	93.1	8	6.1
terpinolene	*136.1*	*121.1*	*8*	136.1	93.1	8	6.5
linalool	*93.1*	*77.1*	*10*	93.1	91.1	5	6.6
isopulegol	*121.1*	*93.1*	*8*	111.1	55.1	10	7.5
geraniol	*69.1*	*41.0*	*5*	69.1	39.0	14	8.9
β-caryophyllene	*133.1*	*91.1*	*8*	133.1	105.1	8	10.9
α-humulene	*93.1*	*77.1*	*10*	93.1	91.1	6	11.3
nerolidol 1	*136.1*	*121.1*	*5*	93.1	77.1	12	11.9
nerolidol 2	*136.1*	*121.1*	*5*	93.1	77.1	12	12.12
caryophyllene oxide	*93.1*	*91.1*	*8*	121.1	93.1	5	12.5
guaiol	*161.1*	*105.1*	*8*	161.1	119.1	8	12.5
α-bisabolol	*109.1*	*67.1*	*7*	119.1	91.1	12	13.2
linalool-d_3	*74.07*	*43.1*	*8*	96.1	79.1	10	6.6

Italic values indicate quantitation ion parameters and non-italic values indicate confirmation ion parameters. Q1 (*m*/*z*) and Q3 (*m*/*z*) are the mass-to-charge ratios of the molecular ion selected in Q1 and the fragment ion selected in Q3, respectively. CE is the collision energy and RT is the chromatographic retention time of each terpene.

6.2.7 Experimental design

A five-level-by-two-variables central composite rotatable statistical design (CCRD) with uniform precision was used to compare cold ethanol extraction at various temperatures (−20°C, −40°C, and room temperature) with respect to total yield of extracted cannabis crude oil, extraction efficiency, and cannabinoid and terpene concentrations. Central composite rotatable design (CCRD) was used for the study because the design consists of five levels and able to test forth-order quadratic models.

As shown in Table 6.4, a total of 13 experimental runs consisting of 4 combinations of factorial values, 4 combinations of axial values, and 5 combinations of central values were generated for the study. Axial points were fixed at a distance ($\alpha = 2^{k/4}$, where k represents the number of variables) from the center to ensure rotatability. Axial combinations additionally allowed for the inclusion of quadratic terms in the response surface model. Replication of central point assures a greater uniformity in the precision of response estimation over the experimental design.

Table 10.4. Rotatable central composite design in the coded and uncoded form of the independent variables for cold ethanol extraction at different temperatures (−20°C, −40°C, and room temperature).

Run	Sample (g) Solvent $(g)^{-1}$ (*w*/*w*)	Sample (g) Solvent $(40\ mL)^{-1}$ (*w*/*v*) (X_1)	Extraction Time (min) (X_2)
1	1/2.93	10.77 (−1.414)	20 (0)
2	1/5	6.31 (−1)	10 (−1)
3	1/5	6.31 (−1)	30 (+1)
4	1/10	3.16 (0)	5.86 (−1.414)
5	1/10	3.16 (0)	20 (0)
6	1/10	3.16 (0)	20 (0)
7	1/10	3.16 (0)	20 (0)
8	1/10	3.16 (0)	20 (0)
9	1/10	3.16 (0)	20 (0)
10	1/10	3.16 (0)	34.14 (+1.414)
11	1/15	2.1 (+1)	10 (−1)

12	1/15	2.1 (+1)	30 (+1)
13	1/17.07	1.85 (+1.414)	20 (0)

[A] Values in parentheses represent coded forms of the variables.

6.2.8 Statistical analysis

Statistical analyses were performed using JMP software (JMP 4.3 SAS Institute Inc.). Least square multiple regression methodology was used to evaluate the relationship between the independent and dependent variables. Two different multiple regression equations were used to fit the second-order polynomial model based on the experimental data for cold ethanol extraction at various extraction temperatures (−20°C, −40°C, and room temperature). The first model (model A) was a full model that included all the independent variables, as well as their respective quadratic and interactions terms (Equation (4). Model B, the second model, was a modification of model A to exclude and control for the extraction time and all interaction and quadratic terms that include the extraction time (Equation (5)).

$$Yj = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1 X_1 + \beta_{22} X_2 X_2 + \beta_{12} X_1 X_2 \quad (4)$$

$$Yj = \beta_0 + \beta_1 X_1 + \beta_{11} X_1 X_1 \quad (5)$$

where Yj represents the predicted response (dependent variables), model intercept (β_0), linear terms (β_1 and β_2), quadratic terms (β_{11} and β_{22}), and interaction term (β_{12}), and X_1 (sample (g) solvent $(g)^{-1}$) and X_2 (extraction time) are the independent variables.

Analysis of variance (ANOVA) was used to investigate the statistical significance of the regression coefficients by conducting the Fisher's F-test at a 95% confidence level. The statistical significance of the model was improved through a "backward elimination" process, deleting non-significant dependent terms ($p > 0.05$). The correlation coefficient (R^2) was used to estimate the quality of fit of each model to the responses. Adjusted R^2 was used to determine the significance of the improved models by estimating the significance of the deleted non-significant dependent terms to the full models. Response surface plot was obtained using the fitted model. The optimal conditions for cold ethanol for the dependent variables were obtained based on modelling and desirability function. All the results from the dependent variables were investigated with multivariate analysis and principal component analysis (PCA) using JMP software (JMP 4.3 SAS Institute Inc.).

6.2.9 Verification of model

Three experiments were conducted using the optimal extraction conditions with the highest desirability used to verify the model. The experimental and predicted values were compared to determine the validity of the model.

6.3 Results and discussion

6.3.1 Preliminary cold ethanol extraction results

The selection of independent variables and their ranges for the extraction systems were based on preliminary experiments and a literature review of the probable effects of the parameters on the yield of cannabis oil, cannabinoids, and terpenes (dependent variables). Major cannabinoid concentrations of the ground cannabis biomass are listed in Table 6.5. Cannabinoid and terpene concentrations were measured using the liquid chromatography-tandem mass spectrometer (LC-MS/MS) and gas chromatography-tandem mass spectrometer (GC-MS/MS), respectively. The total chromatographic run time was 18 min for the cannabinoids and 25 min for the terpenes (Figure 6.1).

Table 10.5. Major cannabinoid and terpene concentrations (g 100 g dry matter^{-1}) present in cryo-ground sample.

Metabolite	Concentration (g 100 g dry matter^{-1})
Tetrahydrocannabinol (Δ9-THC)	0.17 ± 0.11
Tetrahydrocannabinolic acid (THCA)	17.92 ± 6.24
Cannabidiolic acid (CBDA)	0.04 ± 0.03
Cannabigerol (CBG)	0.09 ± 0.05
Cannabigerolic acid (CBGA)	0.27 ± 0.18
Cannabinolic acid (CBNA)	0.02 ± 0.01
Cannabichromenic acid (CBCA)	0.38 ± 0.25
Tetrahydrocannabivarin (THCV)	0.01 ± 0
Tetrahydrocannabivarin acid (THCVA)	1.01 ± 0.4
Total THC	15.87 ± 0.56
Total CBG	0.32 ± 0.02

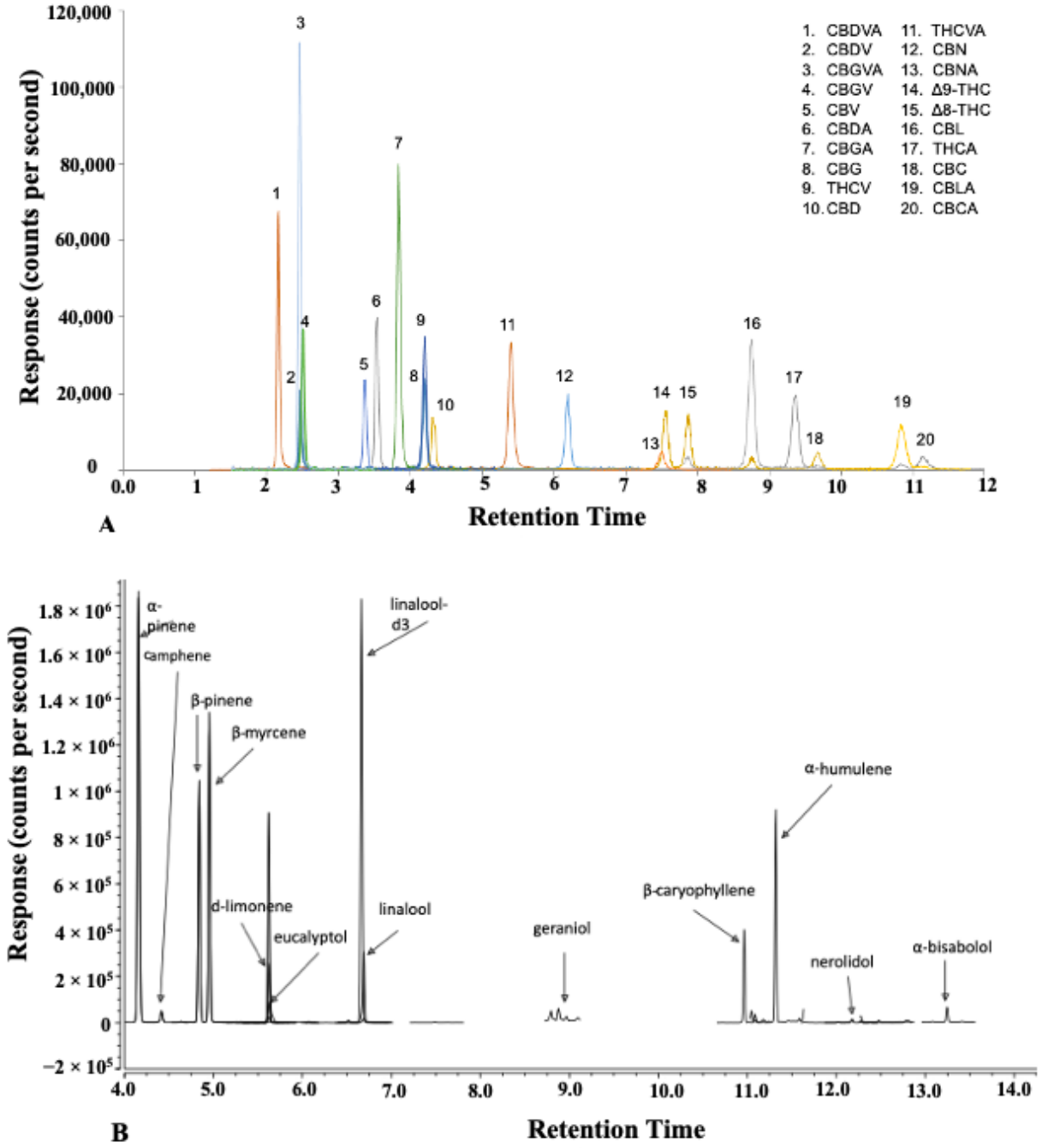

Figure 10.1. LC-MS/MS chromatogram of cannabinoids (A) and GC-MS/MS chromatogram of terpenes (B) for cannabis.

The cryo-ground biomass used for the study contained 17.9 g 100 g dry matter^{-1} (THCA), 0.17 g 100 g dry matter^{-1} (THC), and 0.04 g 100 g dry matter^{-1} (CBDA). The cannabis biomass used for this study can be classified as a Type I chemovar, according to the

classification set by Lewis et al. (2018) based on the high concentration of THCA compared to CBDA. THCVA, which is produced from cannabigerovarinic acid (CBGVA), was 1.01 g 100 g dry matter^{-1} in the cryo-ground sample. CBGVA is produced by the prenylation of divarinolic acid, instead of olivetolic acid, with geranyl diphosphate from terpenoid synthesis (Lewis et al., 2018; Tahir et al., 2021). In contrast to THC, Δ9-tetrahydrocannabivarin (THCV), the decarboxylated form of THCVA, does not cause psychoactive effects and may be a useful metabolite for regulating weight loss and obesity as it decreases appetite and increases satiety and energy metabolism (Abioye et al., 2020; Englund et al., 2016).

Cannabinoid concentration data acquired after cold ethanol extraction at −20°C, −40°C, and room temperature are summarized in Table 6.6. CBD, CBDA, total CBD, and other cannabinoids were not presented as their concentrations were below the limit of detection of the instrumentation and methodology. An observed increase in acidic cannabinoid concentrations indicates that cold ethanol extraction does not cause cannabinoid decarboxylation. This can be explained by the low temperature maintained during the extraction process. The statistical significance of the experimental factors on the cold ethanol extraction process, specifically cannabinoid, terpenes, and extraction yield, for each response, and linear, quadratic, and interaction coefficients of experimental factors are presented in Table 6.7. Regression intercepts of the developed models demonstrate significant ($p < 0.05$) relationships between the cold ethanol extraction independent variables at the set extraction temperatures and corresponding responses of the produced extracts. A positive regression coefficient indicates a positive correlation between the independent variable and the response.

Table 10.6. Concentration of cannabinoids for cold ethanol extraction of cannabis with different experimental conditions.

Cold ethanol extraction	Independent variables		response/dependent variables (g 100 g dry matter^{-1})									
	Sample solvent^{-1}	Extraction time	THC	THCA	Total THC	CBG	CBGA	Total CBG	THCVA	CBCA	Total terpenes	Yield
	(X_1, g 40 mL^{-1})	(X_2, min)	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	Y_7	Y_8	Y_9	Y_{10}
−20°C			0.09	3.95	3.55	0.02	0.05	0.06	0.18	0.07	0.32	4.92
−40°C	10.77	20	0.22	10.07	9.05	0.05	0.14	0.17	0.52	0.18	0.36	8.56
RT			0.31	9.56	8.69	0.05	0.14	0.17	0.48	0.19	0.22	9.84
−20°C			0.34	13.79	12.44	0.07	0.19	0.24	0.72	0.27	0.22	10.92
−40°C	6.31	10	0.33	13.27	11.97	0.06	0.19	0.23	0.69	0.25	0.93	15.85
RT			0.28	9.68	8.77	0.05	0.14	0.17	0.49	0.19	0.05	13.79
−20°C	6.31	30	0.19	7.01	6.34	0.03	0.10	0.12	0.35	0.13	0.77	10.92
−40°C			0.32	12.71	11.46	0.06	0.19	0.23	0.66	0.24	1.13	15.69
RT			0.27	9.37	8.49	0.05	0.13	0.16	0.46	0.19	0.67	11.71
−20°C	3.16	5.86	0.32	12.96	11.68	0.06	0.19	0.22	0.67	0.24	0.06	16.46
−40°C			0.32	12.05	10.89	0.06	0.17	0.21	0.63	0.23	1.13	16.77
RT			0.47	15.32	13.91	0.07	0.23	0.28	0.80	0.30	1.06	17.72
−20°C	3.16	20	0.29	11.16	10.08	0.05	0.16	0.19	0.58	0.21	0.99	16.77
−40°C			0.27	12.58	11.31	0.06	0.18	0.22	0.65	0.24	1.18	16.72
RT			0.36	11.64	10.57	0.06	0.17	0.21	0.60	0.22	1.10	17.78
−20°C	3.16	20	0.35	12.71	11.49	0.06	0.19	0.22	0.66	0.25	1.19	17.41

−40°C			0.41	18.15	16.32	0.09	0.26	0.32	0.94	0.35	1.20	17.72
RT			0.33	12.36	11.17	0.06	0.18	0.22	0.64	0.25	1.02	17.41
−20°C	3.16	20	0.41	13.44	12.20	0.07	0.19	0.24	0.71	0.26	1.09	17.09
−40°C			0.31	13.74	12.36	0.06	0.20	0.24	0.72	0.26	1.17	17.72
RT			0.33	11.97	10.83	0.06	0.17	0.21	0.63	0.24	0.92	18.30
−20°C	3.16	20	0.27	10.59	9.55	0.05	0.15	0.18	0.54	0.20	1.08	17.03
−40°C			0.39	15.59	14.05	0.08	0.23	0.28	0.83	0.30	1.11	17.46
RT			0.33	11.50	10.41	0.06	0.17	0.20	0.60	0.23	0.94	17.35
−20°C	3.16	20	0.26	10.55	9.51	0.05	0.15	0.18	0.53	0.19	1.14	17.41
−40°C			0.30	11.66	10.53	0.06	0.17	0.21	0.61	0.22	1.14	17.72
RT			0.40	13.24	12.01	0.07	0.19	0.24	0.71	0.26	1.13	17.98
−20°C	3.16	34.14	0.31	12.80	11.54	0.06	0.18	0.22	0.65	0.23	1.05	16.51
−40°C			0.33	12.25	11.07	0.06	0.17	0.21	0.63	0.24	1.09	17.09
RT			0.35	11.50	10.43	0.06	0.16	0.20	0.59	0.22	1.09	16.46
−20°C	2.1	10	0.43	15.65	14.16	0.07	0.23	0.27	0.84	0.31	1.13	17.54
−40°C			0.50	19.14	17.28	0.09	0.28	0.34	1.02	0.37	2.00	19.62
RT			0.45	17.03	15.38	0.09	0.25	0.31	0.93	0.34	1.27	18.10
−20°C	2.1	30	0.35	12.70	11.49	0.06	0.18	0.22	0.68	0.26	1.26	18.57
−40°C			0.41	15.94	14.40	0.07	0.23	0.28	0.82	0.30	2.06	18.45
RT			0.44	15.65	14.16	0.08	0.24	0.29	0.82	0.31	1.16	19.05
−20°C	1.85	20	0.36	15.20	13.70	0.07	0.22	0.26	0.80	0.28	1.14	18.82
−40°C			0.45	16.99	15.35	0.08	0.25	0.30	0.92	0.32	2.32	20.11

RT	0.61	20.27	18.39	0.10	0.30	0.37	1.08	0.40	1.23	19.46

Matrix of the central composite rotatable statistical design (CCRD) and observed responses (Yj) for cold ethanol extraction of cannabis biomass at −20°C, −40°C, and room temperature (RT), with different sample-to-solvent ratios and extraction time.

Table 10.7. Regression equation coefficients for cold ethanol extraction of cannabis with different experimental conditions.

Response/dependent variables		Regression model effect parameters					
		Intercept	Linear		Quadratic		Interaction
		β_0	β_1	β_2	β_{11}	β_{22}	β_{12}
Cold ethanol extraction at −20°C							
THC	Coefficient	0.32	0.08	−0.03	−0.03	0.01	0.02
	p value	<0.0001*	0.01*	0.23	0.25	0.6	0.61
THCA	Coefficient	11.69	2.93	−1.24	−0.79	0.86	0.96
	p value	<0.0001*	0.01*	0.13	0.34	0.3	0.38
Total THC	Coefficient	10.57	2.65	−1.12	−0.72	0.77	0.86
	p value	<0.0001*	0.01*	0.13	0.34	0.31	0.39
CBG	Coefficient	0.06	0.01	−0.01	−0.01	0	0.01
	p value	<0.0001*	0.02*	0.16	0.35	0.47	0.23
CBGA	Coefficient	0.17	0.05	−0.02	−0.01	0.01	0.01
	p value	<0.0001*	0.01*	0.1	0.29	0.3	0.51
Total CBG	Coefficient	0.2	0.05	−0.02	−0.02	0.01	0.02
	p value	<0.0001*	0.01*	0.16	0.33	0.35	0.39
THCVA	Coefficient	0.6	0.17	−0.07	−0.04	0.05	0.05
	p value	<0.0001*	0.01*	0.12	0.39	0.32	0.38
CBCA	Coefficient	0.22	0.06	−0.03	−0.01	0.02	0.02
	p value	<0.0001*	0.01*	0.13	0.41	0.36	0.32
Total terpenes	Coefficient	1.1	0.32	0.26	−0.13	−0.22	−0.11
	p value	<0.0001*	0.001*	0.002 *	0.06	0.01 *	0.23
Extraction yield	Coefficient	17.14	4.24	0.14	−2.56	−0.25	0.26
	p value	<0.0001*	<0.0001*	0.63	<0.0001*	0.42	0.53
Cold ethanol extraction at −40°C							
THC	Coefficient	0.34	0.07	−0.01	0.01	0.01	−0.02
	p value	<0.0001*	0.01*	0.62	0.54	0.68	0.52
THCA	Coefficient	14.34	2.36	−0.43	0.2	−0.49	−0.66
	p value	<0.0001*	0.03*	0.63	0.83	0.61	0.60

Total THC	Coefficient	12.91	2.14	−0.39	0.19	−0.42	−0.59
	p value	<0.0001*	0.03*	0.62	0.82	0.62	0.6
CBG	Coefficient	0.07	0.01	0	0	0	−0.01
	p value	<0.0001*	0.04*	0.57	0.89	0.51	0.42
CBGA	Coefficient	0.21	0.04	−0.01	0	−0.01	−0.01
	p value	<0.0001*	0.03*	0.64	0.81	0.53	0.51
Total CBG	Coefficient	0.25	0.04	−0.01	0	−0.01	−0.01
	p value	<0.0001*	0.03*	0.64	0.89	0.56	0.51
THCVA	Coefficient	0.75	0.13	−0.03	0.02	−0.03	−0.04
	p value	<0.0001*	0.02*	0.54	0.75	0.56	0.52
CBCA	Coefficient	0.27	0.05	−0.01	0	−0.01	−0.02
	p value	<0.0001*	0.03*	0.65	0.99	0.69	0.55
Total terpenes	Coefficient	1.16	0.6	0.03	0.17	0.05	−0.04
	p value	<0.0001*	<0.0001*	0.73	0.06	0.52	0.73
Extraction yield	Coefficient	17.47	2.87	−0.09	−1.12	0.18	−0.22
	p value	<0.0001*	0.01*	0.88	0.12	0.78	0.8
Cold ethanol extraction at room temperature							
THC	Coefficient	0.35	0.1	0.02	0.04	0.01	0
	p value	<0.0001*	0.01*	0.24	0.11	0.58	1
THCA	Coefficient	12.14	3.6	−0.9	1.08	0.33	−0.27
	p value	<0.0001*	<0.0001*	0.04*	0.02*	0.42	0.61
Total THC	Coefficient	11	3.25	−0.8	0.98	0.3	−0.24
	p value	<0.0001*	<0.0001*	0.04*	0.03*	0.4	0.63
CBG	Coefficient	0.06	0.02	0	0.01	0	0
	p value	<0.0001*	<0.0001*	0.05	0.01*	0.55	0.22
CBGA	Coefficient	0.18	0.06	−0.01	0.02	0.01	0
	p value	<0.0001*	<0.0001*	0.03*	0.02*	0.42	1
Total CBG	Coefficient	0.22	0.07	−0.02	0.02	0.01	0
	p value	<0.0001*	<0.0001*	0.04*	0.03*	0.43	0.81
THCVA	Coefficient	0.64	0.21	−0.05	0.06	0.01	−0.02
	p value	<0.0001*	<0.0001*	0.02*	0.03*	0.51	0.47

CBCA	Coefficient	0.24	0.07	−0.02	0.02	0.01	−0.01
	p value	<0.0001*	<0.0001*	0.04*	0.02*	0.53	0.47
Total terpenes	Coefficient	1.02	0.39	0.07	−0.18	0	−0.18
	p value	<0.0001*	<0.0001*	0.14	0.01*	0.97	0.02*
Extraction yield	Coefficient	17.76	3.16	−0.36	−1.61	−0.39	0.76
	p value	<0.0001*	<0.0001*	0.04*	<0.0001*	0.04*	0.01*

Effects are statistically significant if p value * < 0.05. Model intercept (β_0), linear terms (β_1 and β_2), quadratic terms (β_{11} and β_{22}), and interaction term (β_{12}) are the model effect parameters.

6.3.2 Effects of the extraction factors on experimental responses

6.3.2.1 Effect of sample (g)-to-solvent (g) ratio

Central composite rotatable design (CCRD) is highly efficient in providing useful information on the effects of process parameters for optimization purposes with a reduced number of total experimental runs compared to factorial designs (Khuri and Mukhopadhyay, 2010). Effects of sample (g)-to-solvent (g) ratio and extraction time (min) on the concentration (g 100 g dry matter^{-1}) of total THC (A, D and G), total terpenes (B, E, and H), extraction yield (C, F, and I) for cold ethanol extraction of cannabis at −20°C (A–C), −40°C (D,E), and room temperature (G–H) are presented in Figure 6.2.

Sample-to-solvent ratio was the most important factor affecting cold ethanol extraction of cannabis, demonstrating a significant ($p < 0.05$) effect on extraction of cannabinoids, terpenes, and extraction yields for all experimental conditions. Decreasing the sample (g)-to-solvent (g) ratio significantly ($p < 0.05$) increased the extraction yield and concentration of cannabinoids and terpenes, likely by providing an increased surface area for the extraction of secondary metabolites from the trichomes. This is evident by the positive sample-to-solvent ratio coefficient values (β_1). Krishnaswamy et al. (2013) showed that decreasing the mass of grape seeds (*Vitis vinifera*) in ethanol during microwave-assisted extraction, increased the total extracted phenols by 7%. Similar findings have been reported for microwave-assisted extraction of antioxidants from olive (*Elaeagnus angustifolia*) (Darvishzadeh and Orsat, 2022).
Table 6.7 and Figure 6.2 showed that sample-to-solvent ratio had a significant ($p < 0.05$) interaction effect with extraction time only for extraction yield for cold ethanol extraction at room temperature. Response surface plots (Figure 6.2) showed that the extraction yield increased

by 37.7%, 19.2%, and 23.8% when the sample mass in 40 mL of ethanol was reduced from 6.31 g to 2.1 g using 10 min extraction time for cold ethanol extraction at −20°C, −40°C, and room temperature, respectively. Similar observations were observed for the extraction time of 30 min used for this study.

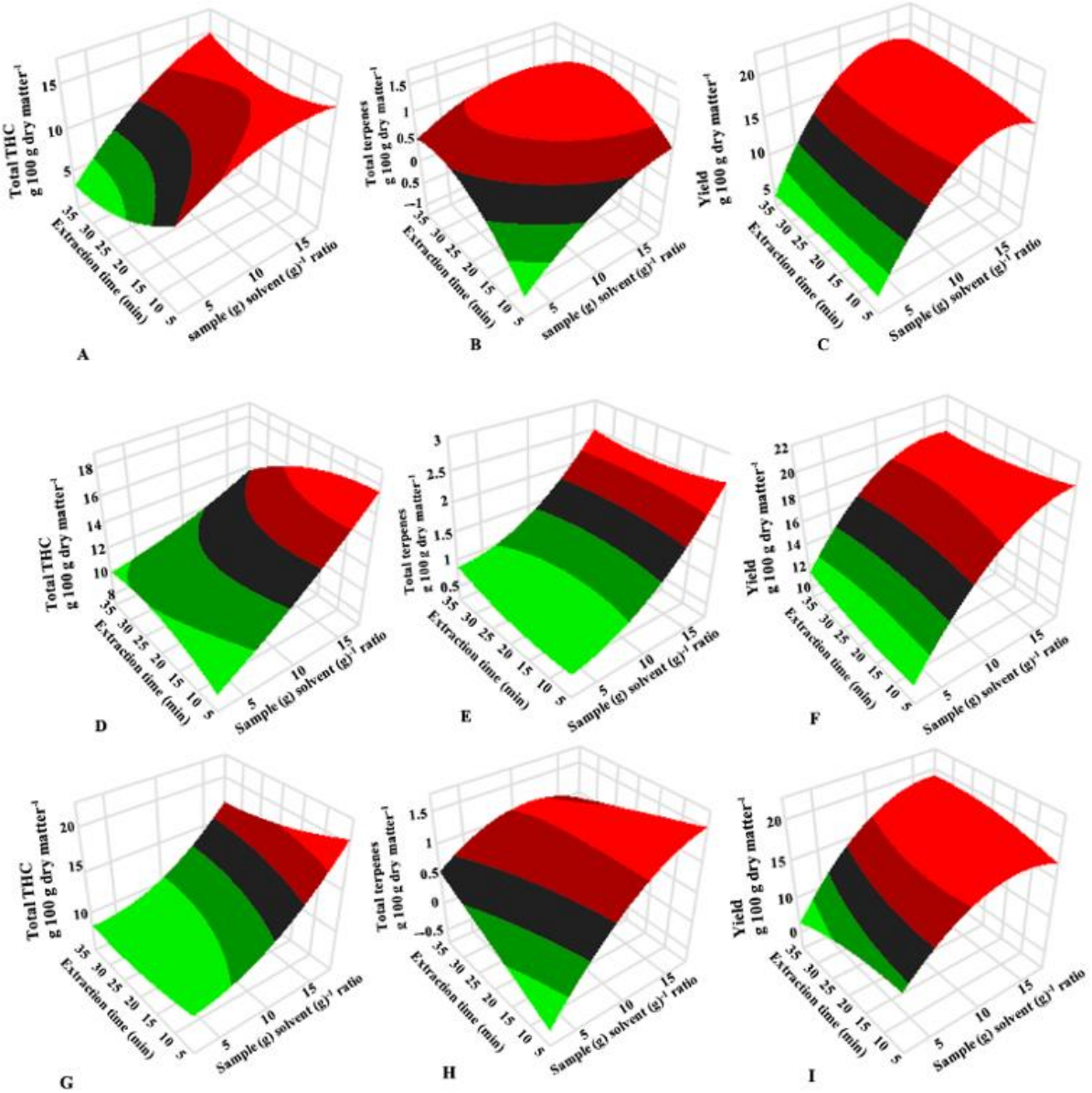

Figure 10.2. 3D plots showing the combined effects of independent variables for cold ethanol extractions.

Quadratic effect, β_{11} (sample-to-solvent^2), significantly ($p < 0.05$) impacted extraction yield for cold ethanol extraction of cannabis at −20°C (Table 6.7). For cold extraction at room temperature, the quadratic effect, β_{11}, had significant ($p < 0.05$) effects on the THCA, total THC, CBG, CBGA, total CBG, THCVA, CBCA, and extraction yield. Importantly, data showed that improved extraction of cannabinoids and cannabis oil can be achieved with all three cold ethanol extraction systems used for the study. Significant difference ($p < 0.05$) was not observed for the extraction yield using either cold ethanol extraction at −20°C, −40°C, and room temperature. However, the extracted cannabis oil extracted at room temperature must be winterized to remove residual waxes and other heavier compounds. If a lower sample-to-solvent ratio is optimal, cost analyses of scale-up studies and industrial systems must be done to minimize the high cost of ethanol needed to maximize extraction yield and cannabinoid concentrations. Studies on ethanol recovery from residual biomass after extraction using either a mechanical press, centrifugal system, or vacuum filtration must be conducted.

6.3.2.2 Effect of extraction time

Effect of extraction time for different plant biomass has been reported and the longer the extraction time, the higher the total amount of metabolites extracted according to mass transfer principles (Che Sulaiman et al., 2017; Krishnaswamy et al., 2013; Spigno et al., 2007). Some researchers have reported that extraction time can be reduced by increasing extraction temperature (Pinelo et al., 2005; Spigno and De Faveri, 2007; Spigno et al., 2007). However, metabolite stability can decrease when they are exposed to high temperatures because most phytochemicals are sensitive to heat (Ubeed et al., 2022). Szalata et al. (2022) reported when compared to cold water extraction, hot water extraction significantly ($p < 0.05$) increased the CBD content from 0.01 to 0.06 g 100 g dry matter^{-1} and 0.01 to 0.05 g 100 g dry matter^{-1} in Futura 75 and KC Dora cannabis accessions, respectively. The increase in CBD can be attributed to the decarboxylation of CBDA to CBD due to the increase in extraction temperature. Optimization of extraction time and temperature to improve extraction yield must be based on the desired phytochemicals' stability during extraction and energy cost analyses. Data presented in Table 6.7 and Figure 6.2 indicate that extraction time did not have a significant effect ($p < 0.05$) on the experimental responses during cold ethanol extraction of cannabis at −40°C.

For cold ethanol extraction at −20°C, extraction time significantly ($p < 0.05$) increased the total terpenes extracted from cannabis. Using the same sample-to-solvent ratio, extending the extraction time increased the concentration of total terpenes in the extracted cannabis oil, likely by increasing the contact time of the sample in the solvent. The negative coefficient values showed a negative significant ($p < 0.05$) correlation between extraction time and the concentration of THCA, total THC, CBGA, total CBG, THCVA, CBCA, and extraction yield for cannabis oil when performing cold ethanol extraction at room temperature (Table 6.7). This can be attributed to degradation or isomerization, which can affect analytical quantification (Spigno and De Faveri, 2007; Spigno et al., 2007). Spigno et al. (2007) observed that the concentration of secondary metabolites, anthocyanin, and tannin, in grape (*Vitis vinifera*) diminished beyond 20 h extraction time. Quadratic effects, β_{22} (extraction time^2), were only observed for total terpenes and extraction yield with cold ethanol extraction at −20°C and cold ethanol extraction at room temperature, respectively.

6.3.3 Optimal cold ethanol extraction conditions for cannabis

Based on the observed effects of the independent parameters used for the study, optimal cold ethanol extraction conditions for cannabis at different temperatures and the predicted responses at 95% confidence interval are listed in Table 6.8. Optimization was driven by maximum desirability and yield of cannabinoids, terpenes, and extracted cannabis oil. The desirability function consolidates all the responses into one response with a numerical value varying from 0 (one or more product characteristics are unacceptable) to 1 (all product characteristics on target). Cold ethanol extraction at −20°C, −40°C, and room temperature using a sample-to-solvent of 1:15 for 10 min are presented as the optimal conditions for maximum responses. According to these statistical analyses of the predicted responses, there were no significant ($p < 0.05$) differences between the extraction yields for the cold ethanol extraction performed at different temperatures. However, reducing the temperature of the cold ethanol extraction system from −20°C to −40°C slightly increased cannabinoid concentration by 7.8%. Compared to room temperature, cold ethanol extraction at −40°C slightly increased the extraction yield by 6%.

Table 10.8. Optimal experimental conditions for cold ethanol extraction of cannabis at −20°C, −40°C, and room temperature and the predicted response values.

Extraction method	Cold ethanol extraction at −20°C	Cold ethanol extraction at −40°C	Cold ethanol extraction at room temperature
Desirability	0.83	0.77	0.78
Optimal independent experimental conditions			
Sample (g)-to-solvent (40 mL)	2.1	2.1	2.1
Sample (g)-to-solvent (g)	1/15	1/15	1/15
Extraction time (min)	10	10	10
Predicted response values at optimal conditions (g 100 g dry matter^{-1})			
THC	0.39	0.46	0.52
THCA	14.98	17.51	18.30
Total THC	13.53	15.81	16.56
CBG	0.07	0.08	0.09
CBGA	0.22	0.25	0.27
Total CBG	0.26	0.31	0.33
THCVA	0.79	0.93	0.99
CBCA	0.29	0.34	0.36
Total terpenes	0.91	1.98	1.34
Extraction yield	18.18	19.72	18.53
Extraction efficiency (%)	83.61	97.73	102.14

If a high terpene content is desired, cold ethanol extraction at −40°C is recommended. Concentration of extracted total terpenes was reduced by 54.1% and 32.2% for extraction at −20°C and room temperature, respectively, compared to extraction at −40°C. Cannabinoid concentrations in extracts were not significantly ($p < 0.05$) different between room temperature extraction and extraction at −40°C. Compared to the reference ground sample (Table 6.5), THCA concentration changed from 17.9 (g 100 g dry matter^{-1}) to 15, 17.5, and 18.3 with an extraction efficiency of 83.6%, 97.7%, 102.1% for −20°C, −40°C, and room temperature,

respectively. Extraction efficiency was calculated based on the concentration of THCA in extracts compared to the concentration of THCA in the reference cryo-ground biomass used for the study. Extraction efficiency greater than 100% for cold ethanol extraction at room temperature can be explained by the biosynthesis or the conversion of other cannabinoids such as CBGA to THCA during the extraction process or variance due to the analytical method (Szalata et al., 2022). Preliminary studies conducted showed that postharvest processing of cannabis can influence the biosynthesis of cannabinoids. The results showed a significant ($p < 0.05$) increase in the total THC (24.2 g 100 g dry matter^{-1}) and THCA (27.2 g 100 g dry matter^{-1}) concentrations in pre-frozen, undried samples compared to fresh, undried samples. Further studies evaluating the effect of cold temperature on biosynthesis of secondary metabolites, cannabinoids, and terpenes, at the molecular level must be conducted to explain the differences observed in this study.

6.3.4 Model fitting

JMP software (JMP 4.3 SAS Institute Inc., Cary, NC, USA) was used for the least square multiple regression analysis of the data and model building. Summary of fit for the experimental data to each model is presented in Table 6.9. Results show non-significant ($p > 0.05$) lack-of-fit values for model A (full model), except for extracted oil and total terpenes for both extraction at −20°C and −40°C. Using model B, which excludes extraction time, all interaction and quadratic terms that include extraction time, only showed non-significant ($p > 0.05$) lack-of-fit values for total terpenes extracted at −20°C and −40°C. This indicates that there is a satisfactory level of accuracy of model B for explaining the relationship between the total terpene content in extracted cannabis using cold ethanol at either −20°C or −40°C and prediction of the corresponding responses. However, both proposed models do not adequately explain the extracted cannabis oil yield using cold ethanol extraction at −20°C and −40°C, and other extraction parameters such as particle size of cannabis biomass must be considered to improve the extraction models. Significant ($p < 0.05$) ANOVA *p*-values indicated significant differences between the extraction conditions. Coefficients of determination (R^2) and adjusted R^2 values of the developed model A ranged from 0.55 to 0.99 and 0.22 to 0.98. Higher R^2 and adjusted R^2 values imply that the experimental data successfully fit the equation with a low deviation from

mean values. However, model A should be used when predicting responses for ethanol extraction at room temperature and model B for cold ethanol extraction at −20°C and −40°C.

Table 10.9. Analysis of variance (ANOVA) analyses of responses for cold ethanol extraction at different temperatures.

Response (g 100 g dry matter^{-1})	Source						F Ratio	Prob > F	Lack-of-Fit (Prob > F)	R^2	Adjusted R^2
	Model			Residual							
	df	SS	MS	df	SS	MS					
Cold ethanol extraction at −20°C											
THC	5	0.07	0.01	7	0.03	0.00	3.1	0.09 (0.01*)	0.41 (0.31)	0.69	0.47
THCA	5	95.79	19.16	7	29.17	4.17	4.6	0.04*	0.1	0.77	0.6
Total THC	5	78.22	15.64	7	24.05	3.44	4.55	0.04*	0.1	0.76	0.6
CBG	5	0.002	0.0004	7	0.001	0.0001	3.2	0.08 (0.03*)	0.21 (0.4)	0.7	0.48
CBGA	5	0.02	0.004	7	0.01	0.001	5.23	0.03*	0.14	0.79	0.64
Total CBG	5	0.03	0.01	7	0.01	0.001	3.99	0.04*	0.13	0.74	0.56
THCVA	5	0.3	0.06	7	0.09	0.01	4.69	0.03*	0.13	0.77	0.61
CBCA	5	0.04	0.01	7	0.01	0.002	4.24	0.04*	0.16	0.75	0.57
Total terpenes	5	1.82	0.36	7	0.17	0.02	14.46	0.001* (0.03*)	0.03* (0.98)	0.91	0.85
Extraction yield	5	189.89	37.98	7	4.23	0.6	62.81	<0.001*	0.01*	0.98	0.96
Cold ethanol extraction at −40°C											
THC	5	0.05	0.01	7	0.02	0.00	2.74	0.11 (0.01*)	0.54 (0.24)	0.66	0.42
THCA	5	50.01	10	7	40.58	5.80	1.73	0.25 (0.03*)	0.6 (0.53)	0.55	0.23
Total THC	5	41.09	8.22	7	32.74	4.68	1.76	0.24 (0.03*)	0.6 (0.52)	0.56	0.24
CBG	5	0.001	0.0002	7	0.001	0.0001	1.55	0.29 (0.04*)	0.84 (0.88)	0.53	0.19
CBGA	5	0.01	0.002	7	0.01	0.001	1.83	0.23 (0.03*)	0.52 (0.48)	0.57	0.26
Total CBG	5	0.02	0.003	7	0.01	0.002	1.78	0.24 (0.03*)	0.55 (0.48)	0.56	0.24

THCVA	5	0.16	0.03	7	0.11	0.02	2.05	0.19 (0.03*)	0.61 (0.59)	0.59	0.31
CBCA	5	0.02	0.004	7	0.02	0.002	1.68	0.26 (0.03*)	0.6 (0.52)	0.55	0.22
Total terpenes	5	3.05	0.61	7	0.28	0.04	15.38	0.001*	<0.001* (0.21)	0.92	0.86
Extraction yield	5	75.73	15.15	7	19.30	2.76	5.49	0.02*	0.01*	0.80	0.65
Cold ethanol extraction at room temperature											
THC	5	0.09	0.02	7	0.03	0	6.5	0.01*	0.96	0.82	0.7
THCA	5	118.43	23.69	7	6.98	1	23.74	<0.001*	0.13	0.94	0.9
Total THC	5	96.78	19.36	7	6	0.86	22.58	<0.001*	0.12	0.94	0.9
CBG	5	0.003	0.001	7	0.0001	0.00001	41.58	<0.001*	0.86	0.97	0.94
CBGA	5	0.03	0.01	7	0.002	0.0002	23.54	<0.001*	0.06	0.94	0.9
Total CBG	5	0.04	0.01	7	0.003	0.0004	21.9	<0.001*	0.18	0.94	0.9
THCVA	5	0.39	0.08	7	0.02	0.003	28.1	<0.001*	0.28	0.95	0.92
CBCA	5	0.05	0.01	7	0.003	0.0004	23.64	<0.001*	0.22	0.94	0.9
Total terpenes	5	1.62	0.32	7	0.1	0.01	23.32	<0.001*	0.21	0.94	0.90
Extraction yield	5	101.31	20.26	7	1.25	0.18	113.67	<0.001*	0.39	0.99	0.98

* Statistically significant ($p < 0.05$). p-values for ANOVA and Lack-of-fit for the revised model, model B, which excludes the extraction time and all interaction and quadratic terms that include the extraction time are shown in parenthesis. Degree of freedom (df), Sum of squares (SS), and Mean square (MS).

6.3.5 Principal component analysis

An exploratory principal component analysis (PCA) was performed to help identify correlation and dependencies between the two independent variables, cannabis biomass sample-to-solvent ratio and extraction time. The scree plot, loadings plot, scores plot, and scatterplot for the different extraction systems are presented in Figure 6.3. A scree plot (Figure 6.3A) is a line plot of the eigenvalues of principal components and is used to determine the number of principal components that are responsible for variations in the data during PCA (Beattie and Esmonde-White, 2021). The scree plot indicated that the first two principal components (PC1 and PC2) on different axes account for 95.3% of the total variance (PC1 = 88% and PC2 = 7.3%). The loading plot (Figure 6.3B) provides information on how the responses contribute to the variations accounted for by the principal components (Huang et al., 2021). The axes on the loading plot range from 1 to −1. The closer the value of the response on the graph to either −1 or 1 describes how strongly the response influences the component. A positive value on the loading plot indicates a positive correlation between the response and the PC. According to the loading plots, parameters positioned close to each other indicate a high positive correlation between them. An increase in the THCA content of an extract can be an indicator of an increase in THCVA. The major cannabinoids identified in the extracts are important contributors to PC1. The loading plot showed that total CBG, CBG, THCV, and THC account for most of the variation of PC1 and not for PC2. PC2 and PC1 can be explained by the total terpenes and the yield of extracts. Scatter plots (Figure 6.3C) did not show any variation in cold ethanol extraction at different temperatures (−20°C, −40°C, and room temperature). This is evident by the overlap of responses for cold ethanol extraction at different temperatures.

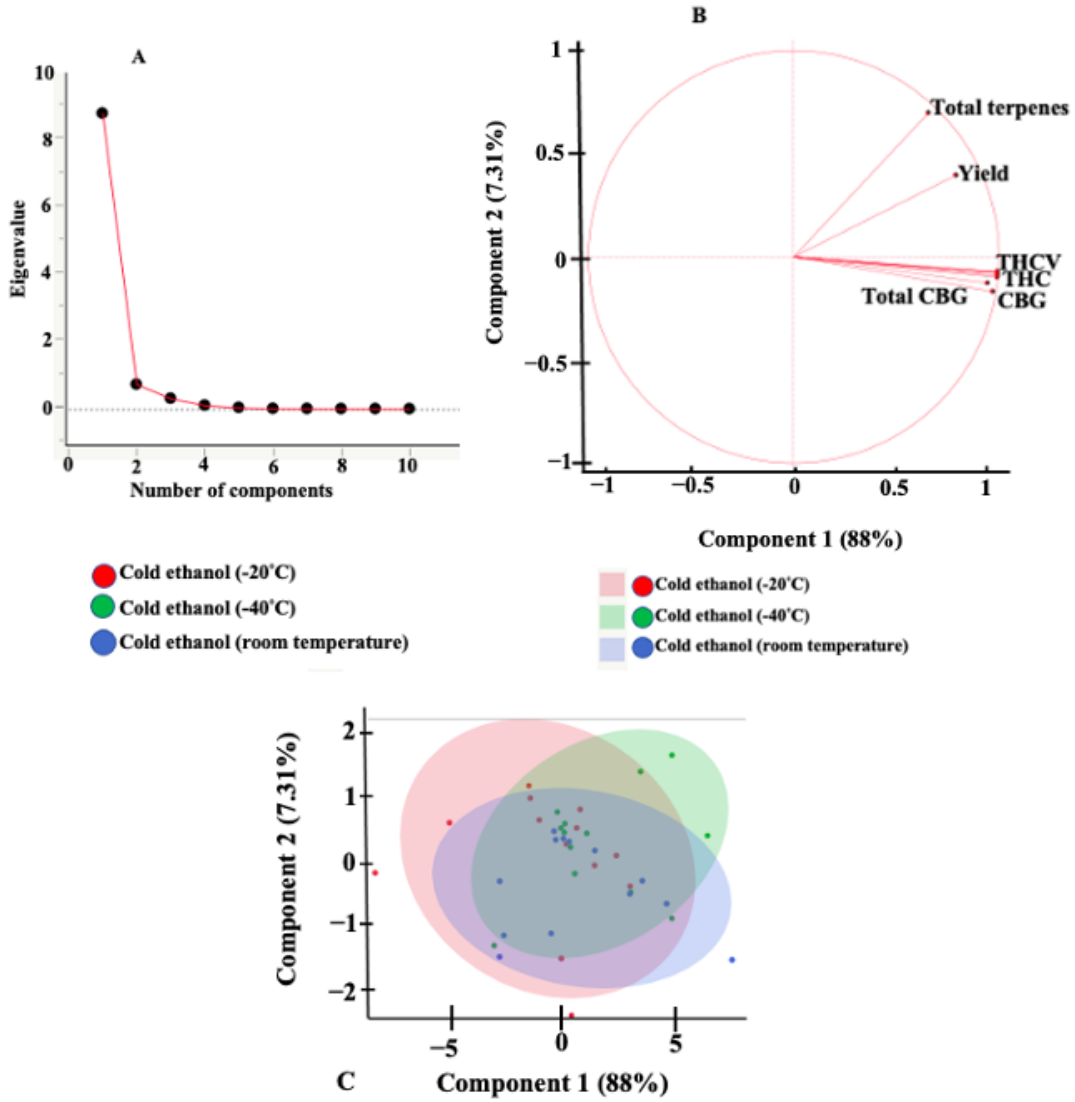

Figure 10.3. Scree (A), loadings (B), and scatter (C) plots for cold ethanol extraction of cannabis at −20°C, −40°C, and room temperature.

6.3.6 Verification of models

Generated models for cold ethanol extraction of cannabis at various temperatures (−20°C, −40°C, and room temperature) were verified by conducting an extraction process using the optimal conditions, sample-to-solvent ratio of 1:15 for 10 min. The corresponding experimental values for cannabinoid content, total terpenes, and extraction yields were determined and compared to predicted results. Data show a strong correlation ranging from 0.87 to 0.93 between the predicted and experimental values, which indicates suitability of the models in predicting

cannabinoid/terpenes profiles and extract yield for cannabis for optimum cold ethanol extraction at −20°C, −40°C, and room temperature.

6.4 Conclusion

Cold ethanol extraction conditions were evaluated to increase the extraction yield and the concentration of cannabinoids and terpenes at different temperatures (−20°C, −40°C, and room temperature). CCRD was used to optimize two independent factors namely samples (g)-to-solvent (g) ratio (1:2.93 to 1:17.07) and extraction time (5.86 to 34.14 min). Developed predictive models for all responses yielded predictable and reproducible results, and the verification of the models showed a close agreement between the experimental values and the predicted values with a strong correlation ranging from 0.87 to 0.93. CCRD predicted that a set sample-to-solvent ratio of 1:15 over 10 min at the different extraction temperatures would provide the optimum conditions for the extraction of cannabis oil with maximum desirability ranging between 0.77–0.83%. At these optimized conditions, extraction yields (g 100 g dry matter^{-1}) were 18.2%, 19.7%, and 18.5% for −20°C, −40°C, and room temperature, respectively, according to the desirability function (0.77 to 0.83%). Compared to the reference ground sample, the THCA concentration changed from 17.9 (g 100 g dry matter^{-1}) to 15, 17.5, and 18.3 with an extraction efficiency of 83.6%, 97.7%, 102.1% for −20°C, −40°C, and room temperature, respectively at the optimal condition. Total terpene was reduced by 54.1% and 32.2% for extraction at −20°C and room temperature, respectively, compared to extraction at −40°C. The scree plot from PCA analyses indicated that the first two principal components account on different axes for 95.3% of the total variance (PC1 = 88% and PC2 = 7.3%) although no significant differences in cold ethanol extraction at different temperatures were observed. Further research studies on ethanol recovery using centrifugation, press system, and vacuum filtration must be conducted to help reduce the operational cost for cannabis industries.

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Connecting text

Chapter 6 showed that the sample-to-solvent ratio of 1:15 over 10 min for different cold ethanol extraction temperatures would provide the optimum conditions for the extraction of cannabis oil with maximum desirability. Based on the findings, Chapter 7 compared the optimal ethanol extraction conditions for cannabis using ultrasound-assisted extraction and microwave-assisted extraction.

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Chapter 7: Microwave- and ultrasound-assisted extraction of cannabinoids and terpenes from cannabis using response surface methodology

Abstract

Limited studies have explored different extraction techniques that improve cannabis extraction with scale-up potential. Ultrasound-assisted and microwave-assisted extraction were evaluated to maximize the yield and concentration of cannabinoids and terpenes. A central composite rotatable design was used to optimize independent factors (sample-to-solvent ratio, extraction time, extraction temperature, and duty cycle). The optimal conditions for ultrasound- and microwave-assisted extraction were the sample-to-solvent ratios of 1:15 and 1:14.4, respectively, for 30 min at 60°C. Ultrasound-assisted extraction yielded 14.4% and 14.2% more oil and terpenes, respectively, compared with microwave-assisted extracts. Ultrasound-assisted extraction increased cannabinoid concentration from 13.2–39.2%. Considering reference ground samples, tetrahydrocannabinolic acid increased from 17.9 (g 100 g dry matter^{-1}) to 28.5 and 20 with extraction efficiencies of 159.2% and 111.4% for ultrasound-assisted and microwave-assisted extraction, respectively. Principal component analyses indicate that the first two principal components accounted for 96.6% of the total variance (PC1 = 93.2% and PC2 = 3.4%) for ultrasound-assisted extraction and 92.4% of the total variance (PC1 = 85.4% and PC2 = 7%) for microwave-assisted extraction. Sample-to-solvent ratios significantly ($p < 0.05$) influenced the secondary metabolite profiles and yields for ultrasound-assisted extracts, but not microwave-assisted extracts.

7.1 Introduction

The extraction of secondary compounds from cannabis presents several challenges. Cannabinoids and terpenoids decompose with light and heat, making them unstable during sample preparation, extraction, and testing methods (Addo et al., 2021; Atkins, 2019; Das et al., 2022a). Additionally, differences in the quality and quantity of the extracted crude oil can be attributed to factors such as cannabis plant type (drug or fibre), pollination, sex, age, plant parts, method of plant cultivation (indoor or outdoor), harvest conditions, drying, and storage (Al Bakain et al., 2020; Namdar et al., 2018; Rodriguez Garcia and Raghavan, 2022). Extraction techniques for cannabis biomass have evolved quickly, resulting in diverse methodologies that have not been properly validated (Ubeed et al., 2022). Frequently used techniques in industries for quality assurance and control include cold ethanol extraction, supercritical CO_2 extraction, conventional Soxhlet extraction, ultrasound-assisted extraction, and microwave-assisted extraction (Brighenti et al., 2017; Yang et al., 2007). Most researchers report that microwave-assisted extraction and ultrasound-assisted extraction are comparably efficient when compared with traditional solvent methods (Chemat et al., 2017; Chemat et al., 2004; Lucchesi et al., 2004; Lucchesi et al., 2007).

Microwaves are non-ionizing irradiation that excites molecules in the essential oil, thereby increasing the rate of extraction (Chandrasekaran et al., 2013; Rodríguez García and Raghavan, 2022). Microwaves may be used in conjunction with solvent extraction, Soxhlet extraction, and distillation (Lucchesi et al., 2007; Luque-Garcıa and De Castro, 2004; Routray and Orsat, 2012; Stashenko et al., 2004). Importantly, microwave-assisted extraction is a safe and environmentally friendly method, as it reduces solvent use and energy consumption, along with various environmental hazards such as chemical wastes. Research studies have concluded that the concentration of secondary metabolites in extracts can be increased using a microwave-assisted extraction system (Chang et al., 2017; Darvishzadeh and Orsat, 2022; Krishnaswamy et al., 2013).

Compared with some other novel extraction techniques, the ultrasonic device is less expensive and is very easy to use (Yolmeh et al., 2014). Ultrasound-assisted extraction is a rapid, simple, and eco-friendly method for extracting bioactive metabolites from plants, with reduced initial production costs due to the low energy and process time required (Moradi et al., 2018). Ultrasound-assisted extraction uses acoustic cavitation to produce cavitation bubbles which

implode and exert mechanical forces which improve the extraction process by increasing solvent penetration into the plant matrix (Vilkhu et al., 2008). Extraction rates are increased by the macroturbulence and high-velocity inter-particle collisions that are caused by the implosion of the gas bubbles (Ji et al., 2006; Rastogi, 2011). Ultrasound and microwaves are considered improved extraction techniques compared with conventional systems, with several advantages, such as shortened extraction time, decreased solvent volumes, and increased extract yield (Azmir et al., 2013; Azwanida, 2015; Chang et al., 2017; Irakli et al., 2018). However, both techniques have not been fully explored for cannabis extraction.

The aim of this study was to determine and compare the optimal extraction conditions for cannabis using ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). Microwave- and ultrasound-assisted extraction were used for the study, as both systems are perceived as 'green' technologies and efficient solutions that industry stakeholders may find advantageous. However, there is inadequate relevant data on optimum extraction conditions and the effect of microwaves and ultrasound on cannabinoid and terpene yield. The effects of several independent variables, including samples-to-solvent ratio (s: s), extraction temperatures, extraction times, and duty cycles, on crude oil yield and concentration of cannabinoids and terpenes were examined. Response surface methodology (RSM) was used to optimize conditions as the established models evaluated and compared the effects of the dependent variables using quantitative results.

7.2 Materials and methods

7.2.1 Sample preparation

Harvested inflorescence from three cannabis accessions, Qrazy Train, Qrazy Apple, and Qrazy Angel, that were cultivated indoors under the same growing conditions were obtained from EXKA Inc. (Mirabel, QC, Canada). Inflorescences were pre-frozen at −20°C for 24 h before transferring to a laboratory-scale vacuum freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH Gamma 1–16 LSCplus, Osterode, Lower Saxony, Germany) with a condenser temperature of −55°C. Freeze-drying was carried out at 10°C for 24 h at 0.85 mbar. The initial moisture content of the inflorescence ranged from 78.52 to 80.48% (wb). Using a previously described method for hops (Addo et al., 2022c), the freeze-dried inflorescences of the different accessions were mixed and cryo-ground to uniform particle size (0.25–0.5 mm)

using liquid nitrogen and a mortar and pestle. Ground samples were kept in clean plastic bags, homogenized by hand mixing and shaking, and stored at either −20°C before extraction and analysis.

7.2.2 Reagents

As described in Chapter 6 section 6.2.2, food-grade ethanol was purchased from Commercial Alcohols (Brampton, Ontario, Canada). Reference standards of cannabinoids and isotopically labeled cannabinoids were purchased from Cerilliant (Round Rock, TX, USA). All neutral cannabinoids including Δ9-THC (tetrahydrocannabinol), Δ8-THC, CBD (cannabidiol), CBG (cannabigerol), CBN (cannabinol), CBC (cannabichromene), THCV (tetrahydrocannabivarin), CBDV (cannabidivarin), CBGV (cannabigerivarin), and CBV (cannabivarin) were provided at 1.0 mg mL^{-1} in methanol. CBL (cannabicyclol) was provided at 1.0 mg mL^{-1} in acetonitrile. The acidic cannabinoids, including Δ9-THCA (tetrahydrocannabinolic acid), CBDA (cannabidiolic acid), CBGA (cannabigerolic acid), CBNA (cannabinolic acid), CBCA (cannabichromenic acid), THCVA (tetrahydrocannabivarin acid), CBDVA (cannabidivarinic acid), and CBGVA (cannabigerovarinic acid), were provided at 1.0 mg mL^{-1} in acetonitrile. CBLA (cannabicyclolic acid) was provided at 0.5 mg mL^{-1} in acetonitrile.

Isotopically labeled cannabinoids, including Δ9-THC-d_3, CBD-d_3, CBN-d_3, and CBG-d_3, were provided at 0.1 mg mL^{-1} in methanol while Δ9-THCA-d_3, CBGA-d_3, and CBCA-d_3 were provided at 0.1 mg mL^{-1} in acetonitrile. THC-d_3 was used as internal standard for Δ9-THC, Δ8-THC, THCV, CBC, and CBL. THCA-d_3 was used for THCA, CBNA, and THCVA. CBD-d_3 was used for CBD, CBDA, CBDV, and CBDVA. CBN-d_3 was used for CBN and CBV. CBG-d_3 was used for CBG and CBGV. CBGA-d_3 was used for CBGA and CBGVA and CBCA-d_3 was used for CBCA and CBLA. Ultrapure water was collected from a Millipore Milli-Q Advantage A10 mixed bed ion exchange system fed with reverse osmosis domestic water (Jaffrey, NH, USA). Optima® grade acetonitrile, methanol, and formic acid were procured from Fisher Scientific (Fair Lawn, NJ, USA).

Terpene reference standards were purchased from Restek (Bellefonte, PA, USA) and provided at 2.5 mg mL^{-1} in isopropanol. Isotopically labeled terpene (±)-linalool-d3 (vinyl-d3) was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada) and used as an internal

standard. Hexane (HPLC Plus, ≥95%) was purchased from Millipore-Sigma (Oakville, ON, Canada).

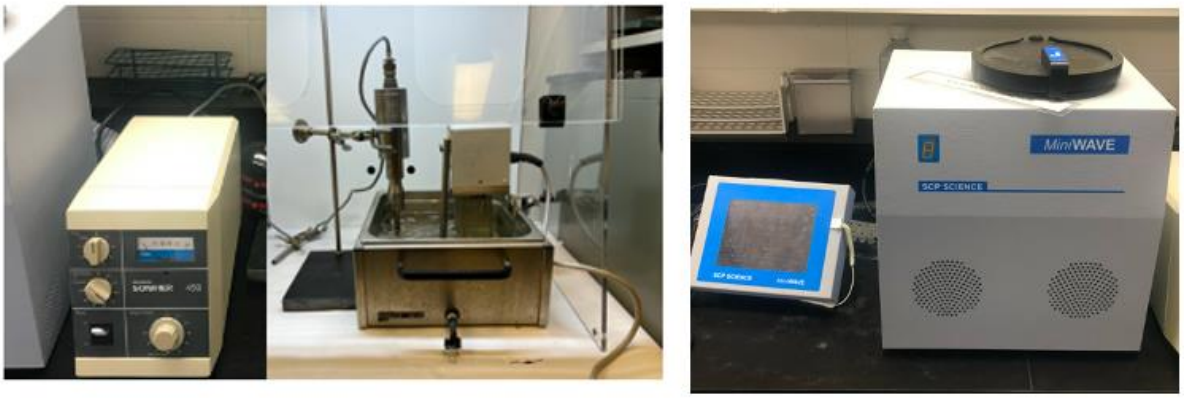
7.2.3 Extraction procedures

Ultrasound-assisted (UAE) and microwave-assisted (MAE) extractions were carried out with different sample (g)-to-solvent (g) ratios, extraction temperatures, and extraction times. The influence of the duty cycle of the ultrasound was used as an independent variable for the ultrasound-assisted extraction of cannabis oil. Sample (g)-to-solvent (g) ratios used for this study were calculated by varying the mass of cannabis biomass into 40 mL ethanol with Equation (1).

$$\text{Mass of cannabis biomass} = 40\ \text{mL} \times \frac{\text{density of ethanol } (0.789 \frac{\text{g}}{\text{mL}})}{\text{mass of ethanol (g)}} \quad (1)$$

7.2.3.1 Ultrasound-assisted extraction

A Branson Sonifier 450 ultrasound system (Marshall Scientific, Hampton, VI, USA) with a fixed working frequency of 20 kHz and an electric power output of 450 W was used for the UAE of crude cannabis oil (Figure 7.1A). The ultrasound system consisted of an ultrasound generator, a transducer, and an ultrasound probe. Cannabis biomass mixed with ethanol was placed in a 50-mL beaker positioned in a water bath with a heating coil system to maintain the extraction temperature. The ultrasonic emitter was immersed 1 cm into the solution, as previously described, (Dey and Rathod, 2013) and turned on. Acoustic cavitation (creation, growth, and implosion of gas bubbles under the ultrasonic treatment) was observed, and the duty cycle was set at the desired level, ranging from 20 to 100%. The duty cycle is the percentage of the total ultrasound extraction time during which the ultrasound signal and power are “on”.



A B

Figure 11.1. Branson Sonifier 450 ultrasound (A) and mini-wave microwave (B) extraction systems used for the study.

7.2.3.2 Microwave-assisted extraction

Microwave-assisted extraction of crude cannabis oil was performed in a multi-mode (closed) mini-wave microwave unit (SCP Science, Baie-D'Urfe, QC, Canada). The system consists of a touchscreen controller that is USB-connected with the microwave module (digestion chamber) (Figure 7.1B). The magnetron is located at the base of the module to ensure even distribution of the microwave energy across the digestion chamber. The module has six equidistant and radially constructed 75-mL vessels in a non-rotating digestion rack. Quartz vessels were used for the microwave extraction process. The average real-time operating temperature was monitored using six infrared sensors located on the side walls of the oven. Irradiation frequency and power were 2.45 GHz and 1000 W, respectively. The duration of irradiation included ramp time (time to reach the target process temperature, set at 5 min for all experiments) and hold time (elapsed time while irradiating the sample at a set temperature). The unit had a forced air ventilation system for cooling.

7.2.4 Calculation of extraction yield and efficiency

After extraction, each extract containing the solvent and cannabis biomass mixture was subjected to vacuum filtration using Whatman 4 filter paper (Sigma Aldrich, St. Louis, MO, USA) to remove any residual biomass. Vacuum rotary evaporator operating at 35 rpm and 50°C was used to evaporate the ethanol present in the extract to determine the yield of crude cannabis oil.

Extraction yield of the crude cannabis oil was calculated using Equation (2). Extraction efficiency at the optimal condition was calculated based on THCA concentration using Equation (3).

$$\text{Yield (g 100 g dry matter}^{-1}) = \frac{\text{mass of extracted crude cannabis oil (g)}}{\text{mass of dried sample (100 g)}} \quad (2)$$

$$\text{Efficiency (\%)} = \frac{\text{Concentration of THCA in extract } \left(\frac{\text{g}}{\text{100 g dry matter}}\right)}{\text{Concentration of THCA in cryo-ground sample } \left(\frac{\text{g}}{\text{100 g dry matter}}\right)} \times 100\% \quad (3)$$

7.2.5 Cannabinoid analyses using liquid chromatography-tandem mass spectrometer (LC-MS/MS)

As described in Chapter 6 section 6.2.5, a cannabinoid analysis method developed and described previously by the National Research Council of Canada was modified and used for this study (Addo et al., 2022b; McRae and Melanson, 2020; Meija et al., 2021). Extracted crude cannabis oil samples were centrifuged at 5000 rpm for 5 min. An aliquot of the supernatant was diluted in methanol based on the initial sample biomass (Table 6.1) used for the extraction (this sample is referred to as the diluted cannabis extract). Samples, standards, and quality control (QC) samples (100 µL) were transferred to high-pressure liquid chromatography (HPLC) vials containing glass inserts. The internal standard (50 µL, 500 ng mL^{-1} in methanol) was added prior to injection onto the liquid chromatography tandem mass spectrometer (LC-MS/MS) system. The LC-MS/MS system consisted of a HPLC (Ultimate3000; Thermo Fisher Scientific, Waltham, MA, USA) coupled to a triple quadrupole mass spectrometer (TSQ Quantiva; Thermo Fisher Scientific, MA, USA). Chromatographic separation was carried out on C_{18} bonded phase column (Accucore C_{18}, 150 mm × 2.1 mm i.d. with 2.6 µm particle size; Thermo Fisher Scientific, MA, USA) maintained at 40°C and the mobile phases consisted of water/formic acid and acetonitrile/formic acid both mixed in a 1000:1 volume ratio. An injection volume of 1 µL was used for the study.

The MS/MS detection of cannabinoids was performed via electrospray ionization in positive ion mode using quasi-molecular ion to product ion transitions (McRae and Melanson, 2020). The LC-MS/MS method includes both acidic and neutral forms of the cannabinoids. The neutral forms ionize only in positive mode while the acidic forms ionize equally well in both positive and negative mode. Using positive ionization mode for both neutral and acidic cannabinoids produced more consistent and more similar signal responses for all cannabinoids and resulted in a simplified method, relative to a polarity-switching method. External calibration

standard solutions containing 20 cannabinoids were prepared in methanol at concentrations of 10, 20, 100, 1000, 6000, 9000 and 10,000 ng mL^{-1} with quality control samples prepared at 30, 1500 and 8 000 ng mL^{-1}. Linear regression, weighted $1/x^2$, was used for calibration with peak area ratio of cannabinoid and internal standard as the response variable.

7.2.6 Terpene analysis using gas chromatography-tandem mass spectrometer (GC-MS/MS)

As described in Chapter 6 section 6.2.6, extracted crude cannabis oil samples were centrifuged at 5000 rpm for 5 min. An aliquot of the supernatant was diluted in hexane based on the initial sample biomass (Table 6.1) used for the extraction (referred to as the diluted cannabis extract). Samples, standards, and QC samples (150 µL) were transferred to HPLC vials containing glass inserts and the internal standard (50 µL, 1 µg mL^{-1} of linalool-d_3 in hexane) was added before injection onto the gas chromatography-tandem mass spectrometer (GC-MS/MS) system (Trace 1310 GC coupled to a TSQ 9000 Triple Quadrupole MS/MS; Thermo Fisher Scientific, MA, USA). An injection volume of 1 µL was used for the study.

Chromatographic separation of the analytes was obtained using the TraceGOLD TG-5SilMS column (30 m x 0.25 mm i.d. with 0.25 µm film thickness; Thermo Fisher Scientific, MA, USA) and helium as the carrier gas. The inlet temperature was held at 250°C with a deactivated splitless quartz wool single taper liner (78.5 mm × 4 mm i.d. × 6.3 mm o.d.; Thermo Fisher Scientific, MA, USA). A constant inlet flow of 1.5 mL min^{-1} with a split flow of 15 mL min^{-1} and a split ratio of 10 was used. Selected reaction monitoring (SRM) scan type with electron impact ionization mode was used for the tandem mass spectrometer, while the ion source temperature and MS transfer line temperature were held at 300°C and 250°C, respectively. The temperature program for the GC oven can be found in Table 6.2.

Calibration curves (0.005–2.5 µg mL^{-1}) were generated using weighted linear regression (1/x) of the peak area ratios (analyte/internal standard) versus the concentration of the calibration standards. The concentration of individual terpenes in extracts was determined using the appropriate calibration curve for the metabolite using the resulting peak area ratios. Monitored ions, ion transitions, and mass spectrometer voltage parameters are listed in Table 6.3.

7.2.7 Experimental design

A five-level-by-four-variables and five-level-by-three-variables central composite rotatable statistical design (CCRD) with uniform precision was used for ultrasound-assisted extraction and microwave-assisted extraction, respectively. CCRD was used to assess and compare the effects of the different extraction conditions on the total yield of cannabis crude oil, cannabinoids, and terpenes. It comprised 16 combinations of factorial values, 8 combinations of axial values, and 7 combinations of central values for ultrasound-assisted extraction (Table 7.1). For MAE, combinations for the factorial, axial, and central values were 8, 6, and 6, respectively (Table 7.1); this was due to the reduced number of variables for this extraction method. Axial points were fixed at a distance ($\alpha = 2^{k/4}$, where k represents the number of variables) from the center to ensure rotatability. Axial combinations allow for the inclusion of quadratic terms in the response surface model. Replication of a central point ensures a greater uniformity in the precision of response estimation over the experimental design.

Table 11.1. Uncoded and coded levels of the independent variables for ultrasound- and microwave-assisted extraction of cannabis.

Ultrasound-assisted extraction						
Independent variables	Symbol	Coded levels				
		−2	−1	0	+1	+2
Sample (g) solvent $(mL)^{-1}$	X_1	0/40	6.31/40	3.16/40	2.1/40	1.58/40
Sample (g) solvent $(g)^{-1}$	X_1	1/0	1/5	1/10	1/15	1/20
Extraction time (min)	X_2	0	10	20	30	40
Duty cycle (%)	X_3	20	40	60	80	100
Extraction temperature (°C)	X_4	30	40	50	60	70
Microwave-assisted extraction						
Independent variables	Symbol	Coded levels				
		−1.682	−1	0	+1	+1.682
Sample (g) solvent $(mL)^{-1}$	X_1	19.84/40	6.31/40	3.16/40	2.1/40	1.71/40
Sample (g) solvent $(g)^{-1}$	X_1	1/1.59	1/5	1/10	1/15	1/18.41
Extraction time (min)	X_2	3.18	10	20	30	36.82
Extraction temperature (°C)	X_4	33.18	40	50	60	66.82

7.2.8 Statistical analysis

The analysis of the independent variables' effect was assessed using JMP software (JMP 4.3 SAS Institute Inc.). The least-square multiple regression method was used to evaluate the relationship between the independent and dependent variables. Four and three multiple regression equations were used to fit the second-order polynomial model based on the experimental data for ultrasound-assisted extraction and microwave-assisted extraction, respectively (Table 7.2). Models A and E represent the full regression model for UAE and MAE, respectively. It includes all the independent terms, their interactions, and quadratic terms. The reduced regression models for UAE (models B, C, and D) and MAE (models F and G) were evaluated by controlling one independent parameter. The analysis of variance (ANOVA) was used to investigate the statistical significance of the regression coefficients by conducting the Fisher's F-test at a 95% confidence level. The statistical significance of the model was improved through a "backward elimination" process, deleting non-significant dependent terms ($p > 0.05$). A response surface plot was obtained using the fitted model. Optimal conditions for MAE and UAE for the dependent variables were determined based on modelling and desirability function and principal component analysis (PCA) using JMP software (JMP 4.3 SAS Institute Inc.).

Table 11.2. Multiple regression equation for ultrasound-assisted extraction and microwave-assisted extraction.

Model	Multiple regression equation	Equation no.
Ultrasound-assisted extraction		
Model A	$Y_j = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_1X_1 + \beta_{22}X_2X_2 + \beta_{33}X_3X_3 + \beta_{44}X_4X_4 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4$	4
Model B	$Y_j = \beta_0 + \beta_1X_1 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_1X_1 + \beta_{33}X_3X_3 + \beta_{44}X_4X_4 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{34}X_3X_4$	5
Model C	$Y_j = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_4X_4 + \beta_{11}X_1X_1 + \beta_{22}X_2X_2 + \beta_{44}X_4X_4 + \beta_{12}X_1X_2 + \beta_{14}X_1X_4 + \beta_{24}X_2X_4$	6
Model D	$Y_j = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1X_1 + \beta_{22}X_2X_2 + \beta_{33}X_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3$	7
Microwave-assisted extraction		

Model E	$Y_j = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_4X_4 + \beta_{11}X_1X_1 + \beta_{22}X_2X_2 + \beta_{44}X_4X_4 + \beta_{12}X_1X_2 + \beta_{14}X_1X_4 + \beta_{24}X_2X_4$	8
Model F	$Y_j = \beta_0 + \beta_1X_1 + \beta_4X_4 + \beta_{11}X_1X_1 + \beta_{44}X_4X_4 + \beta_{14}X_1X_4$	9
Model G	$Y_j = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_{11}X_1X_1 + \beta_{22}X_2X_2 + \beta_{12}X_1X_2$	10

Where Y_j represents the predicted response (dependent variables), the model intercept (β_0), linear terms (β_1, β_2, β_3, and β_4), interaction terms (β_{11}, β_{22}, β_{33}, and β_{44}) and quadratic terms (β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34}), and X_1 (Sample (g) solvent $(g)^{-1}$), X_2 (Extraction time (min)), X_3 (Duty cycle (%)), and X_4 (Extraction temperature (°C)) are the independent variables.

7.2.9 Model verification

To verify the model, three experiments were conducted using optimal extraction conditions with the highest desirability. The experimental and predicted values were compared which determined the validity of the model.

7.3 Results and discussions

7.3.1 Preliminary ultrasound-assisted extraction and microwave-assisted extraction data

Microwave- and ultrasound-assisted extraction methods were studied and compared. The selection of independent variables and their ranges for the extraction systems were based on preliminary experiments and a literature review of the probable effects of microwaves and ultrasound on the yield of cannabis oil, cannabinoids, and terpenes. The influence of the independent variables on the extraction of cannabis oil and the secondary metabolite profile by UAE and MAE was studied using the central composite rotatability design (CCRD). The central composite rotatable design was used because it consisted of five levels for each independent variable and was able to test fourth-order quadratic models.

Major cannabinoid concentrations of the ground cannabis biomass are listed in Table 6.5. Cannabinoid and terpene chromatographs and concentrations for the biomass used in this work and a parallel study (Figure 6.1) were measured using the liquid chromatography-tandem mass spectrometer (LC-MS/MS) and gas chromatography-tandem mass spectrometer (GC-MS/MS), respectively. The total chromatographic run time was 18 min for the cannabinoids and 25 min for the terpenes. Cryo-ground biomass used for the study contained 17.9 g 100 g dry $matter^{-1}$

(THCA), 0.17 g 100 g dry matter^{-1} (THC), 0.04 g 100 g dry matter^{-1} (CBDA), and 1.01 g 100 g dry matter^{-1} (THCVA).

The results of 31 and 20 experimental runs for UAE and MAE, respectively, carried out under the CCRD matrix for cannabis oil yields, cannabinoid concentration, and terpene concentration are presented in Tables 7.3 and 7.4. Extraction conditions with 0 yield indicate that no extraction procedure was performed either due to a high concentration of sample or 0 extraction time. The reproducibility of the extraction data was verified through results obtained by the replication of the central points. No significant differences were observed in the responses of the central points for both extraction methods. Seven major cannabinoids, namely tetrahydrocannabinol (THC), tetrahydrocannabinolic acid (THCA), tetrahydrocannabivarin (THCVA), cannabigerol (CBG), cannabigerolic acid (CBGA), and cannabichromene acid (CBCA), were observed in all extracted samples (Tables 7.3 and 7.4). Cannabidiol (CBD) and total CBD were not presented, as the concentration of CBD was below the limit of detection of the instrument and methodology. The findings demonstrate that the extracted cannabis oil yield ranged from 21.8 to 30.6 g 100 g dry matter^{-1} and 16.6 to 24.6 g 100 g dry matter^{-1} for UAE and MAE, respectively. Preliminary experiments showed that UAE extracted 16.6% more oil compared with MAE for samples extracted at 60°C with a s: s of 1 g of cannabis biomass-to-15 mL of ethanol for 30 min. This significant effect ($p < 0.05$) in cannabis oil can be attributed to the structural damages and the improved solvent penetration into the plant matrix caused by the acoustic vibrations in UAE. Similar observations were made for the THCA (10.5%) and total terpenes (10.7%).

Table 11.3. Crude cannabis oil yield and concentration of cannabinoids and total terpenes obtained from cryo-ground cannabis subjected to ultrasound-assisted extraction.

Independent variables				Response/Dependent variables (g 100 g dry matter^{-1})									
X_1	X_2	X_3	X_4	THC	THCA	Total THC	CBG	CBGA	Total CBGA	THCVA	CBCA	Total terpenes	Yield
1/0	20	60	50	0	0	0	0	0	0	0	0	0	0
1/5	10	40	40	0.44	17.06	15.4	0.08	0.23	0.29	0.80	0.09	0.98	21.80
1/5	10	40	60	0.47	18.4	16.61	0.08	0.23	0.28	0.87	0.09	0.93	23.42
1/5	10	80	40	0.57	19.34	17.53	0.09	0.24	0.31	0.96	0.10	0.99	24.49
1/5	10	80	60	0.59	20.21	18.31	0.1	0.26	0.33	0.99	0.11	0.97	25.63
1/5	30	40	40	0.55	20.36	18.4	0.09	0.27	0.33	1.16	0.12	0.96	24.41
1/5	30	40	60	0.57	22.58	20.38	0.11	0.23	0.32	1.18	0.12	0.92	25.4
1/5	30	80	40	0.59	24.24	21.85	0.12	0.25	0.34	1.29	0.11	0.89	25.99
1/5	30	80	60	0.61	25.22	22.73	0.12	0.24	0.33	1.32	0.11	0.99	26.11
1/10	0	60	50	0	0	0	0	0	0	0	0	0	0
1/10	20	20	50	0.63	26.14	23.55	0.13	0.24	0.34	1.31	0.13	1.01	26.35
1/10	20	60	30	0.66	26.34	23.75	0.14	0.35	0.44	1.37	0.15	1	26.5
1/10	20	60	50	0.74	26.4	22.04	0.15	0.36	0.44	1.42	0.13	1.05	26.9
1/10	20	60	50	0.79	26.31	24.74	0.15	0.4	0.47	1.47	0.15	1.04	27.53
1/10	20	60	50	0.72	26.44	22.93	0.16	0.37	0.45	1.48	0.14	1.05	27.22
1/10	20	60	50	0.77	26.67	20.58	0.15	0.37	0.4	1.42	0.12	1.05	26.58

1/10	20	60	50	0.78	25.66	23.29	0.16	0.39	0.48	1.43	0.14	1.03	26.5
1/10	20	60	50	0.77	26.05	23.52	0.15	0.39	0.47	1.41	0.14	1.05	26.9
1/10	20	60	50	0.73	26.53	23	0.15	0.37	0.45	1.47	0.14	1.03	27.44
1/10	20	60	70	0.84	26.3	23.91	0.16	0.38	0.49	1.62	0.14	1.17	27.85
1/10	20	100	50	0.80	25.82	23.44	0.16	0.37	0.48	1.6	0.13	1.11	27.13
1/10	40	60	50	0.79	26.1	23.68	0.15	0.36	0.47	1.63	0.14	1.1	26.81
1/15	10	40	40	0.89	27.15	24.7	0.17	0.47	0.58	1.64	0.14	1.28	27.96
1/15	10	40	60	0.91	27.77	25.27	0.16	0.47	0.58	1.68	0.24	1.22	28.57
1/15	10	80	40	0.93	27.95	25.45	0.17	0.45	0.56	1.73	0.14	1.19	28.71
1/15	10	80	60	0.96	28.43	25.89	0.17	0.48	0.6	1.76	0.15	1.05	29.25
1/15	30	40	40	0.93	28.12	25.59	0.18	0.49	0.6	1.75	0.16	1.12	28.91
1/15	30	40	60	0.97	28.21	25.7	0.18	0.44	0.57	1.79	0.17	1.12	29.38
1/15	30	80	40	1.14	28.45	26.1	0.18	0.45	0.57	1.81	0.17	1.16	29.52
1/15	30	80	60	1.15	28.74	26.35	0.19	0.47	0.6	1.88	0.19	1.12	29.86
1/20	20	60	50	1.22	29.19	26.82	0.2	0.55	0.68	2.05	0.21	1.6	30.63

X_1 (Sample (g) solvent $(g)^{-1}$), X_2 (Extraction time (min)), X_3 (Duty cycle (%)), and X_4 (Extraction temperature (°C)) are the independent variables.

Table 11.4. Crude cannabis oil yield and concentration of cannabinoids and total terpenes obtained from cryo-ground cannabis subjected to microwave-assisted extraction.

Independent variables			Response/dependent variables (g 100 g dry matter^{-1})									
X_1	X_2	X_4	THC	THCA	Total THC	CBG	CBGA	Total CBGA	THCVA	CBCA	Total terpenes	Yield
1/1.59	20	50	0	0	0	0	0	0	0	0	0	0
1/5	10	40	0.39	13.17	11.94	0.06	0.19	0.23	0.70	0.24	0.73	16.64
1/5	10	60	1.32	16.12	15.45	0.09	0.24	0.30	0.87	0.33	0.79	19.18
1/5	30	40	0.45	11.95	10.93	0.06	0.18	0.21	0.67	0.25	0.73	16.35
1/5	30	60	0.63	13.42	12.40	0.07	0.20	0.24	0.73	0.24	0.69	14.72
1/10	3	50	0.44	15.29	13.85	0.08	0.22	0.27	0.83	0.30	0.92	23.97
1/10	20	33	0.38	16.68	15.01	0.08	0.25	0.30	0.91	0.31	0.97	22.01
1/10	20	50	0.56	17.16	15.60	0.09	0.25	0.30	0.93	0.32	1.02	24.13
1/10	20	50	0.61	14.48	13.31	0.07	0.20	0.25	0.80	0.29	0.90	23.42
1/10	20	50	0.74	19.56	17.90	0.10	0.28	0.35	1.10	0.39	0.67	24.05
1/10	20	50	0.46	12.81	11.70	0.06	0.19	0.23	0.71	0.25	0.98	23.81
1/10	20	50	0.71	21.03	19.16	0.10	0.30	0.37	1.20	0.42	1.07	23.66
1/10	20	50	0.53	18.05	16.36	0.08	0.26	0.31	1.00	0.35	0.97	24.61
1/10	20	67	1.10	14.16	13.52	0.08	0.21	0.27	0.79	0.28	0.83	24.05
1/10	37	50	0.78	15.04	13.96	0.08	0.23	0.28	0.87	0.30	0.96	25.00
1/15	10	40	0.35	16.48	14.80	0.08	0.23	0.28	0.90	0.32	0.37	25.36

1/15	10	60	0.83	18.18	16.78	0.09	0.27	0.33	1.03	0.35	0.89	23.70
1/15	30	40	0.56	22.42	20.22	0.11	0.32	0.39	1.27	0.45	0.96	24.76
1/15	30	60	1.11	25.26	23.27	0.13	0.39	0.47	1.43	0.50	1.00	24.40
1/18.41	20	50	0.39	18.00	16.18	0.09	0.26	0.32	1.04	0.35	1.12	24.56

X_1 (Sample (g) solvent $(g)^{-1}$), X_2 (Extraction time (min)), and X_4 (Extraction temperature (°C)) are the independent variables.

7.3.2 Effect of ultrasound-assisted and microwave-assisted extraction parameters on cannabis oil yield

Optimizing the extraction yield is critical to the development of medicinal cannabis products, as increasing extract yield can reduce the overall production cost. The effects of four and three independent variables for UAE and MAE on the cannabis extraction yield were evaluated according to the significant coefficient ($p < 0.05$) of the full quadratic polynomial equation. The cannabis extraction yield for UAE was significantly ($p < 0.05$) influenced by sample (g) solvent $(g)^{-1}$ and extraction temperature with first-order linear and second-order quadratic effects (extraction time^2). A linear effect of sample (g) to solvent $(g)^{-1}$ and a quadratic effect (s: s^2) was observed for the extraction yield with MAE. According to these data, extending the UAE time from 10 min to 30 min resulted in a higher extraction yield (3.3%).
Positive coefficient values (Tables 7.5 and 7.6) for sample (g) to solvent $(g)^{-1}$ for UAE and MAE showed that increasing the s: s significantly ($p < 0.05$) increases the extraction yield. A similar observation was made for the UAE extraction time. Thus, at a constant temperature of 40°C, increasing the sample (g) solvent $(g)^{-1}$ from 1:5 to 1:15 increases the yield by 15.6% and 33.8% for UAE and MAE, respectively, when samples were extracted for 30 min. Various extraction studies report that increasing sample (g) solvent $(g)^{-1}$ can facilitate the mass transfer of compounds from the plant matrix into the solvent (Sharma and Orsat, 2022; Sulaiman et al., 2017; Zakaria et al., 2021). Sulaiman et al. (2017) showed that increasing the ratio of lindau (*Clinacanthus nutans*) leaves to ethanol from 70/30 (% *v*/*v*) to 90/10 (% *v*/*v*) increased the extraction yield by 20.8%.

Table 11.5. Matrix of the central composite rotatable statistical design (CCRD) and observed responses (Yj) for ultrasound-assisted extraction using model A.

Response/Dependent Variables		Regression Model Effect Parameters														
		Intercept	Linear				Interaction						Quadratic			
		β_0	β_1	β_2	β_3	β_4	β_{12}	β_{13}	β_{23}	β_{14}	β_{24}	β_{34}	β_{11}	β_{22}	β_{33}	β_{44}
THC	Coefficient	0.76	0.25	0.1	0.05	0.02	0.02	0.01	0.01	0.001	−0.001	−0.002	−0.01	−0.07	0.01	0.02
	p value	<0.0001*	<0.0001*	0.01*	0.11	0.43	0.66	0.79	0.82	0.99	0.99	0.96	0.65	0.02*	0.59	0.38
THCA	Coefficient	26.29	4.82	2.99	0.51	0.28	−0.95	−0.52	0.11	−0.25	0.02	−0.1	−2.17	−2.56	0.68	0.76
	p value	<0.0001*	0.0001*	0.01*	0.59	0.77	0.42	0.66	0.92	0.83	0.99	0.93	0.02 *	0.01*	0.44	0.39
Total	Coefficient	22.87	4.48	2.72	0.50	0.27	−0.82	−0.44	0.11	−0.22	0.01	−0.01	−1.68	−2.07	0.84	0.93
THC	p value	<0.0001*	<0.0001*	0.01*	0.57	0.76	0.45	0.68	0.92	0.84	0.99	0.93	0.05	0.02*	0.3	0.26
CBG	Coefficient	0.15	0.04	0.02	0.01	0.003	−0.002	−0.003	0.001	−0.002	0.002	0.001	−0.01	−0.02	0.001	0.003
	p value	<0.0001*	<0.0001*	0.01*	0.17	0.52	0.73	0.57	0.91	0.73	0.73	0.91	0.02 *	0.01*	0.76	0.54
CBGA	Coefficient	0.38	0.12	0.03	0.01	0.001	−0.003	−0.003	−0.003	0.002	−0.01	0.01	−0.01	−0.04	−0.01	0.01
	p value	<0.0001*	<0.0001*	0.05	0.45	0.93	0.86	0.86	0.86	0.92	0.66	0.61	0.32	0.01*	0.65	0.52
Total	Coefficient	0.45	0.15	0.04	0.02	0.01	−0.01	−0.01	−0.003	0.003	−0.004	0.01	−0.01	−0.04	0.004	0.02
CBG	p value	<0.0001*	<0.0001*	0.02*	0.4	0.76	0.8	0.8	0.89	0.89	0.84	0.71	0.42	0.03*	0.80	0.29
THCVA	Coefficient	1.44	0.4	0.21	0.06	0.03	−0.06	−0.01	−0.002	0.002	−0.001	−0.001	−0.07	−0.12	0.04	0.05
	p value	<0.0001*	<0.0001*	0.01*	0.23	0.48	0.35	0.81	0.98	0.98	0.99	0.99	0.14	0.01*	0.39	0.28
CBCA	Coefficient	0.14	0.04	0.02	−0.002	0.01	−0.003	−0.004	0.004	0.01	−0.01	−0.004	−0.003	−0.01	0.002	0.01
	p value	<0.0001*	<0.0001*	0.03*	0.75	0.41	0.69	0.58	0.58	0.31	0.48	0.58	0.53	0.05*	0.68	0.3
Total	Coefficient	1.04	0.2	0.08	0.001	0.004	−0.01	−0.02	0.02	−0.01	0.02	0.003	−0.03	−0.09	0.03	0.04
terpenes	p value	<0.0001*	0.002*	0.17	0.98	0.95	0.92	0.81	0.82	0.83	0.79	0.96	0.55	0.08	0.5	0.43

Yield	Coefficient	27.01	4.01	2.64	0.47	0.36	−0.21	−0.29	−0.18	−0.12	−0.12	−0.1	−1.89	−2.37	0.96	1.07
	p value	<0.0001*	0.0045*	0.04*	0.70	0.77	0.89	0.85	0.9	0.94	0.93	0.95	0.11	0.05*	0.4	0.35

Model A ($Y_j = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_1X_1 + \beta_{22}X_2X_2 + \beta_{33}X_3X_3 + \beta_{44}X_4X_4 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4$).

Where Y_j represents the predicted response (dependent variables), the model intercept (β_0), linear terms (β_1, β_2, β_3, and β_4), interaction terms (β_{11}, β_{22}, β_{33}, and β_{44}) and quadratic terms (β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34}), and X_1 (Sample (g) solvent $(g)^{-1}$), X_2 (Extraction time (min)), X_3 (Duty cycle (%)), and X_4 (Extraction temperature (°C)) are the independent variables.* Independent effects are statistically significant if $p < 0.05$.

Table 11.6. Matrix of the central composite rotatable statistical design (CCRD) and observed responses (Yj) for microwave-assisted extraction using model E.

Response/Dependent Variables		Regression Model Effect Parameters									
		Intercept	Linear			Interaction			Quadratic		
		β_0	β_1	β_2	β_3	β_{12}	β_{13}	β_{23}	β_{11}	β_{22}	β_{33}
THC	Coefficient	0.6	0.05	0.03	0.25	0.14	−0.01	−0.09	−0.1	0.04	0.09
	p value	<0.0001*	0.33	0.55	<0.0001*	0.06	0.88	0.23	0.07	0.40	0.1
THCA	Coefficient	17.05	4.24	0.64	0.35	2.12	0.02	−0.04	−2.01	0.17	0.26
	p value	<0.0001*	0.002*	0.53	0.73	0.13	0.99	0.97	0.06	0.86	0.79
Total THC	Coefficient	15.55	3.78	0.59	0.55	2.00	0.01	−0.12	−1.87	0.19	0.32
	p value	<0.0001*	0.002*	0.53	0.55	0.12	0.93	0.92	0.06	0.83	0.73
CBG	Coefficient	0.08	0.02	0.003	0.01	0.01	−0.001	−0.001	−0.01	0.003	0.003
	p value	<0.0001*	0.002*	0.48	0.32	0.11	0.85	0.85	0.07	0.57	0.57

CBGA	Coefficient	0.24	0.06	0.01	0.01	0.03	0.01	0.002	−0.03	0.01	0.01
	p value	<0.0001*	0.002*	0.40	0.58	0.12	0.80	1	0.07	0.73	0.64
Total CBG	Coefficient	0.30	0.08	0.01	0.01	0.04	0.004	−0.001	−0.04	0.01	0.01
	p value	<0.0001*	0.001*	0.45	0.46	0.1	0.87	0.96	0.06	0.76	0.61
THCVA	Coefficient	0.95	0.25	0.05	0.02	0.12	0.01	−0.01	−0.11	0.01	0.01
	p value	<0.0001*	0.001*	0.39	0.68	0.13	0.92	0.89	0.07	0.86	0.86
CBCA	Coefficient	0.33	0.08	0.01	0.01	0.05	0.003	−0.01	−0.04	0.005	0.003
	p value	<0.0001*	0.002*	0.47	0.69	0.11	0.9	0.7	0.06	0.81	0.89
Total	Coefficient	0.94	0.16	0.05	0.03	0.1	0.07	−0.07	−0.14	−0.003	−0.02
terpenes	p value	<0.0001*	0.02*	0.42	0.68	0.22	0.40	0.37	0.03*	0.96	0.77
Yield	Coefficient	23.92	5.32	−0.21	0.17	0.61	−0.37	−0.36	−3.92	0.39	−0.12
	p value	<0.0001*	<0.0001*	0.73	0.78	0.46	0.65	0.66	<0.0001*	0.52	0.84

Model E ($Y_j = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_4X_4 + \beta_{11}X_1X_1 + \beta_{22}X_2X_2 + \beta_{44}X_4X_4 + \beta_{12}X_1X_2 + \beta_{14}X_1X_4 + \beta_{24}X_2X_4$).

Where Y_j represents the predicted response (dependent variables), the model intercept (β_0), linear terms (β_1, β_2, and β_4), interaction terms (β_{11}, β_{22}, and β_{44}) and quadratic terms (β_{12}, β_{14}, and β_{24}), and X_1 (Sample (g) solvent $(g)^{-1}$), X_2 (Extraction time (min)), and X_4 (Extraction temperature (°C)) are the independent variables.* Independent effects are statistically significant if $p < 0.05$.

Effects of sample (g) solvent $(g)^{-1}$ and duty cycle (%) for ultrasound-assisted extraction (A–C) and sample (g) solvent $(g)^{-1}$ and extraction temperature (^{o}C) for microwave-assisted extraction (D–F) on the concentration (g 100 g dry $matter^{-1}$) of total THC (A and D), total terpenes (B and E), and extraction yield (C and F) are illustrated in three-dimensional (3D) response surface plots (Figure 7.2). The extraction temperature insignificantly ($p < 0.05$) influenced the extraction yield for both UAE and MAE. An increase in the extraction temperature would confer either a negative or positive effect on extraction yield. This is evident with the slow linear increase in the extract yield from 28 to 28.6 g 100 g dry $matter^{-1}$ for UAE and a slight decrease from 24.7 to 24.4 g 100 g dry $matter^{-1}$ for MAE as the temperature rose from 40 to 60°C using a sample (g)-to-solvent (g) ratio of 1:15. Increasing extraction temperature reduces solvent density, promoting an increase in the mass transfer rate and solute solubility, which improves the extraction yield. Irakli et al., (2018) showed that total phenolic compounds increased as the ultrasound extraction temperature increased from 25 to 60°C for olives (*Olea europaea*). However, excessive extraction temperature degrades certain phytochemical compounds such as antioxidants (tannins, oxalate, etc.) and should be avoided (Onyebuchi and Kavaz, 2020; Serea et al., 2022).

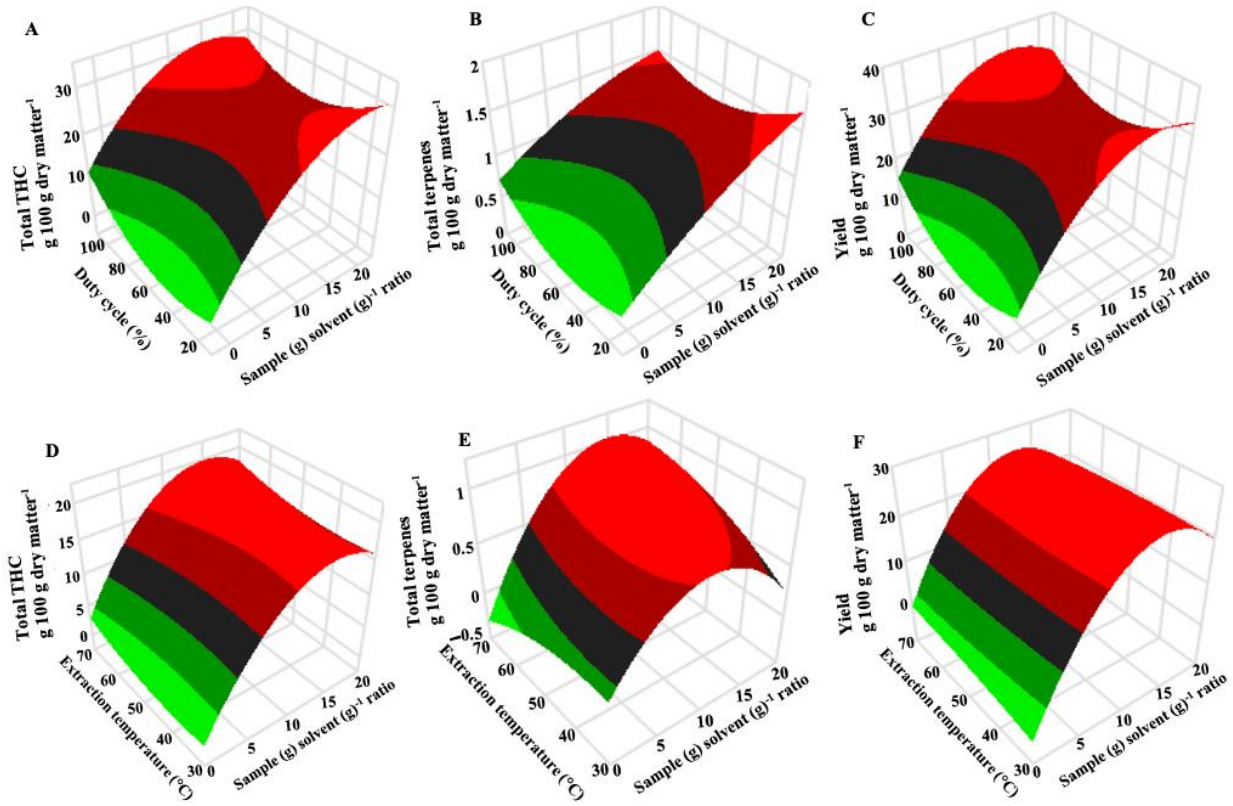


Figure 11.2. 3D response surface plots illustrating the combined effects of independent variables for microwave- and ultrasound-assisted extractions.

7.3.3 Effect of ultrasound-assisted and microwave-assisted extraction parameters on cannabinoids

Cannabinoids are mainly responsible for the therapeutic effects of cannabis (Tahir et al., 2021). Sample (g)-to-solvent (g) ratio had a linear significant ($p < 0.05$) effect on the major cannabinoids analyzed for both extraction systems, except for THC content after MAE (Tables 7.5 and 7.6). This is supported by the low correlation (0.41) between MAE extraction yield and THC concentration and the parabolic shape of the curve (Figure 7.2). The THC content in MAE extracts was influenced by extraction temperature. For UAE, the quadratic effect of extraction time affected all cannabinoids; however, the quadratic effect of sample (g)-to-solvent (g) ratio only influenced THCA and CBG content. A positive coefficient value for sample (g)-to-solvent (g) ratio showed that increasing the sample (g)-to-solvent (g) ratio significantly ($p < 0.05$) increased cannabinoid concentration in extracts. Increasing the ratio from 1:5 to 1:15 increased the total THC in extracts by 37.7% and 19.3% for UAE and MAE, respectively, when samples

were extracted for 10 min at 40°C. This is likely due to cavitation bubbles with UAE and volumetric heating properties with MAE.

Ultrasound-assisted extraction involves mechanical oscillating sound waves ranging from 20 kHz to 2 MHz that produce acoustic cavitation (Chemat et al., 2017). Acoustic cavitation is affected by sample (g)-to-solvent (g) ratio and physical properties of the solvent, such as viscosity, saturation vapor pressure, and surface tension (Rastogi, 2011; Sousa et al., 2021). Decreasing the sample (g)-to-solvent (g) ratio and viscosity of the solvent intensifies molecular interactions and thus hinders cavitation. The mechanical effect caused by the cavitation increases the permeability of the plant's cell walls and improves the yield of cannabinoids (Agarwal et al., 2018; Albero et al., 2019; Chemat et al., 2017). Zakaria et al., 2021 showed that increasing the ratio of havil (*Mitragyna speciosa*) leaves to methanol by 66% increased the extraction yield and total phenolic content by 36.1% and 6.7%, respectively.

Microwave-assisted extraction uses microwaves to create heat and mass gradients (Chandrasekaran et al., 2013; Darvishzadeh and Orsat, 2022). Microwaves increase the kinetic energy of the solvent and improve the rate of penetration of the solvent into the solid matrix. Cannabinoids dissolve in the solvent and the solution diffuses to the surface of the solid. By natural or forced convection, the solution is transferred from the surface of the solid to the bulk medium. Increasing the sample-to-solvent ratio increases the amount of solvent diffusing into the solid matrix and hence, improves the concentration of secondary metabolites in the extracts (Darvishzadeh and Orsat, 2022; Krishnaswamy et al., 2013).

7.3.4 Effect of ultrasound-assisted and microwave-assisted extraction parameters on the total terpenes

Terpenes are mainly responsible for the aroma of cannabis plants (Ashour et al., 2018). The greater terpene content of 23.8 to 25.8% (Tables 7.5 and 7.6) observed with UAE extracts compared with MAE extracts can be attributed to the simultaneous action of the sonication that promoted the hydration and fragmentation reaction while expediting the rate of mass transfer of solutes to the extraction solvent and avoiding substantial solvent degradation. The sample-to-solvent ratio had a linear effect on total terpene content for both UAE and MAE. However, a quadratic effect (sample-to-solvent^2) was only observed for MAE (Table 7.6). The main terpenes identified in the cannabis-extracted oil in this investigation were pinene, myrcene, eucalyptol,

limonene, linalool, caryophyllene, and humulene. The observed terpenes are reported to have peppery, citrus, and hoppy mixed aroma (Surendran et al., 2021; Vieira et al., 2018). As indicated in Figure 7.2, the duty cycle did not have a significant ($p < 0.05$) effect on terpene content. Increasing the duty cycle from 40 to 80% at a constant sample (g)-to-solvent (g) ratio of 1:5 and extraction temperature of 40°C, caused a non-significant ($p > 0.05$) increase from 0.98 to 0.99 g 100 g dry matter^{-1}. Extraction time and extraction temperature had similar minimal effects on the terpenes extracted. Terpenes have a low molecular mass and boiling point compared with other plant secondary metabolites (Sommano et al., 2020). They undergo thermal degradation with prolonged extraction time and increased temperature. Response surface plots (Figure 7.2) show that increasing the sample-to-solvent ratio from 1/5 to 1/15 caused a significant increase in terpenes by 11.2% and 23.8% for MAE and UAE, respectively, at a constant temperature (60°C) and time (10 min).

7.3.5 Model fitting for ultrasound-assisted and microwave-assisted extraction systems

Responses consisting of THC, THCA, total THC, CBG, CBGA, total CBGA, THCVA, CBCA, total terpene content, and extraction yield for cannabis extracts for UAE and MAE were optimized using CCRD. Four and three second-order polynomial regression models were used to fit the experimental data for UAE and MAE, respectively. The models were included in the study to help explain the correlations between the independent variables and dependent variables and assist scale-up purposes. Regression coefficients for the intercept, linear, quadratic and interaction terms of the models were statistically analyzed and are presented in Tables 7.5, 7.6 and 11.1–11.5 (Appendix). Based on ANOVA (Table 7.7) and the lack-of-fit data (0.05 to 0.98), models B and F are the best models for explaining the experimental data acquired for UAE and MAE, respectively. F ratios ranging from 1.5 to 5.8 and 3 to 30.5 for UAE and MAE, respectively, imply the significance of all the models. Notably, model E was not significant ($p < 0.05$) and does not explain the THCA and extract yield data. This is evident by the strong correlation (0.97) between THCA and the extraction yield.

Table 11.7. Analysis of variance (ANOVA) of responses for ultrasound- assisted and microwave-assisted extractions.

Response	Source	df	SS	MS	F Ratio	Prob > F	Lack-of-Fit	R^2	Adj. R^2
Ultrasound-assisted extraction using model B (sample (g) solvent $(g)^{-1}$, duty cycle (%), and extraction temperature (°C) as independent parameters)									
THC	Model	9	1.57	0.17	5.57	0.001*	0.79	0.70	0.58
	Error	21	0.66	0.03					
THCA	Model	9	750.49	83.39	2.32	0.05	0.77	0.51	0.28
	Error	21	755.83	35.99		(0.005*)	(0.33)		
Total THC	Model	9	637.95	70.88	2.49	0.04*	0.74	0.52	0.31
	Error	21	598.79	28.51					
CBG	Model	9	0.05	0.01	4.67	0.002*	0.98	0.67	0.52
	Error	21	0.02	0.00					
CBGA	Model	9	0.36	0.04	5.68	0.001*	0.82	0.71	0.58
	Error	21	0.15	0.01					
Total CBG	Model	9	0.53	0.06	5.81	0.0004*	0.83	0.71	0.59
	Error	21	0.22	0.01					
THCVA	Model	9	4.23	0.47	4.10	0.004*	0.89	0.64	0.48
	Error	21	2.41	0.11					
CBCA	Model	9	0.04	0.00	3.56	0.008 *	0.66	0.60	0.43
	Error	21	0.03	0.00					
Total	Model	9	1.13	0.13	1.68	0.16	0.18	0.62	0.47
terpenes	Error	21	1.56	0.07		(0.02*)	(0.05)		
Yield	Model	9	582.84	64.76	1.52	0.20	0.37	0.69	0.44
	Error	21	893.61	42.55		(0.04*)	(0.06)		
Microwave-assisted extraction using model F (sample (g) solvent $(g)^{-1}$ and extraction temperature (°C) as independent parameters)									
THC	Model	5	1.16	0.23	5.28	0.01*	0.22	0.65	0.53
	Error	14	0.62	0.04					
THCA	Model	5	310.11	62.02	4.99	0.01 *	0.1	0.64	0.51
	Error	14	174.18	12.44					

Total THC	Model	5	253.77	50.75	4.81	0.01*	0.09	0.63	0.5
	Error	14	147.7	10.55					
CBG	Model	5	0.01	0	4.67	0.01*	0.12	0.63	0.49
	Error	14	0	0					
CBGA	Model	5	0.07	0.01	4.59	0.01*	0.08	0.62	0.49
	Error	14	0.04	0.00					
Total CBG	Model	5	0.1	0.02	4.91	0.01*	0.11	0.64	0.51
	Error	14	0.06	0					
THCVA	Model	5	1.03	0.21	5.21	0.01*	0.14	0.65	0.53
	Error	14	0.56	0.04					
CBCA	Model	5	0.12	0.02	4.67	0.01*	0.15	0.63	0.49
	Error	14	0.07	0.01					
Total terpenes	Model	5	0.66	0.13	2.96	0.04*	0.05	0.51	0.34
	Error	14	0.63	0.04					
Yield	Model	5	616.93	123.4	30.46	<0.001*	<0.001*	0.92	0.89
	Error	14	56.71	4.05					

Effects are statistically significant if p-value * < 0.05. p-values for ANOVA and lack-of-fit for the revised model B, which has only sample (g) solvent $(g)^{-1}$ and extraction temperature (°C) as independent parameters. Degree of freedom (df), Sum of squares (SS), and Mean square (MS). p-values for ANOVA and Lack-of-fit for the revised model, model E, which include only the sample-to-solvent (g) ratio and extraction temperature (°C) as independent parameters are shown in parenthesis.

Revising model E to include only the sample-to-solvent (g) ratio and extraction temperature (°C) as independent parameters, was the ideal model for THCA and the yield. R^2 values above 0.5 demonstrated a significant correlation between the CCRD design and the developed models. Apart from R^2 values, the lack-of-fit analysis determines the validity of the models in which a *p*-value > 0.05 indicates that the model fits accurately with the experimental data. Since the lack-of-fit was only significant ($p < 0.05$) for the MAE extraction yield, this means that the quadratic polynomial model F does not accurately predict extract yield for cannabis oil using MAE. Further studies exploring other factors, such as microwave power,

could be conducted and included in the model to expand our understanding of this method. The low coefficient of determination (R^2) values for both models B and F showed that the models can be improved by considering the effects of other independent variables, such as particle size of biomass and ultrasound and microwave power densities on the extraction of cannabis oil, cannabinoids, and terpenes.

7.3.6 Optimal experimental conditions for ultrasound-assisted and microwave-assisted extraction systems for cannabis

Ultrasound- and microwave-assisted extraction for cannabis were successfully optimized with a response surface methodology when evaluating the effects of the independent parameters of this study. All independent parameters were kept within the range for both extraction systems. Optimization was based on the maximum desirability function for the maximum yield of cannabinoids, total terpenes, and extracted cannabis oil. The desirability function consolidates all the responses into one response with a numerical value varying from 0 (one or more product characteristics are unacceptable) to 1 (all product characteristics are on target). The optimal independent experimental conditions for UAE and MAE at various conditions and the predicted responses at 95% confidence interval are presented in Table 7.8. UAE and MAE extractions of cannabis using a sample-to-solvent of 1:15 and 1:14.4, respectively, for 30 min at 60°C were presented as the optimal conditions for maximum responses. Statistical analyses of the predicted responses showed significant ($p < 0.05$) differences between the extraction yields and secondary metabolite profiles for UAE and MAE. Under the optimal conditions, UAE extracts resulted in 14.4% more oil from cannabis biomass compared with MAE (Table 7.8). The concentration of total terpenes extracted was reduced by 14.7% when MAE was used. Compared with the reference ground sample (Table 6.5), the THCA concentration increased from 17.9 (g 100 g dry matter^{-1}) to 28.5% and 20% with extraction efficiencies of 159.2% and 111.4% for ultrasound-assisted and microwave-assisted extraction, respectively. Extraction efficiency greater than 100% can be explained by the biosynthesis or conversion of other cannabinoids to THCA during the extraction process or variance due to the analytical method used.

Table 11.8. Optimal experimental conditions for ultrasound-assisted and microwave-assisted extraction systems and predicted response values.

Extraction Method	Ultrasound-assisted extraction	Microwave-assisted extraction
Desirability	0.83	0.75
Sample (g) solvent (40 mL)$^{-1}$	2.1	2.19
Sample (g) solvent (g)$^{-1}$	1/15	1/14.43
Duty Cycle (%)	80	NA
Extraction temperature (°C)	60	60
Extraction time (min)	30	30
Concentration of cannabinoids and total terpenes (g 100 g dry matter^{-1})		
THC	1.06	0.92
THCA	28.52	19.95
Total THC	26	18.42
CBG	0.18	0.1
CBGA	0.48	0.3
Total CBG	0.6	0.37
THCVA	1.86	1.13
CBCA	0.17	0.39
Total terpenes	1.2	1.03
Extraction yield	29.81	25.52

NA: not applicable.

7.3.7 Verification of models for ultrasound-assisted and microwave-assisted extraction systems for cannabis

Generated models for UAE and MAE for cannabis were verified by performing cannabis extraction using the optimal conditions (Table 7.8). The corresponding experimental values for the cannabinoid content, total terpenes, and extraction yields were determined and compared with the predicted results. The results showed a strong correlation ranging from 0.81 to 0.89 between the predicted and experimental values, which indicates the suitability of the models in

predicting cannabinoid and terpenes profiles and extract yield for cannabis produced by the optimum UAE and MAE conditions.

7.3.8 Principal component analysis for ultrasound-assisted and microwave-assisted extraction systems for cannabis

An exploratory principal component analysis (PCA) was performed to help identify correlation and dependencies between the independent variables and understand their effects on the responses. The scree plots, loading plots, score plots, and scatterplots for the different extraction systems are presented in Figures 7.3 and 7.4. Scree plots are line plots of eigenvalues of principal components and are used to determine the number of principal components that are responsible for variations in the data during PCA (Beattie and Esmonde-White, 2021). Scree plots indicate that the first two principal components (PC) account for 96.6% of the total variance (PC1 = 93.2% and PC2 = 3.4%) for UAE and 92.4% of the total variance (PC1 = 85.4% and PC2 = 7%) for MAE. The loading plots provide information on how the various responses contribute to the variations accounted for by the principal components. Axes on the loading plot (1 to −1) describe how strongly the response influences the principal component. A positive value on the loading plot indicates a positive correlation between the response and the PC. Total THC, THCA, total terpenes, and the extraction yield directly influenced the variation observed by PC2 for UAE (Figure 7.3) and inversely affected the variation accounted by PC2 for MAE. All dependent variables/responses identified in the extracts are important contributors to PC1 for both UAE and MAE, except the THC concentration under MAE. According to the loading plots, parameters positioned close to each other indicate a high positive correlation. Figures 7.3 and 7.4 showed a strong correlation between all the dependent variables for both extraction systems except the THC concentration under MAE. The score and the scatter plots did not show any variation in the sample-to-solvent ratio for MAE. For UAE, however, there was a significant ($p < 0.05$) variation caused by the sample-to-solvent ratios.

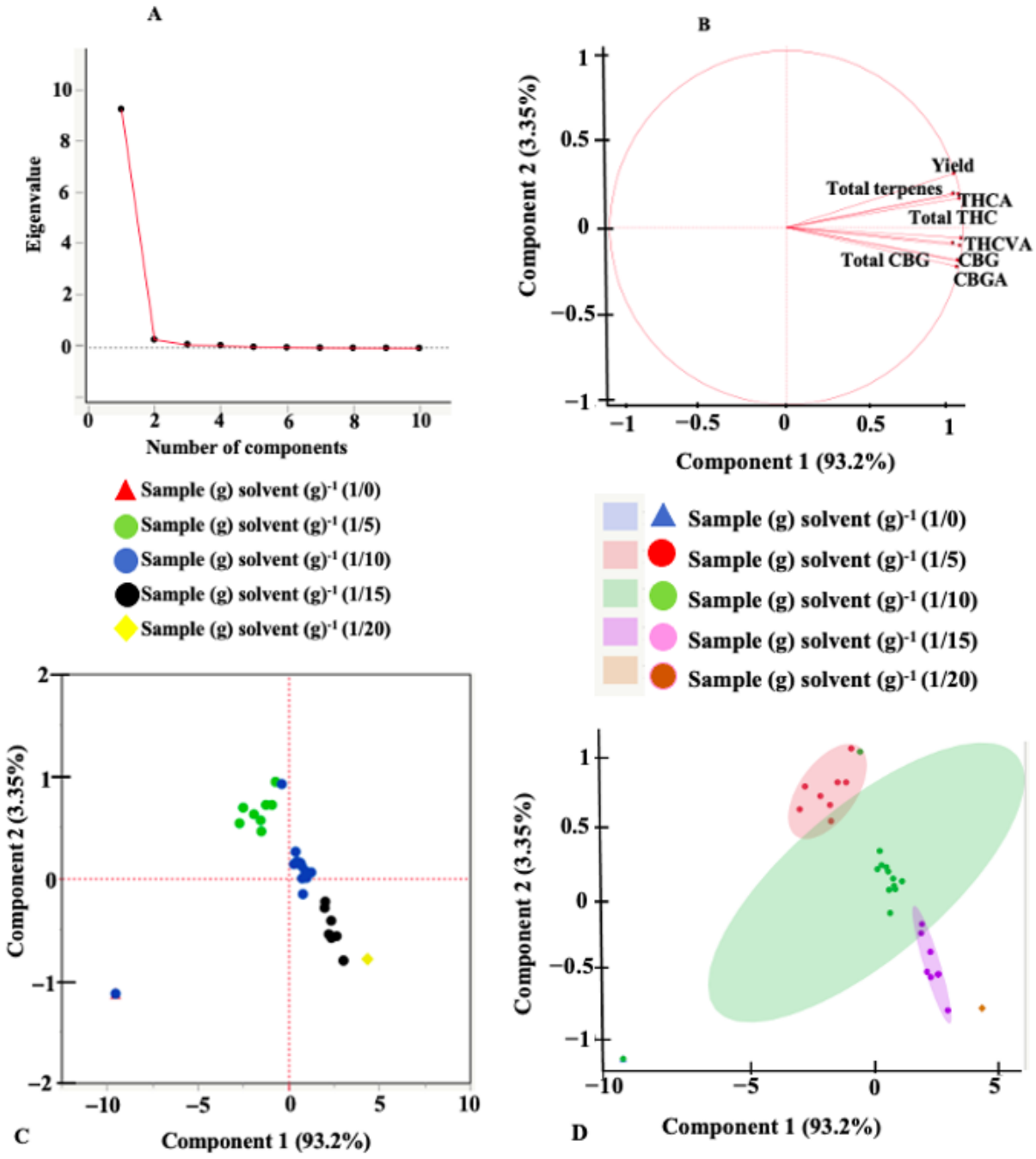


Figure 11.3. Scree (A), loadings (B), and scatter (C) for ultrasound-assisted extraction.

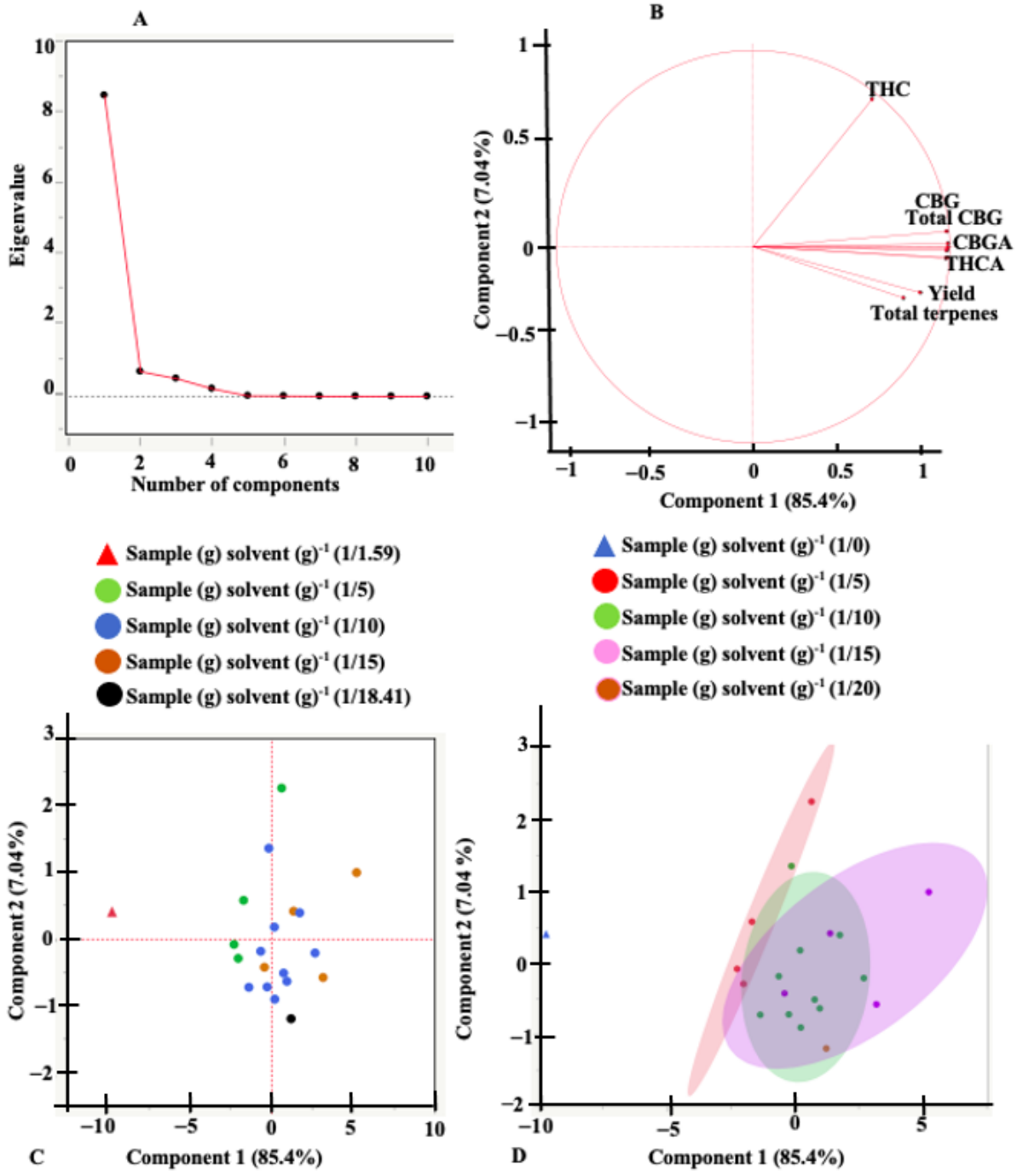


Figure 11.4. Scree (A), loadings (B), and scatter (C) for microwave-assisted extraction.

7.4 Conclusion

Legalization of the cannabis industry in Canada has intensified cannabis production and driven sales of cannabis and cannabis products for medical and recreational adult use. Although some commercial entities have developed efficient extraction systems to improve the safety and potency of cannabis, most of these novel systems have not been optimized for maximum extraction yield and concentration of secondary metabolites. This study optimized for maximum extraction efficiency, using CCRD as a function of several independent variables, namely samples-to-solvent ratio, extraction temperatures, extraction times, and duty cycles. Cannabis samples were extracted using ultrasound-assisted and microwave-assisted extraction. UAE and MAE extraction of cannabis using a sample-to-solvent ratio of 1:15 and 1:14.4, respectively, for 30 min at 60°C were presented as the optimal conditions for maximum responses with maximum desirability of 0.83% and 0.75% for UAE and MAE, respectively. UAE increased the crude oil yield, cannabinoid concentration, and total terpene extracted by 14.4%, 13.2–39.2%, and 14.7% respectively, compared with MAE. Developed predictive models for all responses yielded predictable and reproducible results, and the verification of the models showed a close agreement between the experimental values and the predicted values, with a strong correlation ranging from 0.81 to 0.89. Scree plots under PCA indicated that the first two principal components account for 96.6% of the total variance (PC1 = 93.2% and PC2 = 3.4%) for UAE and 92.4% of the total variance (PC1 = 85.4% and PC2 = 7%) for MAE. The data showed a significant ($p < 0.05$) variation caused by the sample-to-solvent ratios for only the UAE. Further research studies on ethanol recovery using centrifugation, mechanical press system, and vacuum filtration must be conducted to help reduce the operational cost for cannabis industries.

7.5 References

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Connecting text

Chapter 3 - 7 carefully explained the research studies conducted to improve and optimize the postharvest processing of medicinal plants. Chapter 8 addresses preliminary studies conducted in the determination of processing parameters for the various projects. This chapter reintroduces discussion arguments and elaborates on the importance of the published findings to industries.

Chapter 8: Comprehensive scholarly discussion

The doctoral research described in this thesis aimed to investigate and optimize the post-harvest processing practices for medicinal plants, and in particular, to improve and innovate methods along the cannabis product value chain in a nascent legal industry for adult use cannabis and cannabis products. This project aimed to improve and optimize the postharvest processing of medicinal plants through the conservation of important secondary metabolites. Preservation and extraction of secondary metabolites is important as they have beneficial health benefits such as anticancer, antimicrobial, antifungal, antiviral, analgesic, anti-inflammatory, and antiparasitic activities (Ojeda-Sana et al., 2013; Rufino et al., 2015; Schieber and Wüst, 2020).

Model medicinal plants used included hops (*Humulus lupulus*) and cannabis (*Cannabis sativa*) since they have comparable physiological traits and belong to the same family, Cannabaceae. Storage studies were conducted by freezing hops and cannabis to investigate the effects of freezing temperature on the formation of ice crystals, secondary metabolites, cellular structural damages, drying behaviour, and extraction efficiency. Different drying systems (hot air, microwave assisted hot air, and freeze drying) were explored to investigate the effects of drying temperature and pre-temperature on the drying kinetics of hops and cannabis. Extraction efficiencies were improved by investigating the effects of extraction temperature, sample-to-solvent ratio, extraction time, and ultrasound duty cycle.

In the agricultural industry, the freezing process is one of the widely used techniques to preserve products (Hu et al., 2022). Freezing of plant material, a thermodynamic process, is characterized as the phase transition of liquid components of the material to solid when the temperature is reduced below the freezing point (Nowak and Jakubczyk, 2020). Pre-freezing not only improves the shelf life of biomass but is required for efficient drying and extraction, as it influences the number, and size of the ice crystals. Pre-freezing affects the drying performance, residual moisture content, extraction efficiency, and the extraction of heavier compounds such as waxes (Kasper and Friess, 2011). Hot air drying of cannabis at 35°C and 50°C showed that pre-freezing significantly reduced the drying time by 9.1% and 21.6% respectively. This can help improve the processing rate and efficiency of medicinal plants and reduce waste caused by microbial or chemical degradation. Pre-freezing can help reduce the production costs due to the low energy and time required for processing.

Preliminary studies to determine the freezing rate curves of hop buds submitted to different pre-freezing temperatures, -20°C, -80°C, and liquid nitrogen (LN), temperature changes were plotted against freezing time. Total of 100 grams of harvested hop buds were placed on plastic trays and pre-frozen at -20°C, and -80°C for 24 h in chest freezers to obtain various freezing rates. For LN pre-freezing, 100 grams of harvested hop buds were placed on plastic trays and immersed in liquid nitrogen until the representative hop bud in the center of the tray reached -20°C. Temperature changes data were collected every minute using an Onset 12-bit temperature/relative humidity smart sensor (Onset Computer Corporation, Bourne, MA, US) connected to a Hobo U30 USB weather station data logger (Onset Computer Corporation, Bourne, MA, US). The sensor was placed in a representative hop bud in the center of the tray before pre-freezing.

The total freezing time was defined as the time taken for the temperature at the centre of a representative hop bud to reach -20°C, as commercially frozen foods are kept at a temperature of -18°C to -20°C. The total freezing time required for samples to reach -20°C when pre-frozen at -20°C, -80°C, and immersed in liquid nitrogen were 190 min, 31 min and 4 min, respectively, and all total freezing times differed significantly from each other ($p < 0.05$) (Figure 8.1). As discussed in Chapter 4.3.1, although freezing curves of agricultural products can be divided into three stages: the cooling stage, the phase-change stage, and the sub-cooling stage (Figure 4.2), the freezing curve for hop bud samples pre-frozen at −80°C and immersed in liquid nitrogen were almost linear and had no evident characteristics of the three stages seen in the -20°C freezing curve. This is evident by the higher freezing rates of 1.30°C min^{-1} and 11.48°C min^{-1} for -80°C and liquid nitrogen, respectively. The results show that −80°C and liquid nitrogen can be considered as a quick-freezing step because of the high freezing rate. Similar results have been reported for different vegetables (Nowak et al., 2016; Vallespir et al., 2019).

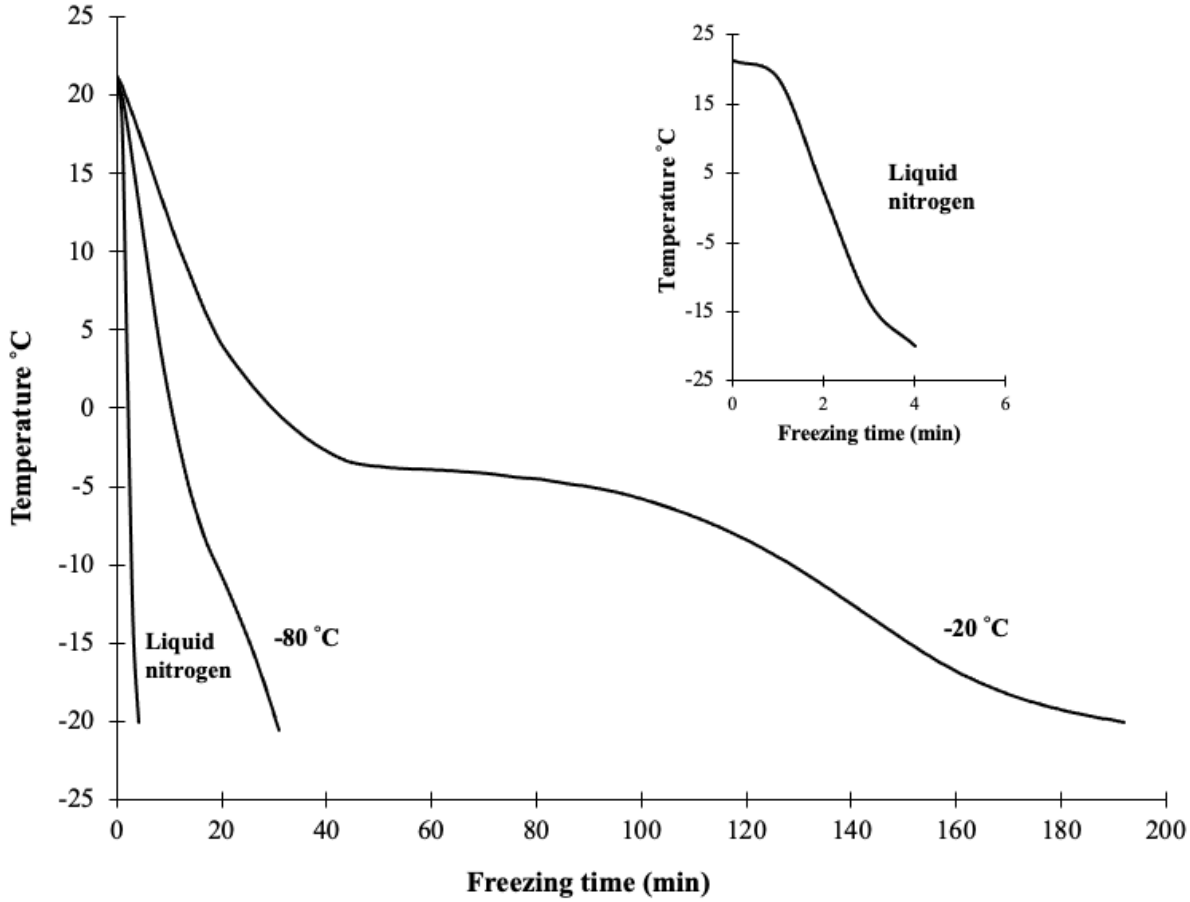

Figure 13.1. Freezing curves for hop bud samples submitted to different pre-freezing conditions.

The effect of pre-freezing temperature on the drying kinetics was evaluated using freeze-drying. Hop bud drying curves when freeze-drying at 10°C and 20°C is shown in Figure 8.2. Drying curves were significantly ($p < 0.05$) affected by the freeze-drying temperature but not by pre-freezing conditions. As expected, the moisture ratio reduced exponentially with time. For hop buds pre-frozen at -20°C, it took only 523 ± 8.49 min at 10°C, to reach the desired final moisture content of 7% (db). As such, the drying time was reduced by 23.6% when compared to hop bud samples dried at 20°C. Similar results were obtained for hop bud samples pre-frozen at -80 ˚C and those immersed in LN, with drying times reduced by 26.8% and 30.1%, respectively, for hops dried at 20°C when compared to 10°C.

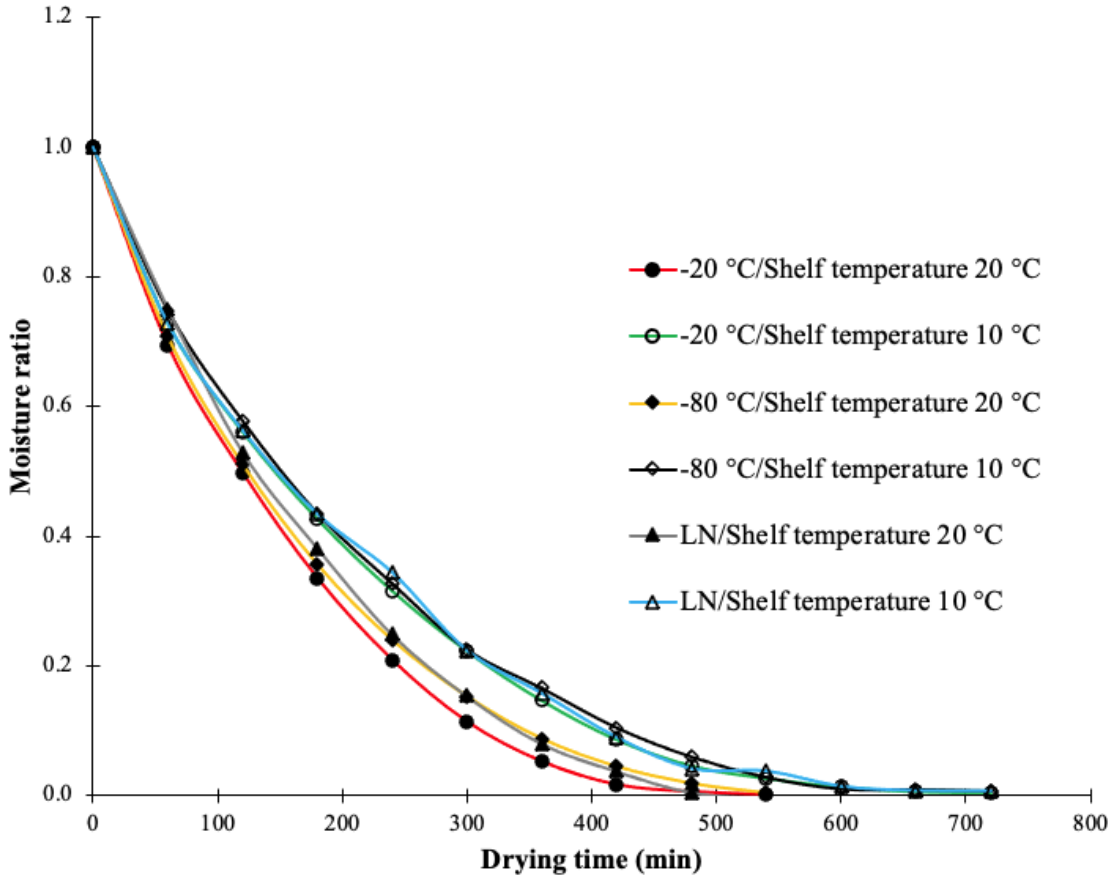

Figure 13.2. Moisture ratio curves of for hop buds.

To determine the effective moisture diffusion coefficients, moisture ratios as a function of drying time were plotted in semi-log graphs for hop buds dried at the different drying temperatures. The effective moisture diffusivities were determined from the linear regression of these plots. The effective diffusion coefficients of hops dried at different temperatures are shown in Table 8.1. D_{eff} values significantly ($p < 0.05$) increased with an increase in freeze-drying temperature for the same pre-freezing condition, ranging between 2.43×10^{-7} m^2 s^{-1} and 3.99×10^{-7} m^2 s^{-1}. At the same freeze-drying temperature, there were no significant differences in D_{eff} values for hop buds pre-frozen under different conditions. ANOVA analyses confirmed the hypothesis that although pre-freezing significantly ($p < 0.05$) improves the drying rate, pre-freezing temperature does not significantly ($p < 0.05$) affect the drying rate of biomass. Hence, cannabis processors could save considerable by investing in -20°C to -40°C freezers compared to -80°C freezers.

Table 13.1. Effective moisture diffusion coefficient of drying of hops at different drying conditions.

Pre-freezing and drying conditions	R^2	Slope	Moisture diffusivity ($m^2\ s^{-1}$)
-20 ˚C/Shelf temperature 20 ˚C	0.9344	0.0118	3.9853×10^{-7}
-20 ˚C /Shelf temperature 10 ˚C	0.9735	0.0076	2.5668×10^{-7}
-80 ˚C/Shelf temperature 20 ˚C	0.9298	0.0093	3.1410×10^{-7}
-80 ˚C/Shelf temperature 10 ˚C	0.9682	0.0072	2.4317×10^{-7}
LN/Shelf temperature 20 ˚C	0.8934	0.0097	3.2761×10^{-7}
LN/Shelf temperature 10 ˚C	0.9722	0.0073	2.4655×10^{-7}

Scanning electron microscopy was done to evaluate the effect storage pre-freezing temperature on the lupulin glands of hops. Lupulin glands (glandular trichomes) contain the secondary metabolites, α- and β-bitter acids, and prenylated flavonoids, and essential oils composed mainly of myrcene, α-humulene and β-caryophyllene, which are responsible for flavor and bitterness of beer (Patzak et al., 2015; Raut et al., 2020a). Evaluating the structure of the samples (Figure 8.3), it can be concluded that pre-freezing caused significant structural damages to the lupulin glands. Pre-freezing caused shrinkage and disruption of the cell structure of the glands. This can be attributed to the formation of ice crystals during freezing (Ando et al., 2019; Nowak et al., 2016; Vallespir et al., 2019). Although all the pre-freezing conditions caused significant damages to the cell structure, the damages were more prominent at -20°C pre-freezing (Figure 8.3D). This is because of pre-freezing at -20°C is considered to be a slow freezing process and produces large ice crystals, which are detrimental to cellular structures (Zhang and Ertbjerg, 2019).

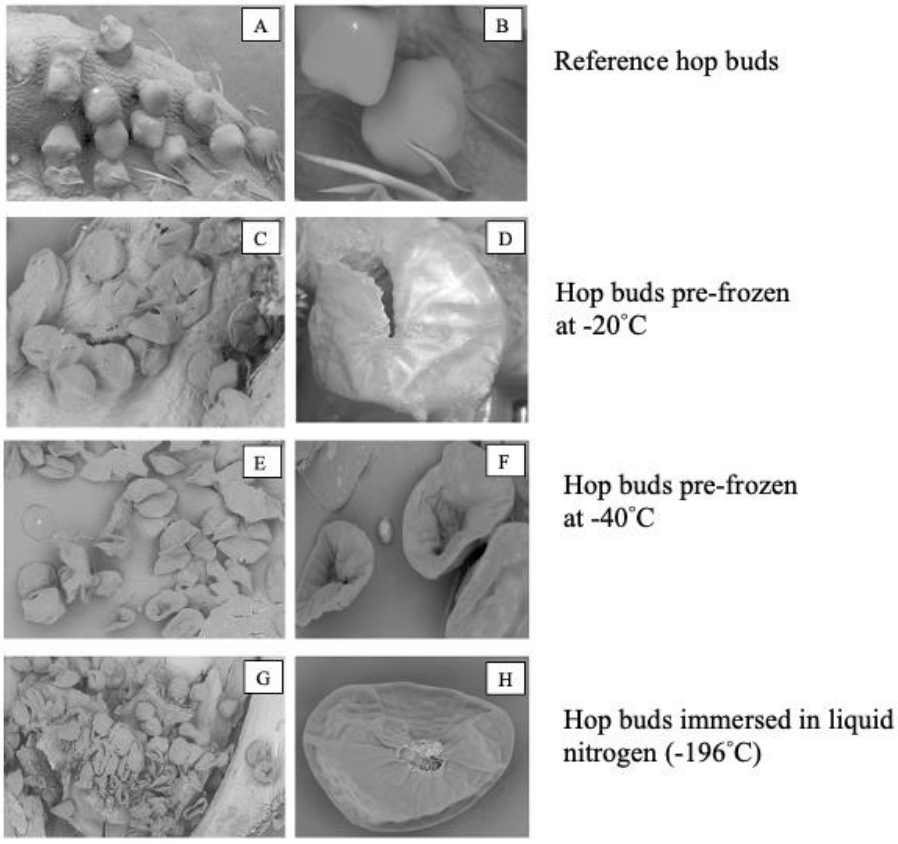

Figure 13.3. Scanning electron microscopy of hop buds prior stored at different freezing temperatures.

Preliminary analyses and published research studies played important roles in the selection of the drying conditions for the project. The effect of microwave power on the drying rate and time and quality attributes were also considered. The main disadvantage of microwave drying is the uneven distribution of heat and negative sensory changes. Microwave drying affect both the colour and organoleptic properties of the food material (Ahrné et al., 2007). Preliminary experiments showed that the visual color degradation (from green to dull green yellow) was greater during microwave drying compared to hot air drying. At 35°C, drying with a microwave power of 100 W caused a significant increase in the total colour change by 33.9% compared to hot air drying. Uneven distribution of heat can be solved by the rotation of the sample in the drying unit. However, care must be taken to prevent damage to the product structure. As products dry, they become more bristle and the least force can cause them to break into smaller pieces.

Various studies showed that increasing microwave power improves the drying rate thereby reducing the drying time (Kumar et al., 2014; Sarimeseli, 2011; Soysal, 2004). However, this can affect the quality of products. The preliminary analyses conducted showed that increasing the microwave power above 200 W at 65°C caused the charring of hops and cannabis and arcing in the system. The rapid burning of the hop and cannabis buds at 300 W can also be caused by the nonuniform distribution of microwaves in the drying chamber as the sample tray was not attached to a rotating device. Reducing the microwave power below 100 W (50 W, air temperature 35°C) had a similar drying time value to hot air drying at 35°C. Results showed that at the same drying temperature (50°C), increasing the microwave power density from 1 to 2W g^{-1} significantly ($p < 0.05$) reduced the drying of cannabis from 126.5 min to 76.5 min although there was a significant ($p < 0.05$) increase in total color change (16.3%). Similar observations were made for hops and the different drying temperatures (35°C and 65°C).

Kwaśnica et al., (2020) showed that although the drying time was reduced by 60% when the microwave power was increased from 240 W to 480 W in a microwave dryer, there was a 14.3% decrease in the concentration of volatile compounds. The study recommended 240 W microwave power as the optimal for the retention of aroma-active compounds. Similar results were observed for the drying of green peas (*Pisum sativum*). Drying using a microwave power of 100 W had the highest phenolic content (Chahbani et al., 2018).

Kumar et al., (2014) studied the effect of microwave power, air temperature, and airflow rate on the drying of okra (*Abelmoschus esculentus*) using a central composite rotatable design. The drying parameters were optimized based on the quality of dried okra and the energy used during the drying process. The study showed that air temperature and microwave power have significant effects on all drying parameters and energy consumption, although the predominant factor was microwave power. The optimal drying conditions of 1.51 m s^{-1} air velocity, 52.09°C air temperature, and 241 W microwave power were found optimum for microwave-convective drying of okra. Based on all the above published data and preliminary studies, 100 W and 200 W were used to evaluate the effect of microwave power on the drying kinetics of hops and cannabis.

Measuring the microwave power intensities showed that during the drying process, the reflected power was low and gradually increased (Figure 8.4). This can be attributed to the reduced moisture content. Statistical analyses showed a negative correlation (-0.5) between

moisture content and reflected power. This showed that that when the moisture content of the sample decreases, the amount of reflected power increases. One limitation of the microwave unit used for the study is that the microwave power is set from the start of the experiment and remain constant throughout drying. Control of the product temperature is done by switching ON/OFF the microwave generator. A better system would be to control the amount of incident power, reducing it as the drying progress, to maintain the product temperature while matching what can be absorbed by the moisture left in the product. A system with zero reflected power would be preferred.

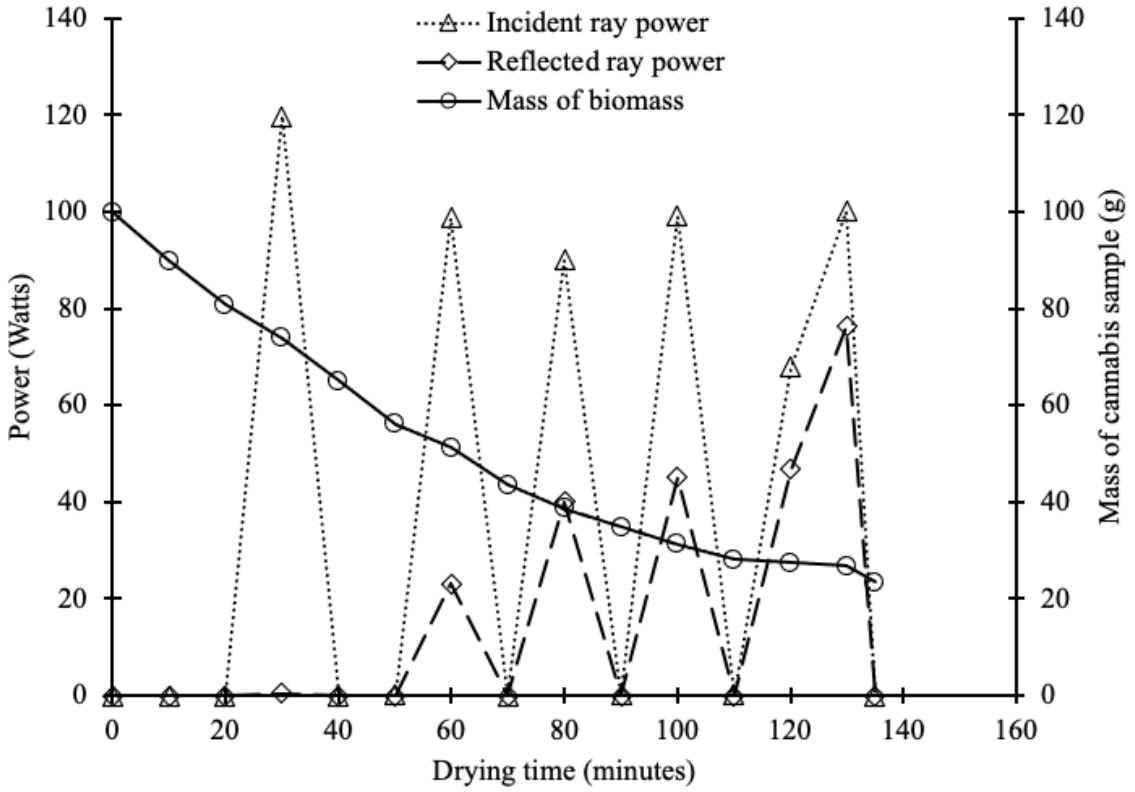

Figure 13.4. Changes in the mass of samples, incident power and reflected power during microwave drying.

The use of relative humidity sensor as an indicator for the end of the freeze-drying process for cannabis was verified using hops. In an industrial setting, relative humidity sensors in a drying system will make it convenient to determine whether a material has been sufficiently dried. This

will help prevent over or under drying of biomass and limit the cross contamination of products by personnel. In this study, relative humidity placed in the center of the drying tray near a representative bud was monitored when freeze-drying at 10°C and 20°C, to determine optimal drying time and energy-efficient drying conditions. Changes in relative humidity within the freeze dryer were recorded when freeze-drying hops samples pre-frozen at different temperatures (-20°C, -80°C, and immersion in LN). Figure 8.5 shows an exponential decrease in relative humidity during the drying process. The relative humidity in the dryer quickly reduced to 0 when no sample was in the dryer, indicating that the recorded relative humidity values when drying at 10°C and 20°C were due to the sublimation of water molecules from the hop bud samples (Bradford et al., 2016; Phitakwinai et al., 2019). As expected, these data confirmed a strong relationship between changes in relative humidity and sample mass during freeze-drying. Mathematical model analyses showed that the data best fit the Richard's sigmoidal and linear regression models, with a high R^2. To verify these findings, ANOVA were performed using predicted hops sample mass changes during drying, using the models as a function of the measured mass changes. High correlation coefficients (0.97 - 0.99) and R^2 values were recorded (0.98 - 0.99). Wang et al. (2022) reported similar findings using citrus (*Citrus reticulata*) peels that the relative humidity reduced in the drying system with the increasing of sample temperature. However, the study was not able to correlate the changes in the mass of the sample and relative humidity during the drying process. This can be attributed to the fluctuation in the relative humidity measurements affecting by the simultaneous measurement of the moisture content (Wang et al., 2022).

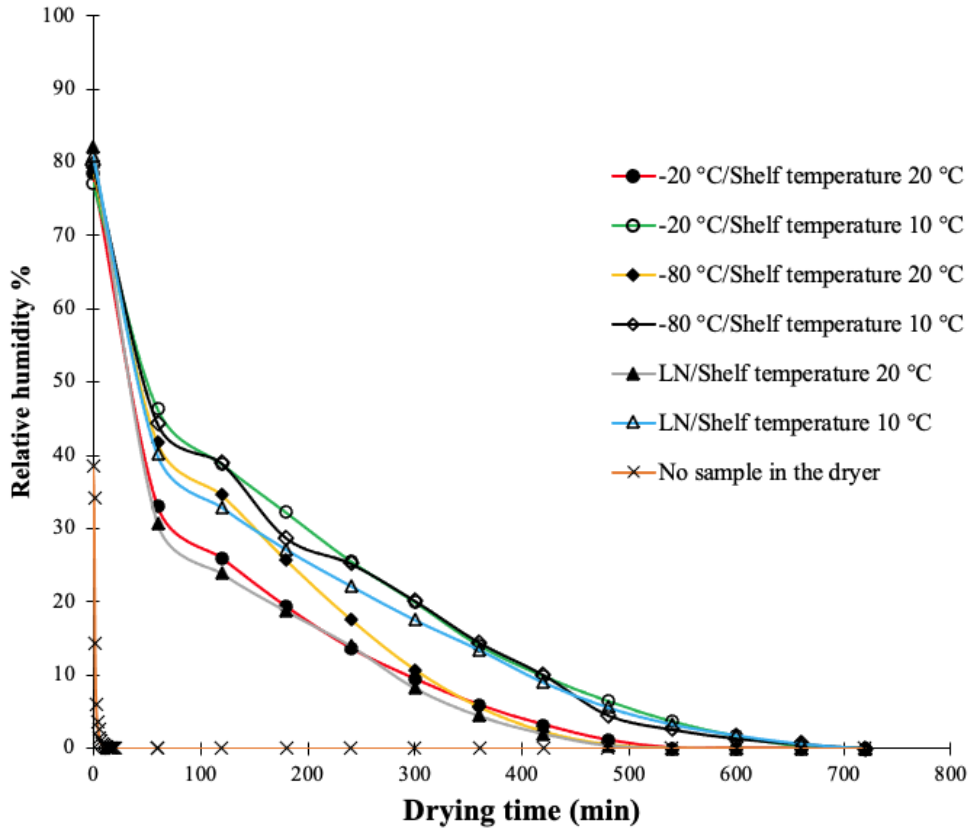

Figure 13.5. Relative humidity values recorded for hop bud samples.

The effect of pre-freezing and drying conditions on the secondary metabolites present in cannabis and hops were evaluated by analysing the cannabinoids, terpenes, and antioxidant activities. Cannabis believed to have originated from China is mostly cultivated for its medicinal and psychotropic properties (Jin et al., 2020; Ren et al., 2019). Jin et al., (2020) showed that cannabis inflorescence was characterized by cannabinoids (15.77–20.37%), terpenoids (1.28–2.14%), and flavonoids (0.07–0.14%). Hops are mostly used in the brewing industries due to their unique chemical compounds which contribute greatly to the bitterness, flavour and aroma (Patzak et al., 2015).

Results showed an increased antioxidant activity in the pre-frozen hops and cannabis samples. Significant ($p < 0.05$) increase in total THC and THCA concentrations in cannabis can be attributed to the pre-freezing step. Pre-freezing exerts positive effects on the quality and functional properties of plant material since a frozen state allows the release of bioactive compounds as bound phenolic acids and anthocyanins, resulting in increased antioxidant activity

(Leong and Oey, 2012; Mullen et al., 2002). The high temperature used during microwave assisted hot air drying (MAHD) and freeze-drying caused significantly ($p < 0.05$) reduced in the antioxidant activity in hops using the DPPH and FRAP assays compared to the pre-frozen samples. Significant ($p < 0.05$) differences were observed between the antioxidant activity values for pre-frozen, freeze-dried and MAHD-dried cannabis samples using the DDPH and not the FRAP method, likely due to the presence of iron-chelating compounds such as cannabinoids. Non-enzymatic decarboxylation process was observed in MAHD-dried cannabis samples (Das et al., 2022b). ANOVA analyses showed a significant ($p < 0.05$) increase in THC for MAHD-dried samples compared to fresh, undried (64.7%) and pre-frozen, undried (57%). Freeze-drying did not cause a significant ($p < 0.05$) change in the concentration of THC and THCA in cannabis and can be used to preserve the secondary metabolites present in medicinal plants during drying. Further studies exploring the concentration of secondary metabolites and the antioxidant activities of different parts of the plant such as the stem must be conducted to help waste.

Preliminary extraction studies using hops and cannabis showed the effect extraction temperature on the extraction efficiencies. Increased concentration of waxes and other heavier compounds were visually observed during extraction at room temperature compared to cold extraction. Sample-to-solvent ratio was the most important factor affecting cold ethanol, ultrasound-assisted and microwave-assisted extraction of cannabis, demonstrating a significant ($p < 0.05$) effect on extraction of cannabinoids, terpenes, and extraction yields for all experimental conditions. One limitation of the extraction studies was that the microwave power and ultrasound power of the microwave and ultrasound extraction systems respectively, were set throughout the extraction process. This was due to a system limitation. The effect of changes in microwave power and ultrasound power on the extraction or degradation of cannabinoids and terpenes were not explored.

Decreasing the sample (g)-to-solvent (g) ratio significantly ($p < 0.05$) provided an increased surface area for the extraction of secondary metabolites from the trichomes. Optimal conditions for ultrasound- and microwave-assisted extraction were sample-to-solvent ratios of 1:15 and 1:14.4, respectively, for 30 min at 60°C. Ultrasound-assisted extraction yielded more oil (14.4%) and terpenes (14.4%) compared with microwave-assisted extracts. This can be attributed to the mechanical effect caused by cavitation. This increased the permeability of the plant's cell walls and improved the solubility of secondary metabolites (Agarwal et al., 2018;

Albero et al., 2019; Chemat et al., 2017). Ultrasound-assisted extraction increased cannabinoid concentration from 13.2–39.2%.

Predicted optimal conditions at different extraction temperatures for cold ethanol extraction were a cannabis-to-ethanol ratio of 1:15 and a 10 min extraction time. Yields with cold ethanol extraction were 18.2, 19.7, and 18.5 g 100 g dry matter^{-1} for −20°C, −40°C and room temperature, respectively. Considering reference ground samples, tetrahydrocannabinolic acid (THCA) increased from 17.9 to 28.5 and 20 g 100 g dry matter^{-1} with extraction efficiencies of 159.2% and 111.4% for ultrasound-assisted and microwave-assisted extraction, respectively. For cold ethanol extraction, compared to the reference ground sample, THCA changed from 17.9 (g 100 g dry matter^{-1}) to 15, 17.5, and 18.3, with an extraction efficiency of 83.6%, 97.7%, 102.1% for −20°C, −40°C, and room temperature, respectively.

These findings add new and important industry-relevant knowledge to the growing body of evidence that can support and optimize postharvest processes for this regulated crop by reducing drying time. This project has contributed to our understanding of the effect of pre-freezing, drying, and extraction conditions for medicinal plants. Results showed that pre-freezing is an important additional step for optimizing extraction of secondary metabolites and drying of biomass. Freeze-drying is the preferred drying system due to a higher metabolite retention compared to other traditional systems. Although, increasing the sample-to-solvent will increase the extraction efficiency, research studies on solvent recovery using centrifugation, press system, and vacuum filtration must be conducted to help reduce the operational cost for the medicinal industries. More research examining the effect of freeze-drying on secondary metabolites should be conducted to further explore cannabinoid and terpene biosynthesis at the molecular level postharvest.

Chapter 9: Conclusion and summary

9.1. General conclusion

The aim of this doctoral research was to evaluate and optimize post-harvest activities for two medicinal plant species in the Cannabaceae family, namely hops and cannabis. Within the context of a new regulatory framework involving the legalization of recreational cannabis in Canada, knowledge acquired through this work is intended as a starting point with scale-up potential that could help innovate licensed producers' operations in this industrial sector. Initial experiments performed on hops served as a stepping stone for licensed research performed with cannabis. The studies evaluated the effects of pre-freezing, drying conditions, and extraction techniques on the valued secondary metabolites present in the biomass, drying and extraction kinetics, as well lupulin glands' and trichomes' physical properties. In doing so, the relationship between relative humidity and sample mass reduction during freeze-drying was investigated to help develop sensors that could determine the end of a drying process and subsequently reduce processing time.

Pre-freezing is beneficial for oil extraction and reducing drying times, as SEM analyses show evidence of structural damage incurred by lupulin glands and cannabis trichomes caused by the ice crystals. Pre-freezing prior to drying significantly reduced the drying time by 0.2% and up to 85.9% by improving the drying rates with freezing times of 51.4–63.2 min (-40˚C) and 189.1–196.4 min (-20˚C). Although freezing inflorescence is primarily a preservation technique, pre-freezing hops and cannabis samples before drying additionally increased antioxidant values by 13% (DPPH assay) and 29.9% (FRAP assay) for hops, and by 7.7% (DPPH assay) and 19.4% (FRAP assay) for cannabis.

Data showed a strong correlation (0.97-0.99) between changes in sample mass and relative humidity during drying that may be explained using the rational regression model. The study showed that relative humidity can be used as an indicator to determine the end of the drying process when using a freeze-dryer to prevent over- and under-drying. However, the relative humidity sensor must be placed near a representative inflorescence in the center of the sample tray.

Comparing the drying techniques, freeze drying and MAHD, investigated in this study, the low temperatures used during freeze-drying successfully preserved biomass quality and its flavoring molecules. Freeze-drying increased the concentration of CBDA, CBGA, and CBG in

all dried samples by 73.3% to 87.7%, 23.4% to 42.4%, and 6.9% to 51.9%, respectively, compared to their respective fresh, undried samples. Non-enzymatic decarboxylation was observed by the significant ($p < 0.05$) increase in the THC in MAHD-dried cannabis samples compared to fresh, undried (64.7%) and pre-frozen, undried (57%). Although both drying systems showed a significant loss in the total terpene concentration, freeze-drying had higher terpene retention compared to MAHD. Freeze-drying should be used as the drying system for medicinal plants to reduce postharvest losses of secondary metabolites and decarboxylation of cannabinoids.

Extraction studies showed that the developed predictive models for all responses yielded predictable and reproducible results, and verification of the models showed a close agreement between the experimental values and the predicted values with a strong correlation ranging from 0.81 to 0.93. Statistical analyses for cold ethanol extraction showed that a set sample-to-solvent ratio of 1:15 over 10 min at the different extraction temperatures would provide the optimum conditions for the extraction of cannabis oil with maximum desirability ranging between 0.77–0.83%. At these optimized conditions, extraction yields (g 100 g dry matter^{-1}) were 18.2%, 19.7%, and 18.5% for −20°C, −40°C, and room temperature, respectively, according to the desirability function (0.77 to 0.83%).

UAE and MAE extraction of cannabis for 30 min at 60°C using sample-to-solvent ratios of 1:15 and 1:14.4, respectively, were the optimal conditions for maximum responses with maximum desirability of 0.83% and 0.75% for UAE and MAE, respectively. UAE increased the crude oil yield, cannabinoid concentration, and total terpenes extracted by 14.39%, 13.21–39.24%, and 14.67%, respectively, compared with MAE. Together, these findings add new and important industry-relevant knowledge to the growing body of evidence that can support and optimize postharvest processes for this regulated crop by improving drying and extraction efficiencies.

9.2 Further suggested studies

The following recommendations are based on data compiled over the course of this research and are offered as possible future studies in this field:

1. Determine cannabis' and hops' dielectric properties to further understand the effects of microwaves on valued secondary compounds in medicinal plants. Research studies show that factors influencing the microwave heating of biomass are the dielectric properties of the biomass, microwave power, temperature, moisture content, and metabolite compositions. The dielectric properties of a material are specific at a specified microwave frequency and material state. This study will help with the design of microwave dryers and microwave extraction system for the cannabis industry.
2. Explore molecular distillation of CBGA, THCA and CBDA to generate pure cannabinoid crystalline forms for pharmaceutical- and food-grade products. Specific studies on improving the distillation efficiency of cannabinoids for drug development. Research studies on the effect of independent variables such as condensation temperature, evaporation temperature, pressure, and feed flow rate on distillate yield and the concentration of cannabinoids and terpenes in distillate. These studies will help evaluate if decarboxylation of acidic cannabinoids to neutral cannabinoids can be achieved using molecular distillation.
3. Investigate the effect of biomass particle size, type of solvent, and microwave and ultrasound power densities on extraction efficiency and concentration of cannabinoids and terpenes in extracts. Optimization of the best particle size and extraction conditions, including solvent type and temperature is beneficial for the cannabis industry to achieve high yield of cannabis oil containing significant concentration of cannabinoids and terpenes.
4. Research the effect of cryopreservation on cannabinoid and terpene concentration by exploring their biosynthesis at the molecular level. Advanced scanning electron microscopy and biosynthetic labelling can be used to evaluate the effects of freezing temperature on the biosynthesis of secondary metabolites in medicinal plants.
5. Conduct studies on solvent recovery after extraction using centrifugation, press system, and vacuum filtration to help reduce the operational costs for cannabis industries. Although increasing the sample-to-solvent ratio increases the extraction yield, this can

lead to an increase in solvent waste. Solvent recovered from residual biomass after extraction can be used for further extraction to help reduce the production cost. However, distillation of the solvent recovered is recommended to remove residual oils or biomass present in the solvent.

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Appendix

Table 11.1. Ultrasound-assisted extraction of cannabis using model B (sample (g) solvent $(g)^{-1}$, duty cycle (%), and extraction temperature (°C) as independent parameters).

Response/dependent variables		Regression model effect parameters									
		Intercept	Linear			Interaction			Quadratic		
		β_0	β_1	β_3	β_4	β_{13}	β_{14}	β_{34}	β_{11}	β_{33}	β_{44}
THC	Coefficient	0.69	0.25	0.05	0.02	0.01	0.001	-0.002	-0.005	0.02	0.03
	p value	<.0001*	<.0001*	0.2	0.53	0.83	0.99	0.97	0.88	0.52	0.37
THCA	Coefficient	23.68	4.82	0.51	0.28	-0.52	-0.25	-0.1	-1.9	0.95	1.03
	p value	<.0001*	0.001*	0.68	0.82	0.73	0.87	0.95	0.1	0.41	0.36
Total THC	Coefficient	20.76	4.48	0.5	0.27	-0.44	-0.22	-0.1	-1.46	1.06	1.15
	p value	<.0001*	0.0005*	0.65	0.81	0.74	0.87	0.94	0.16	0.3	0.26
CBG	Coefficient	0.14	0.04	0.01	0.003	-0.003	-0.002	0.001	-0.01	0.003	0.004
	p value	<.0001*	<.0001*	0.37	0.67	0.71	0.82	0.94	0.2	0.64	0.5
CBGA	Coefficient	0.34	0.12	0.01	0.001	-0.003	0.002	0.01	-0.01	-0.002	0.01
	p value	<.0001*	<.0001*	0.52	0.94	0.88	0.93	0.66	0.54	0.89	0.42
Total CBG	Coefficient	0.41	0.15	0.02	0.01	-0.01	0.003	0.01	-0.01	0.01	0.02
	p value	<.0001*	<.0001*	0.46	0.80	0.83	0.9	0.75	0.63	0.66	0.25
THCVA	Coefficient	1.32	0.4	0.06	0.03	-0.01	0.002	-0.001	-0.06	0.05	0.06
	p value	<.0001*	<.0001*	0.39	0.62	0.87	0.98	0.99	0.39	0.42	0.34
CBCA	Coefficient	0.12	0.04	-0.002	0.01	-0.004	0.01	-0.004	-0.002	0.004	0.01
	p value	<.0001*	<.0001*	0.78	0.47	0.63	0.37	0.63	0.72	0.57	0.27

Total terpenes	Coefficient	0.95	0.2	0.001	0.003	-0.02	-0.01	0.003	-0.02	0.04	0.05
	p value	<.0001*	0.002*	0.98	0.95	0.81	0.83	0.96	0.69	0.39	0.33
Yield	Coefficient	24.59	4.01	0.47	0.36	-0.29	-0.12	-0.1	-1.64	1.21	1.32
	p value	<.0001*	0.0067*	0.73	0.79	0.86	0.94	0.95	0.19	0.33	0.29

Table 11.2. Ultrasound-assisted extraction of cannabis using model C (sample (g) solvent $(g)^{-1}$, extraction time (min), and extraction temperature (°C) as independent parameters).

Response/dependent variables		Regression model effect parameters									
		Intercept	Linear			Interaction			Quadratic		
		β_0	β_1	β_2	β_4	β_{12}	β_{14}	β_{24}	β_{11}	β_{22}	β_{44}
THC	Coefficient	0.77	0.25	0.10	0.02	0.02	0.001	-0.001	-0.01	-0.07	0.02
	p value	<.0001*	<.0001*	0.002*	0.41	0.64	0.99	0.99	0.59	0.01*	0.39
THCA	Coefficient	26.98	4.82	2.99	0.28	-0.94	-0.25	0.02	-2.24	-2.63	0.69
	p value	<.0001*	<.0001*	0.002*	0.74	0.37	0.82	0.99	0.01*	0.01*	0.38
Total THC	Coefficient	23.73	4.48	2.72	0.27	-0.82	-0.22	0.01	-1.77	-2.16	0.84
	p value	<.0001*	<.0001*	0.003*	0.73	0.41	0.83	0.99	0.02*	0.01*	0.26
CBG	Coefficient	0.15	0.04	0.02	0.002	-0.002	-0.002	0.002	-0.01	-0.02	0.002
	p value	<.0001*	<.0001*	0.001*	0.49	0.71	0.71	0.71	0.01*	0.01*	0.53
CBGA	Coefficient	0.37	0.12	0.03	0.001	-0.003	0.002	-0.01	-0.01	-0.04	0.01
	p value	<.0001*	<.0001*	0.03*	0.93	0.85	0.91	0.62	0.29	0.01*	0.44
Total CBG	Coefficient	0.46	0.15	0.04	0.01	-0.01	0.003	-0.004	-0.01	-0.04	0.02
	p value	<.0001*	<.0001*	0.01*	0.74	0.78	0.88	0.83	0.35	0.01*	0.25
THCVA	Coefficient	1.48	0.4	0.21	0.03	-0.06	0.002	-0.001	-0.07	-0.13	0.04
	p value	<.0001*	<.0001*	0.001*	0.45	0.32	0.97	0.99	0.09	0.01*	0.29
CBCA	Coefficient	0.14	0.04	0.02	0.01	-0.003	0.01	-0.01	0.004	-0.01	0.01
	p value	<.0001*	<.0001*	0.01*	0.36	0.66	0.26	0.44	0.45	0.02*	0.27
Total terpenes	Coefficient	1.08	0.20	0.08	0.003	-0.01	-0.01	0.02	-0.03	-0.1	0.04

	p value	<.0001*	0.001*	0.13	0.94	0.91	0.81	0.76	0.45	0.04*	0.41
Yield	Coefficient	27.99	4.01	2.64	0.36	-0.21	-0.12	-0.12	-2	-2.47	0.97
	p value	<.0001*	0.001*	0.02*	0.75	0.88	0.93	0.93	0.06	0.02*	0.34

Effects are statistically significant if p value * < 0.05

Table 11.3. Ultrasound-assisted extraction of cannabis using model D (sample (g) solvent $(g)^{-1}$, extraction time (min), and duty cycle (%) as independent parameters).

Response/dependent variables		Regression model effect parameters									
		Intercept	Linear			Interaction			Quadratic		
		β_0	β_1	β_2	β_3	β_{12}	β_{13}	β_{23}	β_{11}	β_{22}	β_{33}
THC	Coefficient	0.78	0.25	0.1	0.05	0.02	0.01	0.01	-0.01	-0.07	0.01
	p value	<.0001*	<.0001*	0.001*	0.08	0.62	0.77	0.8	0.55	0.01*	0.62
THCA	Coefficient	27.07	4.82	2.99	0.51	-0.95	-0.52	0.11	-2.25	-2.64	0.6
	p value	<.0001*	<.0001*	0.002*	0.55	0.37	0.62	0.91	0.01*	0.01*	0.45
Total THC	Coefficient	23.82	4.48	2.72	0.5	-0.82	-0.44	0.11	-1.78	-2.17	0.74
	p value	<.0001*	<.0001*	0.002*	0.54	0.41	0.65	0.91	0.02*	0.01*	0.31
CBG	Coefficient	0.16	0.04	0.02	0.01	0.001	-0.002	-0.003	-0.01	-0.02	0.001
	p value	<.0001*	<.0001*	0.001*	0.13	0.7	0.53	0.9	0.01*	0.02*	0.78
CBGA	Coefficient	0.39	0.12	0.03	0.01	-0.003	-0.003	-0.003	-0.01	-0.04	-0.01
	p value	<.0001*	<.0001*	0.03*	0.4	0.85	0.85	0.85	0.24	0.01*	0.56
Total CBG	Coefficient	0.47	0.15	0.04	0.02	-0.01	-0.01	-0.003	-0.02	-0.04	0.002
	p value	<.0001*	<.0001*	0.01*	0.35	0.78	0.78	0.88	0.31	0.01*	0.88
THCVA	Coefficient	1.49	0.4	0.21	0.06	-0.07	-0.01	-0.002	-0.07	-0.12	0.03
	p value	<.0001*	<.0001*	0.01*	0.19	0.31	0.79	0.97	0.08	0.01*	0.41
CBCA	Coefficient	0.14	0.04	0.02	-0.002	-0.003	-0.004	0.004	-0.004	-0.01	0.002
	p value	<.0001*	<.0001*	0.02*	0.74	0.68	0.57	0.57	0.44	0.03*	0.75
Total terpenes	Coefficient	1.08	0.2	0.08	0.001	-0.01	-0.02	0.02	-0.03	-0.1	0.03

	p value	<.0001*	0.0005*	0.13	0.98	0.91	0.78	0.8	0.45	0.04*	0.51
Yield	Coefficient	28.1	4.01	2.64	0.47	-0.21	-0.29	-0.18	-2.01	-2.49	0.85
	p value	<.0001*	0.0014*	0.02*	0.67	0.88	0.83	0.89	0.06	0.02*	0.4

Effects are statistically significant if p value * < 0.05

Table 11.4. Microwave-assisted extraction of cannabis using model F (sample (g) solvent $(g)^{-1}$ and extraction temperature (°C) as independent parameters).

Response/dependent variables		Regression model effect parameters					
		Intercept	Linear		Interaction	Quadratic	
		β_0	β_1	β_4	β_{14}	β_{11}	β_{44}
THC	Coefficient	0.63	0.05	0.25	-0.01	-0.11	0.09
	p value	<.0001*	0.37	0.001*	0.89	0.07	0.14
THCA	Coefficient	17.18	4.24	0.35	0.02	-2.03	0.24
	p value	<.0001*	0.001*	0.72	0.98	0.04*	0.8
Total THC	Coefficient	15.7	3.78	0.55	0.01	-1.89	0.3
	p value	<.0001*	0.001*	0.54	0.96	0.04*	0.73
CBG	Coefficient	0.09	0.02	0.01	-0.001	-0.01	0.002
	p value	<.0001*	0.001*	0.32	0.85	0.06	0.61
CBGA	Coefficient	0.25	0.06	0.01	0.01	-0.03	0.01
	p value	<.0001*	0.001*	0.58	0.8	0.06	0.66
Total CBG	Coefficient	0.3	0.08	0.01	0.004	-0.04	0.01
	p value	<.0001*	0.001*	0.46	0.87	0.05	0.63
THCVA	Coefficient	0.96	0.25	0.02	0.01	-0.11	0.01
	p value	<.0001*	0.0004*	0.67	0.92	0.06	0.87
CBCA	Coefficient	0.34	0.08	0.01	0.001	-0.04	0.002
	p value	<.0001*	0.001*	0.69	0.97	0.05	0.9
Total terpenes	Coefficient	0.93	0.16	0.03	0.07	-0.14	-0.02

	p value	<.0001*	0.02*	0.67	0.38	0.03*	0.77
Yield	Coefficient	24.24	5.32	0.17	-0.37	-3.96	-0.16
	p value	<.0001*	<.0001*	0.76	0.61	<.0001*	0.76

Effects are statistically significant if p value * < 0.05

Table 11.5. Microwave-assisted extraction of cannabis using model G (sample (g) solvent $(g)^{-1}$ and extraction time (min) as independent parameters).

Response/dependent variables		Regression model effect parameters					
		Intercept	Linear		Interaction	Quadratic	
		β_0	β_1	β_2	β_{12}	β_{11}	β_{22}
THC	Coefficient	0.67	0.05	0.03	0.14	-0.11	0.04
	p value	<.0001*	0.54	0.71	0.22	0.19	0.67
THCA	Coefficient	17.26	4.24	0.64	2.12	-2.04	0.14
	p value	<.0001*	0.0002*	0.46	0.07	0.03*	0.86
Total THC	Coefficient	15.81	3.78	0.59	2	-1.9	0.16
	p value	<.0001*	0.0003*	0.46	0.07	0.02*	0.84
CBG	Coefficient	0.09	0.02	0.003	0.01	-0.01	0.003
	p value	<.0001*	0.0004*	0.43	0.07	0.03*	0.57
CBGA	Coefficient	0.25	0.06	0.01	0.03	-0.03	0.004
	p value	<.0001*	0.0003*	0.33	0.07	0.03*	0.72
Total CBG	Coefficient	0.31	0.08	0.01	0.04	-0.04	0.004
	p value	<.0001*	0.0002*	0.38	0.06	0.03*	0.77
THCVA	Coefficient	0.96	0.25	0.05	0.12	-0.11	0.01
	p value	<.0001*	0.0001*	0.32	0.08	0.03*	0.86
CBCA	Coefficient	0.34	0.08	0.01	0.05	-0.04	0.004
	p value	<.0001*	0.0002*	0.4	0.06	0.03*	0.8
Total terpenes	Coefficient	0.92	0.16	0.05	0.1	-0.14	-0.001

	p value	<.0001*	0.01*	0.38	0.18	0.02*	0.98
Yield	Coefficient	23.81	5.32	-0.21	0.61	-3.91	0.4
	p value	<.0001*	<.0001*	0.69	0.39	<.0001*	0.44