Conversion of Limonene to Carvone by Oxidative Biocatalysis

By

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Declaration

I hereby confirm that this dissertation is entirely my own, original work. It is submitted in partial fulfilment of the requirements for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. This dissertation has not been previously submitted for any degree or examination at any other university or institution.

Otisha Govender On __03__ June__2025 at Johannesburg

Dedication

I would like to dedicate this work to:

- My parents, Vanessa Govender and Devin Govender
- My husband, Veolen Govender
- My siblings, Venailin Govender and Deolin Govender

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I would like to express my deepest gratitude to my supervisors, Dr. Kennedy Ngwira and Prof. Dean Brady, for their invaluable guidance, support, and encouragement throughout my research. Their expertise, insightful feedback, and constant motivation have been instrumental in shaping this work.

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To my parents, words cannot fully express how thankful I am for all the sacrifices you have made for me. Your unwavering love, dedication, and encouragement have shaped me into the person I am today. You've always believed in me, even when I doubted myself, and you made countless sacrifices to ensure that I had the opportunities to succeed. Whether it was providing emotional support during difficult times or offering the practical help that allowed me to focus on my studies, your sacrifices have been immeasurable. This achievement is a reflection of all the hard work, love, and commitment you've poured into me, and I will forever be grateful for your never-ending faith in me. Thank you for always being my pillar of strength.

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I would like to express my deepest gratitude to God, whose guidance, strength, and grace have been with me every step of the way. In moments of doubt and uncertainty, I found the resilience and clarity needed to persevere through divine presence and support. I am truly blessed for the many blessings, both big and small, that have led me to this moment. This achievement is a testament to the unwavering love and guidance I have received, and I am forever grateful for the strength and purpose provided throughout this journey.

Abstract

Flavour and fragrances play an important role in our daily lives. *R*-(−)-carvone, an oxygen-containing monoterpene, is the characteristic odour found in spearmint. It is commercially important and used in the baking, cosmetic and pharmaceutical industries. However, demand greatly exceeds the natural supply, and the bulk of carvone is produced synthetically. Unfortunately, the chemical synthesis of carvone from the sustainable natural compound limonene produces harmful by-products such as nitrosyl-chloride. Hence, we aimed to use biocatalysis to convert limonene to carvone in an environmentally friendly manner. We used lipoxygenase (LOX) rich soybean meal to facilitate the conversion of limonene to carvone. Two soybean preparations were compared for efficiency, ground soybean (GSB) and milled defatted soybean (MDS). GSB unfortunately produced excessive emulsions that trapped our product and made the reaction workup difficult. Since both GSB and MDS provided similar conversions, we opted to use MDS throughout going forward for ease of product extraction. Our study sought to characterise the enzyme activity and thereby confirm that LOX was indeed responsible for conversion of limonene to carvone. We found that the maximum enzyme activity occurred at the pH 8.5, using a 0.1 M sodium borate buffer. A temperature study was also conducted, and it was found that the enzyme activity was highest at 50°C and temperatures higher than this causes the enzyme to denature. Reactions excluding oxygen did not proceed, therefore we can confirm that they involve an oxidative biocatalyst that utilises molecular oxygen. The conversion was also demonstrated to require the intermediate peroxidation of fatty acids. These results are consistent with LOX activity. We decided to investigate which isozyme of LOX is responsible for the conversion. Calcium is a known inhibitor of LOX-3 activity but increases the activity of LOX-1 and LOX-2. After adding calcium salts to the reaction, the conversion rate increased. This result, and the optimal pH of 8.5, are consistent with the activity of LOX-1. It was determined that the optimal MDS to substrate ratio was 10:1. The highest conversion obtained for the reaction was at 58% when adding $FeSO_4.7H_2O$ to the reaction, implying a synergistic Fenton reaction. Finally, carvone was successfully isolated and purified using column chromatography and the chemical identity confirmed using GC-MS and NMR.

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EOs – Essential Oils

LOX – Lipoxygenase

GPP - Geranyl pyrophosphate

GRAS - Generally recognized as safe

MEP - Methylerythritol phosphate

DMAPP - Dimethylallyl diphosphate

IPP - Isopentenyl diphosphate

MRSA - Methicillin-resistant Staphylococcus aureus

AE - Atom economy

RME - Reaction mass efficiency

EME - Effective mass efficiency

EF - Environmental factor

PMI - Process mass intensity

ME - Molar efficiency

APIs - Active pharmaceutical ingredients

IREDs – Immine reductases

TBHP - *t*-butyl hydroperoxide

PUFAs - Polyunsaturated fatty acids

HPOD – Hydroperoxides

HRP - Horse radish peroxidase

CDH - Carveol dehydrogenase

GC – Gas Chromatography

NMR – Nuclear Magnetic Resonance

MPCC - Medium pressure column chromatography

TLC - Thin-layer chromatography

MS - Mass spectrometry

GSB – Ground Soybean

MDS – Milled Defatted Soybean

EPR – Electro paramagnetic resonance

UV – Ultra Violet

TMS – Trimethylsilane

PPM – Parts per million

OA – Oleic acid

LA – Linoleic acid

Chapter 1

Introduction

1.1. Background

The rapid expansion of the global economy has fuelled the increasing demand for both flavour and fragrance products. Due to the increasing number of biotechnological methods used in the production of flavour compounds, the chemical oxidation of terpenes has gradually been replaced by the use of natural sources of aromatic compounds. Aroma compounds, which are also referred to as flavours, fragrances and odorants, are chemical substances that are rapidly growing in the chemical industry, more specifically the food, fragrance, cosmetic, pharmaceutical and detergent industries (Sharmeen *et al.*, 2021) These aroma compounds are volatile such as alcohols, esters, ketones, lactones and terpenes. Plants are one of nature's source of aroma compounds and with the growing demand for environmentally friendly methods, extracting these compounds and their precursors from plants has gained much interest in green chemistry (Sharmeen *et al.*, 2021).

When looking at natural fragrances, essential oils (EOs) are very popular. EOs are a blend of aroma compounds and terpenes that are produced as secondary metabolites in aromatic plants (Sharmeen *et al.*, 2021). EOs consist of a vast range of aroma compounds that have very distinct scents that can be identified even at low concentrations (Buckle *et al.*, 2015) . They are found in particular areas of a plant for example some EOs are found in the petals, others in leaves, stems or roots of a flower (Buckle *et al.*, 2015). The popular and in-demand EOs are lavender, eucalyptus, tea tree and limonene (Buckle *et al.*, 2015). Terpenes are responsible for the distinct scent in most plants and in this project the focus is on terpenes, more specifically monoterpenes.

Monoterpenes are the largest class of terpenes, and they are found in many plants. Limonene is known for its citrus-like characteristics and is used extensively in a number of industries, including cosmetics, fragrances, detergents, and flavouring. Limonene can be converted into useful products such as carvone. Carvone is widely utilized across various industries, including baking, cosmetics, and insecticide production. Due to its relatively high cost, producing carvone from limonene adds significant value, making it a more economical and attractive option.

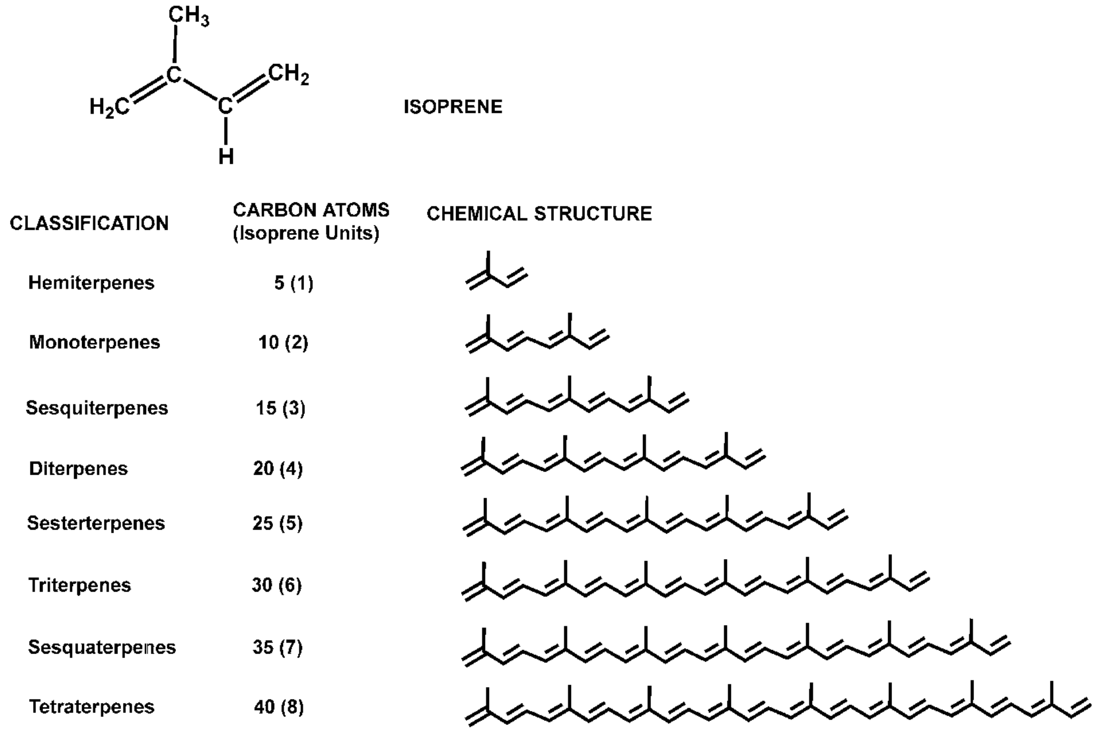
The conversion of limonene to carvone can be accomplished via a chemical route using nitrosyl chloride, however, this method is not environmentally friendly and does not adhere with the green chemistry principles. In this project, we look at the biocatalytic conversion of limonene to carvone using the lipoxygenase (LOX) enzyme, which is extracted from soybeans. This method is environmentally friendly and in line with the green chemistry principles. In previous studies done in our group, crude LOX was obtained from soybean was utilised in the conversion of valencene to nootkatone. LOX was proven to be the responsible enzyme as it was inhibited by a known LOX inhibitor anacardic acid. Literature also provides other examples of other enzymes that have been utilized for this biocatalytic conversion which is explained the subsequent section . With regards to the conversion of limonene to carvone, there are not many reports in the literature citing the use of LOX in this transformation. Hence, this study was conceptualized to evaluate if LOX can be utilized in the oxidative biocatalytic conversion of limonene to carvone.

1.2. Terpenes

Terpenes are natural compounds that consist of hydrocarbons with small isoprene units that are linked to one another to form a chain as shown in **Figure 1** (Mosquera *et al.*, 2021). Terpenes and terpenoids are generally used in the same context, however they have different structures. Terpenes are hydrocarbons (only carbon and hydrogen are present); whereas terpenoids are usually oxygen-containing terpenes. Terpenes are generally found in essential oils, and they have various classifications and importance in the flavour and fragrance industry. Terpenes are secondary metabolites of plants that are produced as a defence mechanism against insects and other threats. Terpenes can help fend off various threats, such as parasites, bacteria, and predators (Cox-Georgian *et al.*, 2019).

1.2.1. Classification of terpenes

Terpenes are made up of isoprene molecules (also referred to as isoprene units). An isoprene unit contains five carbons with a pair of double bonds as shown in **Figure 1**. There are different types of terpenes such as hemiterpenes which contain one isoprene unit, monoterpenes which contain two isoprene units, sesquiterpenes which contain three isoprene units, diterpenes which contain four isoprene units, and so on (Mosquera *et al.*, 2021). Terpenes are also subdivided into groups that are based on their structure, which is acyclic or cyclic. β-myrcene, a monoterpene, is an example of an acyclic terpene with a linear structure (Buckle *et al.*, 2015). In contrast, cyclic terpenes are ring-forming compounds that can be classified as monocyclic, bicyclic, or tricyclic based on whether they have one, two, or three rings. Limonene is an example of a monocyclic monoterpene since it consists of two isoprene units forming a single ring in its structure (Buckle *et al.*, 2015).



CLASSIFICATION	CARBON ATOMS (Isoprene Units)
Hemiterpenes	5 (1)
Monoterpenes	10 (2)
Sesquiterpenes	15 (3)
Diterpenes	20 (4)
Sesterterpenes	25 (5)
Triterpenes	30 (6)
Sesquaterpenes	35 (7)
Tetraterpenes	40 (8)

Figure 1: Classification of terpenes (Mosquera *et al.*, 2021)

1.2.2 Monoterpenes

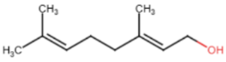
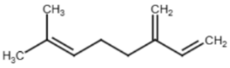
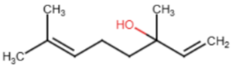
Monoterpenes are compounds present in essential oils, derived from a diverse range of plants such as fruits, vegetables, spices, and herbs. A monoterpene consists of 10 carbon atoms and is part of the isoprenoid class of natural compounds. Monoterpenes consist of two isoprene units. The main precursor for the synthesis of monoterpenes is geranyl pyrophosphate (GPP). Monoterpenes are very useful compounds and there are different structural classes of monoterpenes. Monoterpenes can be either acyclic, monocyclic or bicyclic.

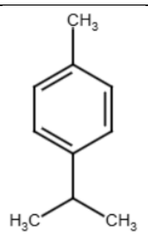
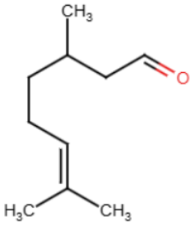
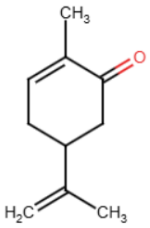
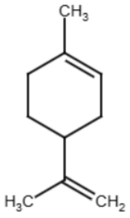
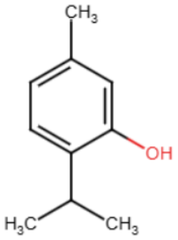
Acyclic monoterpenes are considered linear, and examples are geraniol, myrcene, linalool, and citronella. Monocyclic monoterpenes contain one six-membered ring in their structure whereas bicyclic monoterpenes contain two six-membered rings in their structure. Examples of monocyclic monoterpenes are carvone, limonene, and thymol. Examples of bicyclic monoterpenes are eucalyptol, camphene, carene and thujene. Structures of these linear and monocyclic monoterpenes are exhibited in **Table 1**.

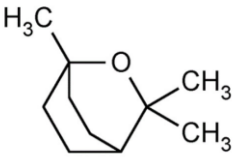
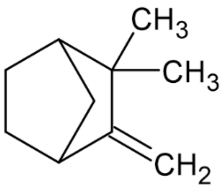
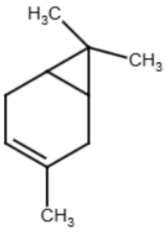
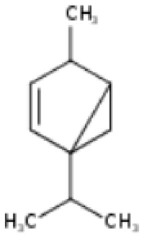
Monoterpenes can also function as volatile substances, which can attract or repel insects. They are usually considered to be nontoxic to mammals, however, some monoterpenes can act as insecticides (Morgan *et al.*, 1999). For example, limonene is toxic to pet fleas and it can be used to control these fleas found on cats and dogs (Morgan *et al.*, 1999). Linalool also has insecticide properties and can be used as sprays for house plants. Menthol is also another example of an insecticide that is used against mites and honeybees (Morgan *et al.*, 1999). Therefore, it is observed that monoterpenes can be used and found in commercial insecticides.

Monoterpenes are widespread in plant life and are accountable for the characteristic scents in plants (Bouwmeester *et al.*, 1998). They have a unique fragrance. For example, citral has a lemon-like smell and is responsible for the scent of citrus fruit. Monoterpenes are used in the medical industry as well. Studies have shown that various forms of monoterpenes can act as effective agents against various diseases, such as cancer and viral infection (Santos *et al.*, 2011). They can also improve the cardiovascular system's functioning and prevent various heart conditions (Santos *et al.*, 2011). Natural monoterpenes, such as limonene, γ-terpinene, and α-phellandrene exhibited analgesic effects (Salakhutdinov *et al.*,2017). Myrcene and (+)-α-pinene, on the other hand, exhibited anti-inflammatory activity (Salakhutdinov *et al.*, 2017). Some of the benefits of monoterpenes are exemplified in **Table 1**

Table 1: Examples of monoterpenes and their benefits

Compound	**Structural Class**	**Chemical Name & Formula**	**Benefits**	**Scent**	**Reference**
Geraniol 	Acyclic	3,7-dimethylocta-trans-2,6-dien-1-ol ($C_{10}H_{18}O$)	Effective insect repellent and flavouring agent for the food industry.	Rose/sweet scent	(Chen *et al.*, 2010)
Myrcene 	Acyclic	7-methyl-3-methylene-1,6-octadiene ($C_{10}H_{16}$)	Effective sedative that promotes sleep and used as a flavouring agent for the food and cosmetic industry.	Clove-like, spicy, musky scent	(Surendran *et al.*, 2021)
Linalool 	Acyclic	2,6-dimethyl-2,7-octadien-6-ol ($C_{10}H_{18}O$)	Effective insect repellent. Has anti-fungal and antimicrobial activity.	Lavender scent	(Russo *et al.*, 2017)
Cymene	Monocyclic	1-Methyl-4-(propan-2-yl)benzene ($C_{10}H_{14}$)	Effective anti-oxidant and has anti-fungal properties.	Woody, earthy-like scent	(Balahbib *et al.*, 2021)

					
Citronellal 	Acyclic	3,7-dimethyloct-6-en-1-al ($C_{10}H_{18}O$)	Effective mosquito repellent.	Lemongrass scent	(Fradin *et al.*, 2019)
Carvone 	Monocyclic	2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl) ($C_{10}H_{14}O$)	Insect repellent and flavouring agent in the food and cosmetic industry.	Caraway and spearmint scent.	(Morcia *et al.*, 2016)
Limonene 	Monocyclic	1-methyl-4-(1-methylethenyl)-cyclohexene ($C_{10}H_{16}$)	Used as an insect repellent. Used in the detergent and cosmetic industry.	Citrus scent	(Kvittingen *et al.*, 2021)
Thymol 	Monocyclic	2-isopropyl-5-methylphenol ($C_{10}H_{14}O$)	Used as an antiseptic in toothpaste and mouthwash.	Strong herb-like scent	(Noma *et al.*,2010)

Eucalyptol 	Bicyclic	1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane ($C_{10}H_{18}O$)	Used in mouthwash and cough syrup.	Camphor -minty like scent.	(Salehi *et al.*, 2019)
Camphene 	Bicyclic	2,2-Dimethyl-3-methylidenebicyclo[2.2.1]heptane	Used as a food additive for flavouring.	Piney-woody scent	(Morgan *et al.*, 1999
Carene 	Bicyclic	3,7,7-trimethylbicyclo[4.1.0]hept-3-ene	Used in the fragrance industry to make perfumes and aroma oils.	Piney and citrusy scent	(Salakhutdinov *et al.*, 2017)
Thujene 	Bicyclic	4-methylidene-1-(1-methylethyl)bicyclo[3.1.0]hexane	Used in insect repellents.	Herbal and woody scent	(Santos *et al.*, 2011)

1.3 Limonene

Limonene, a monoterpene olefin with a distinct citrus aroma, is widely used in products like soaps and detergents (Kazyoba *et al.*, 2008). Limonene is a type of monoterpene that is found in abundance in nature. The status of limonene is generally recognized as safe (GRAS) and it has many applications in industry (Nikfar *et al.*, 2014). (+)-Limonene has a 92 to 96%

concentration that is found in orange and lemon peels (Duetz *et al.*, 2001). It is a promising starting compound for a wide variety of flavour compounds and fine chemicals, such as carvone, perillyl alcohol, and carveol (Duetz *et al.*, 2001).

1.3.1 Biosynthetic Pathway of Limonene

Limonene, a monoterpene compound, is synthesized from the precursor geranyl diphosphate (GPP) through an enzymatic reaction catalyzed by either *d*-limonene synthase or *l*-limonene synthase (Pang *et al.*, 2019). In both plants and *Y. lipolytica*, GPP is generated from the universal isoprenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) as shown in **Figure 2**.

(a) Plant Pathway:

In plants, GPP production occurs via the methylerythritol phosphate (MEP) pathway, which begins with pyruvate and glyceraldehyde-3-phosphate. This pathway involves several key enzymes, including DXP synthase (DXPS), DXP reductoisomerase (DXPRI), MEP cytidylyltransferase (MEPCT), CDP-ME kinase (CDP-MEK), MECDP synthase (MECDPS), (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HMBPPS), and HMBPP reductase (HMBPPR) (Pang *et al.*, 2019).

(b) Yeast Pathway:

In contrast, *Y. lipolytica* utilizes the mevalonate (MVA) pathway to produce GPP from acetyl-CoA. Since this yeast does not naturally express limonene synthase, genes encoding *d*-limonene synthase from *Citrus limon* and *l*-limonene synthase from *Mentha spicata* were introduced to establish a complete biosynthetic route. These heterologous enzymes are indicated in red. Additionally, potential downstream metabolic transformations of limonene within yeast are marked in blue (Pang *et al.*, 2019).

To enhance limonene production, several native enzymes in the MVA pathway were overexpressed in engineered *Y. lipolytica* strains. These include acetyl-CoA C-acetyltransferases (ACOAAT1 and ACOAAT2), hydroxymethylglutaryl-CoA synthase (HMGS), hydroxymethylglutaryl-CoA reductase (HMGR), mevalonate kinase (MK), phosphomevalonate kinase (PMK), and diphosphomevalonate decarboxylase (PMVADO). Additional enzymes involved include isopentenyl diphosphate isomerase (IPPDI), geranyl diphosphate synthase (GPPS), farnesyl diphosphate synthase (FPPS), and geranylgeranyl diphosphate synthase (GGPPS, type III). These native enzymes, shown in purple in **Figure 2**, highlight the yeast's internal capabilities to support and enhance the engineered limonene production pathway (Pang *et al*., 2019).

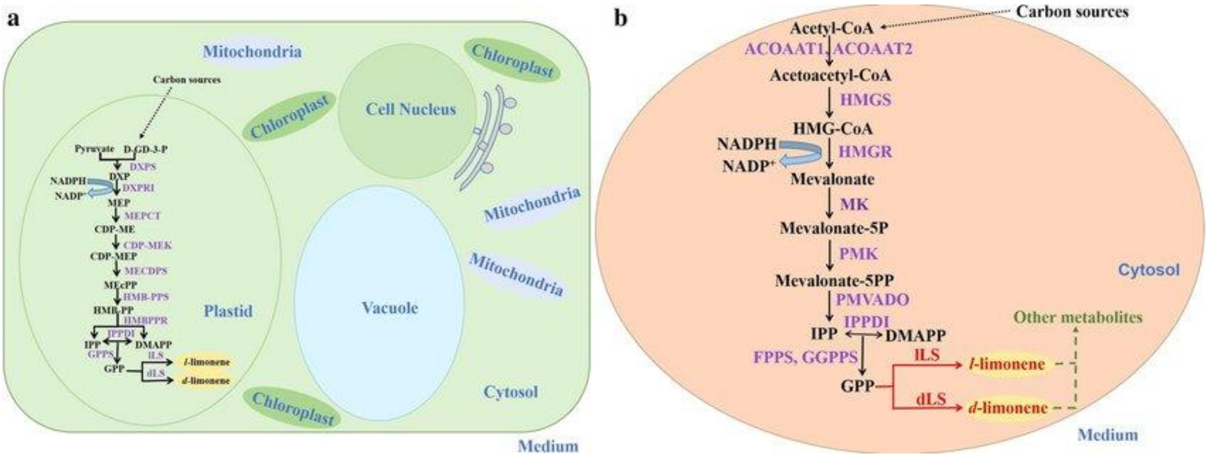


Figure 2: The biosynthetic reaction pathway of limonene (Pang *et al*., 2019).

1.3.2 Stereochemistry of Limonene

Limonene is a chiral molecule due to the presence of a chiral centre, making it optically active. It has two enantiomers, (R)-limonene and (S)-limonene. Stereochemistry helps scientists understand the optical activity of limonene by explaining the behaviour of enantiomers. The (+) enantiomer, also known as dextrorotatory (D), rotates plane-polarized light clockwise, while the (-) enantiomer, or levorotatory (L), rotates light counter-clockwise. In limonene, the D-isomer corresponds to the (R) configuration, and the L-isomer corresponds to the (S) configuration (Isac-García *et al.*, 2016).

1.3.3 Applications of Limonene

Limonene, is a compound that occurs naturally and is found in citrus peels, boasts a wide variety of applications and capability of health benefits. Its antioxidant, anti-inflammatory, and antimicrobial properties make it a valuable agent in the agri-food industry, effective in preventing pest attacks and food spoilage (Viuda-Martos *et al.*,2020). Additionally, limonene has shown neuroprotective effects, potentially helping with neurodegenerative diseases such as Alzheimer's and Parkinson's (Yu *et al.* 2018). Its anti-cancerous, anti-nociceptive pain, and gastroprotective properties have also been demonstrated (Yu *et al.* 2018). Furthermore, limonene has been found to improve lung function and exhibit anti-inflammatory effects in the lungs (Souza *et al.*, 2018). Its anxiolytic effects and anxiety reduction have also been studied (Souza *et al.*, 2018).

The wide range of limonene's applications highlights its versatility and potential. Consequently, it is frequently utilized as a flavoring agent in citrus-flavored products including soft drinks, candies, detergents, and perfumes. Its nutraceutical properties make it a valuable ingredient in healthcare products, while its antibacterial and biopesticide properties render it effective in agricultural and environmental applications (Jongedijk *et al.*, 2016). Moreover, limonene's green extraction solvent properties and fragrance characteristics make it a sought-after additive in the food and beverage industry. Limonene has the potential to serve as a building block for the production of p-cymene, a crucial intermediate in various industrial processes, underscores its significance in modern biorefineries. As demand for limonene continues to grow, optimizing its extraction and conversion processes is essential to fully harness its potential and address the increasing demand (Jongedijk *et al.*, 2016).

1.4 Carvone

Carvone is an essential monoterpene ketone that is used in the flavour industry (Paula Dionísio *et al.*, 2012) It has a boiling point of 230˚C. Carvone has two optical isomers which are *S*-(+)-carvone (also known as D-carvone) and *R*-(-)-carvone (also known as L-carvone) (Morcia *et al.*, 2016). These isomers give rise to different biological responses, mainly towards the olfactory receptors. For example, *S*-(+)-carvone has a medium strength mentholated, spicy aroma of caraway seeds, whereas *R*-(-)-carvone has a minty and sweetish medium strength odour of spearmint (Morcia *et al.*, 2016).

1.4.1 Biosynthetic Pathway of Carvone

According to **Figure 3**, geranyl diphosphate is the starting point for the three-step biosynthetic process that produces limonene and carvone in the caraway plant (*Carum carvi L.*). A monoterpene synthase first converts geranyl diphosphate to (+)-limonene by cyclization. Secondly, this intermediate either undergoes no additional metabolism in the essential oil ducts or is transformed into (+)-trans-carveol by limonene-6-hydroxylase. Third, a dehydrogenase converts (+)-trans-carveol to (+)-carvone. By tracking the accumulation of limonene and carvone during fruit development and comparing it to the changes in the activity of the three enzymes and monoterpene biosynthesis Bouwmeester and co-workers were able to better understand the regulation of monoterpene creation in caraway (Bouwmeester *et al.*, 1998).

The patterns of monoterpene accumulation are effectively explained by the activities of the enzymes, with limonene-6-hydroxylase being a key player in regulating the final product's nature. Only limonene was accumulating at insignificant amounts during the early stages, when limonene-6-hydroxylase was undetectable. The development of limonene-6-hydroxylase corresponds meticulously to the start of carvone buildup. At later phases of fruit growth, the activity of all three enzymes decreased significantly. Even though this strongly correlates with a decline in monoterpene concentration, the latter could possibly be due to competition for substrate with other pathways.

The biosynthetic pathway of carvone involves the transformation of limonene to carveol by the enzyme cytochrome P450 limonene-6-hydroxylase (P450) as shown in **Figure 3** (Abraham *et al.*, 2012). Carveol is then oxidized to carvone by the enzyme carveol dehydrogenase (CDH) (Wouters *et al.*, 2001). The expression levels and enzymatic kinetics of P450 and CDH can impact carvone production (Abraham *et al.*, 2012). An optimal ratio of P450 to CDH expression has been shown to be crucial for maximizing carvone production (Wang *et al.*, 2013).

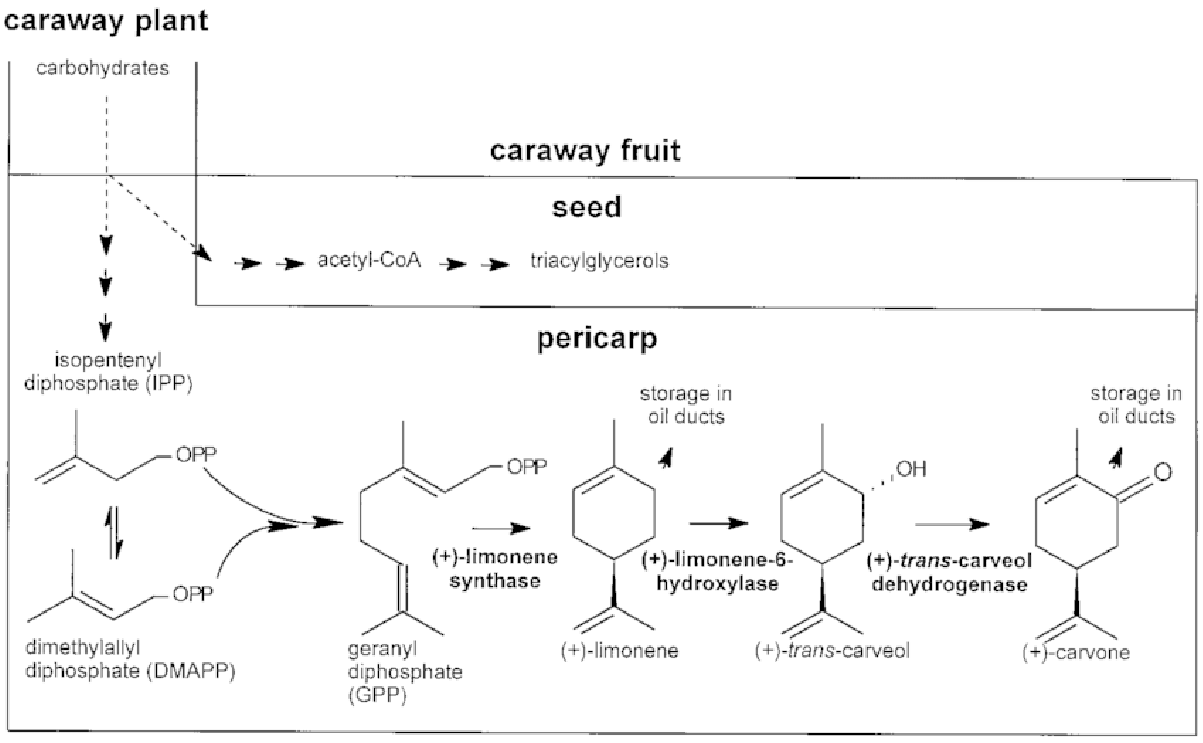


Figure 3: Biosynthetic pathway of (+)-carvone (Bouwmeester et al., 1998).

1.4.2 Applications of Carvone

Carvone has demonstrated various biological properties, including antimicrobial, anticancer, anti-inflammatory, antidiabetic, neurological, and other pharmacological effects (Bouyahya *et al.*, 2021). Carvone also has the capability to act as an antioxidant, insecticide, fungicide, and plant growth regulator (Huang *et al.*, 2022). Carvone has very interesting properties and it undergoes photodegradation in an ethanol-water solution to form carvone camphor (Huang *et al.*, 2022). Carvone is a very important essential oil and it can be found in parsley oil and dill seed oil in the form of the *S*-isomer ((+)-carvone), and spearmint oil in the form of the *R*-isomer((-)-carvone) (Huang *et al.*, 2022). Carvone is being investigated as a component in biodegradable plastics and is used in specialized paints and coatings due to its pleasant scent. Furthermore, it acts as a flavour-masking agent in food and medicine goods (Morcia *et al.*, 2016). Its unique properties make it a valuable compound.

Carvone's versatility and range of applications make it a vital compound across various industries. In the pharmaceutical sector, carvone has shown promise as a neuroprotective agent, preventing neurodegenerative diseases like depression, sedation, and seizure (Bouyahya *et al.*, 2021). Its antidiabetic properties also make it a promising candidate for managing diabetes by improving glucose metabolism and glycoprotein component abnormalities. Furthermore, carvone's antifungal and antibacterial activities make it effective against various fungal and bacterial strains, including methicillin-resistant *Staphylococcus aureus* (MRSA), rendering it a potential treatment for fungal and bacterial infections. Its anticancer properties, attributed to its antioxidant and anti-inflammatory effects, may help prevent cancer cell growth and proliferation (Bouyahya *et al.*, 2021). Moreover, carvone's analgesic and anti-inflammatory properties make it a natural pain reliever and a potential solution for managing conditions like arthritis, allergies, and asthma. Overall, carvone's diverse applications make it a valuable compound for developing therapeutic drugs and treatments (Huang *et al.*, 2022).

1.4.3 Chemical Synthesis of Carvone

Fractional distillation of caraway or spearmint oils are typically used to isolate carvone. However, the more favoured approach was the chemical synthesis using limonene (Morcia *et al.*, 2016; Paula Dionísio *et al.*, 2012).Approximately 3800 tons of (-)-carvone is produced yearly, with the majority (around 2,000 tons) produced through chemical synthesis from (+)-limonene. (Yoshida *et al.*, 2021). The chemical synthesis of converting (+)-limonene to (-)-carvone is shown in **Figure 4** (Demidova *et al.*, 2016).

The chemical conversion of (+)-limonene to (-)-carvone was first reported by Royals et al. in 1951. They obtained an overall yield of between 50-60% (Royals *et al.*, 1951). Their chemical synthesis method consisted of three steps as shown in **Figure 4.** The first step involved the conversion of (+)-limonene to (+)-limonene nitrosochloride and the obtained yield was 80%. The second step involved the dehydrohalogenation of the (+)-limonene nitrosochloride to (-)-carvoxime which had a yield of between 90-95%. The third and final step involved the hydrolysis of (-)-carvoxime by the reflux with 5% aqueous oxalic acid to give rise to (-)-carvone which had a yield between 78-80% (Royals & Horne, 1951). However, this method uses hazardous chemicals such nitrosyl chloride, which is a toxic gas and is not environmentally friendly. Therefore, scientists have endeavoured to utilize a more environmentally friendly method that incorporates the use of oxidative biocatalysis.

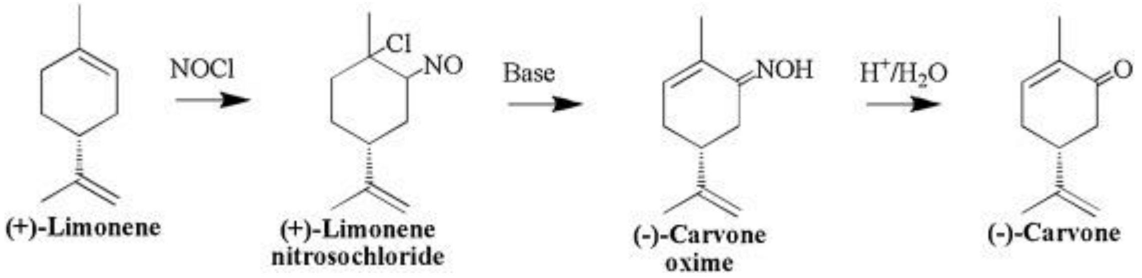


Figure 4: The chemical synthesis of (-)-carvone (Demidova *et al.*, 2016)

1.5 Green Chemistry

Green chemistry is an array of principles that are implemented to minimize or eradicate the use of toxic and hazardous chemical substances. For over 30 years, Green Chemistry has been a framework for chemical engineers and scientists to contribute to the global sustainability of the environment (Ganesh *et al.*, 2021). The main idea behind green chemistry is the development of environmentally friendly chemical substances that can serve the same purpose in a chemical reaction and be energy efficient and selective (Anastas & Eghbali, 2010). There are twelve green chemistry principles that have been designed to sustain and preserve the future generations (Jessop *et al.*, 2008).

1.5.1 The Twelve Green Chemistry Principles

The first principle of green chemistry emphasizes that it's better to prevent waste formation in the first place than to deal with its management and cleanup after it has been created (Ardila-Fierro *et al.*, 2021). In the 20th century waste prevention and pollution control became an important factor in the design of chemical reactions. Researchers have come a long way and have developed green chemistry from nothing into a large body of information that aims to create a greener chemical industry (Ardila-Fierro *et al.*, 2021). The second principle of green chemistry is based on atom economy or atom efficiency. It states that chemical reactions need to be designed to accentuate the use of starting materials in the process of making the end product (Ardila-Fierro *et al.*, 2021).

The third principle of green chemistry is to ensure that all chemical synthetic methods should be less hazardous and not toxic to the environment. The fourth principle is to design chemicals that are safer and environmentally friendly. This principle is in correlation with the third principle of green chemistry. Principle number five is to use more environmentally friendly solvents (Wardencki *et al.*, 2005). Therefore, we can see that the main aim of green chemistry principles is to protect the environment from harmful chemical substances. The sixth principle involves designing chemical procedures that are energy efficient. If possible, the amount of energy used in a reaction should be minimized, for example, reactions should be under ambient conditions (Anastas *et al.*, 2010).

The seventh principle of green chemistry involves using renewable feedstock. Fossil fuels are depleting and shifting towards renewable resources will assist in creating a sustainable future. Principle number eight focuses on reducing derivatives that are used in chemical reactions such as blocking and protecting groups (Jessop *et al.*, 2008). The use of derivatives creates more waste and in organic synthesis these groups are very common and there are many steps involved. These steps cause waste and toxic by-products. This is when the ninth principle of green chemistry comes into play since it focuses on catalysis. A catalyst can take a reaction from having many steps to just one step because it lowers the activation energy which allows the reaction to take place at a faster rate. Catalysts can also be very selective and this will reduce the number of by-products in a reaction (Anastas *et al.*, 2010).

The tenth principle focuses on designing products for degradation, so that they will not persist and cause pollution to the environment. The eleventh principle is based on pollution prevention and this involves real-time analysis of reactions (Wardencki *et al.*, 2005). If reactions are constantly monitored, then this will prevent accidents from occurring. The final and most important principle of green chemistry is to ensure the prevention of accidents in the laboratory and on industrial sites. This principle includes safety practices and eliminating hazardous chemicals. The main goal associated with this principle is to remove or replace hazardous chemicals in laboratories and plants and to create a safe environment to work in (Anastas *et al.*, 2010).

1.5.2 Green Chemistry Metrices

Green chemistry metrics serve as methods for evaluating the environmental sustainability of chemical processes and products. These metrics provide a framework for chemists and industries to design and optimize sustainable chemical processes. Green chemistry metrics have been widely adopted in the chemical industry to reduce waste, improve efficiency, and minimize environmental impact (Anastas *et al.*, 1998). The most used metrics include atom economy (AE), reaction mass efficiency (RME), effective mass efficiency (EME), environmental factor (E-factor), process mass intensity (PMI), and molar efficiency (ME) (Tang *et al.*, 2019).

Atom economy (AE) measures the percentage of atoms from the reactants that remain in the final product (Trost *et al.*, 1991). Reaction mass efficiency (RME) and effective mass efficiency (EME) evaluate the efficiency of a process based on the mass of reactants and non-benign reagents (Constable *et al.*, 2007). The environmental factor (E-factor) measures the ratio of waste to product (Sheldon *et al.*, 1992). Process mass intensity (PMI) assesses the total mass of materials used in a process (Constable *et al.*, 2007). Molar efficiency (ME) is a metric designed for use in discovery-phase medicinal chemistry (Watkins *et al.*, 2014). These metrics have been applied in various industries, including pharmaceuticals (Kjell *et al.*, 2019), catalysis (Huang *et al.*, 2020), and biotechnology (Zhang *et al.*, 2019). The use of green chemistry metrics has led to significant reductions in waste, energy consumption, and environmental impact (Tang *et al.*, 2019).

1.6 Biocatalysis

Biocatalysis can speed up chemical reactions by using enzymes or whole cells. Biocatalysis has now become important in the synthesis of organic compounds (Bell *et al.*, 2021). Enzymes are essential in catalysing numerous processes, including the synthesis of alcohols and ketones, even the fermentation of cheese and the breakdown of proteins found in milk and other substances (Paul *et al.*, 2019). Latest developments in the study of enzymes have assisted many scientists in the understanding of their structure and functional activities. Correct structure of an enzyme confers function, stability and specificity (Paul *et al.*, 2019).

Biocatalysis begins with the process of recognizing the target reaction, obtaining the biocatalyst, characterizing, engineering/modifying, and process of modelling. Biocatalysis is envisioned to continue playing a critical role in future product developments due to the rising ability to use enzymes to catalyse various chemical reactions in industrial processes such as pharmaceutical drugs, flavours, fragrances and polymers (Paul *et al.*, 2019). The use of proteins as catalysts for the synthesis of more complex compounds has become a main attraction in the pharmaceutical industry. Enzymes can be bought commercially or can be found in natural sources. Commercial enzymes are screened against specific substrates to determine if they can be used as a candidate biocatalyst.

When it comes to a biocatalyst, there are factors that need to be considered such as the efficiency and availability. In industrial reactions it is important to obtain high product concentrations and to minimize product recovery cost. Substrate concentration is also an important factor to include, and an ideal biocatalyst's activity should not be restrained by high substrate concentrations (> 50 $g.l^{-1}$). A highly stable biocatalyst under industrial process conditions is also a very important property that makes it a good biocatalyst. Biocatalysis is typically considered as a green and sustainable method as compared to the chemical synthesis approach and is in check with the green chemistry principles.

1.6.1 Significance and Applications of Biocatalysis

Biocatalysis has emerged as a vital tool in the pharmaceutical industry, offering novel options for the synthesis of active pharmaceutical ingredients (APIs). Strauss *et al.* (2022) highlighted the significance of biocatalysis in drug synthesis, demonstrating its potential to revolutionize the industry. The use of biocatalytic approaches has been successfully employed in the synthesis of antibiotics, antivirals, and anticancer agents, showcasing its versatility and efficacy. Furthermore, biocatalysis has been instrumental in developing more efficient and sustainable routes to APIs. For instance, the use of transaminases for the synthesis of chiral amines has been extensively explored, with Abdelraheem *et al.* (2019) demonstrating the potential of engineered imine reductases (IREDs) for the synthesis of chiral imines.

In addition, extreme biocatalysts have been investigated for their potential in biotechnological applications. Petchey *et al.* (2022) provide a comprehensive review of the significance of extreme biocatalysts in industrial processes, highlighting their ability to revolutionize various sectors. Overall, biocatalysis has emerged as a powerful tool in the pharmaceutical industry, offering novel options for API synthesis and sustainable routes to chiral amines. As research continues to advance, it is likely that biocatalysis might play an increasingly essential role in shaping the future of drug synthesis.

Moreover, biocatalysts have been successfully employed in various industrial applications, including chemical synthesis, due to their advantages over traditional chemical catalysts (Schmidt *et al.*, 2018). Recent research has also explored the potential of extreme biocatalysts in biotechnological applications. According to Santos *et al.* (2020), extreme biocatalysts can revolutionize industrial processes across diverse sectors. For example, thermophilic enzymes have been employed in the synthesis of biofuels and biochemicals (Lee *et al.*, 2019).

1.7 Catalytic Conversion of Limonene to Carvone

Catalysis is an inorganic method that uses mainly metal oxides or metal complexes to speed up chemical reactions. In 2017, Becerra and team worked on using a heterogenous catalyst to convert limonene to carvone. In their study they synthesized an iron hexadecachlorinated phthalocyanine immobilized on modified silica catalyst ($FePcCl_{16}$-NH_2-SiO_2) and the oxidizing agent used for the reaction was *t*-butyl hydroperoxide (TBHP) (Becerra *et al.*, 2018). In this study the predominant component of this essential oil was limonene (91–96 weight percent), which was oxidised to create valuable compounds like carvone, limonene 1,2-epoxide, and carveol that are used in the culinary, pharmaceutical, and cosmetic sectors (Becerra *et al.*, 2018).

Becerra and team discovered that after 23 hours, there was a 74% conversion of limonene with a 10% carvone selectivity and an apparent activation energy of 38.6 kJ mol-1; however, after

10 hours, there was an 87% selectivity to carvone at 78% carveol conversion. Therefore, they concluded that the selectivity towards carvone increases with time however, there is a certain maximum of carvone production which then causes carvone to oxidize, hence decreasing its selectivity (Becerra *et al.*, 2018).

In a 2019 study, Yizhou Li et al. used ZIF-67(Co), a cobalt-based zeolitic imidazolate framework, as a heterogeneous catalyst to evaluate the liquid-phase catalytic oxidation of limonene. Benzene was used as the solvent and t-butyl hydroperoxide (t-BHP) as the oxidant in this moderate reaction. According to the findings, ZIF-67(Co) demonstrated a respectable conversion rate of 29.8% and substrate-product selectivity of 55.4%. Notably, X-ray diffraction patterns before and after the reaction proved that the catalyst showed good reusability with negligible loss of activity.

This study explored the potential of ZIF-67(Co) as an efficient and reusable catalyst for the selective oxidation of limonene to carvone. Yizhou Li *et al*. demonstrated that carvone is produced through a free-radical reaction at the allyl group of limonene, leading to the formation of limonene-1,2-oxides and limonene-1,2-diols as byproducts. Additionally, under acidic conditions, the limonene-1,2-oxide undergoes hydrolysis to form limonene-1,2-diol, which can then rearrange and be converted into carvone. The study provides insight into the mechanistic pathway for the selective oxidation of limonene to carvone, showcasing the effectiveness of ZIF-67(Co) as a catalyst in this process.

While traditional catalytic methods have shown promising results in converting limonene to carvone, our focus lies in harnessing the potential of biocatalysis to achieve this transformation. Lipoxygenase enzymes, known for their regio- and stereoselective oxidation capabilities, offer a green alternative to conventional chemical catalysts. By leveraging the enzymatic activity of lipoxygenase, we seek to develop an efficient and selective biocatalytic process for the conversion of limonene to carvone, minimizing the environmental impact and enhancing the overall sustainability of the process. This approach aligns with the principles of green chemistry and biocatalysis, offering a promising route for the production of carvone and other valuable compounds.

1.8 Lipoxygenase Enzyme

The enzyme lipoxygenase (LOX) can be found in plants and animals. LOX is a group of non-heme metal-containing dioxygenases which is known for catalysing the oxygenation of specific polyunsaturated fatty acids (PUFA) to form fatty acid hydroperoxides (Hayward, Cilliers, & Swart, 2017). Oxygen plays an important role in LOX-catalysed reactions because it acts as a terminal electron acceptor. Plants, however, contain numerous types of the LOX enzyme which are different with regards to their substrate preference, optimal pH, product formation and stability (Hayward *et al.*, 2017). There are different types of LOX that can be found in plants and animals.

Plant lipoxygenases (LOXs) are classed into three types: LOX-1, LOX-2, and LOX-3. Their positional specificity determines whether the oxygenation occurs at C-9 or C-13 of the fatty acid's hydrocarbon chain. These enzymes produce two main groups of compounds: 9S-hydroperoxy-HPOD and 13S-hydroperoxy-HPOD. LOXs exhibit high selectivity for their substrates. In plants, linoleic acid is the most common substrate, while in animals, arachidonic acid is the preferred substrate for LOX.(Soler *et al.*, 2016).

LOX was discovered as early as 1932 and it was first reported from soybeans. Soybean seeds have been found to contain the LOX enzyme in the largest amount, and it is usually purified and isolated from defatted soymeal. From previous studies it was found that soybean seeds contain three LOX enzymes which are LOX-1, LOX-2 and LOX-3 (Shibata *et al.*, 1987). These enzymes show diversity in their behaviour, regiospecificity and pH optima. LOX-1 shows selection for the carbon 13 as the site of hydro-peroxidation and LOX-2 and LOX-3 utilize either carbon 9 or 13 (Shibata *et al.*, 1987).

When it comes to their secondary reaction, these enzymes differ. For example, LOX-1 will form a 13-hydroperoxide that is in stoichiometric ratio when acting on linoleic acid in the presence of oxygen. When oxygen is used up, the hydroperoxide product is attacked and it forms ketodienes, split products, dimers and various other products. With LOX-3, the secondary reaction will proceed readily even when oxygen is present. LOX-2, however, exhibits minimal propensity to react with hydroperoxide, regardless of the presence of oxygen. These enzymes are very interesting, and they show different substrate specificities as well. For example, LOX-1, unlike LOX-2 and LOX-3 prefer an anionic substrate (Shibata *et al.*, 1987).

The kinetics of these isozymes of LOX are generally linked to the availability of the substrate and the extraction procedures. Previous studies suggest that defatting inhibits the activity of LOX isozymes, indicating that LOX activity can potentially be modulated by adjusting the enzyme-to-substrate ratios and pH within the enzyme's environment. (Mandal *et al.*, 2014). LOX-3 has the highest protein concentration followed by LOX-1, which is almost as concentrated. The ideal pH range for LOX-1 is between 8 and 9, whereas for LOX-2 and LOX-3, the optimal pH is 6.5 (Murphy *et al.*, 2008). LOX-1 has peak activity with linoleic acid at pH 9. LOX-2 and LOX-3 exhibit greater activity with methyl esters of fatty acids and triglycerides than with free fatty acids (Murphy *et al.*, 2008).

The activation and catalytic activity of soybean lipoxygenase (LOX), particularly LOX-1, relies on the presence of non-heme iron in its active site (Undeland *et al.*, 2016). The enzyme becomes catalytically active when the iron is in the ferric state (Fe^{3+}), whereas the ferrous form (Fe^{2+}) is inactive in initiating the primary oxygenation reaction (Undeland *et al.*, 2016). Upon activation, ferric LOX abstracts a hydrogen atom from a bis-allylic methylene group of a polyunsaturated fatty acid such as linoleic acid, forming a pentadienyl radical as shown in **Figure 5**. This radical then reacts with molecular oxygen to produce a fatty acid hydroperoxide, such as 13-hydroperoxy-octadecadienoic acid (13-HPOD). This oxygenation reaction is central to LOX's function and is referred to as its dioxygenase activity (Hayward *et al.*, 2017). In addition to this, LOX enzymes can also exhibit hydroperoxide isomerase activity, particularly when the iron is in the ferrous form. Under such conditions, the enzyme can convert fatty acid hydroperoxides into epoxyalcohols and ketones as shown **Figure 5**. This dual functionality allows LOX to play multiple roles in lipid metabolism. The enzyme cycles between its ferric and ferrous states during catalysis, with the ferrous form being reoxidized by another hydroperoxide molecule to regenerate the active ferric state and continue the catalytic cycle as shown in **Figure 5** (Hayward *et al.*, 2017).

Zheng and Brash (2010) discovered that molecular oxygen is not directly involved in the hydroperoxide isomerase cycle but rather it was involved in the direct activation of LOX (Zheng *et al., 2010*). LOX-based isoenzymes from soybean seed are composed of 94 to 97 kDa and have distinct isoelectric point values ranging from 5.7 to 6.4. They can be distinguished from one another due to their substrate specificity, stability, and pH. The smallest of these is LOX-1, which exhibits a maximal activity at pH 9.0 and converts free linoleic acid into the 13 hydroperoxides. LOX-2 exhibits a peak activity of around pH 6.8 and forms equal quantities of the 9- and 13-hydroperoxides. It also oxygenates the unsaturated fatty acids found in membranes. LOX-3, on the other hand, exhibits a moderate preference to produce 9-HPOD at approximately pH 7.

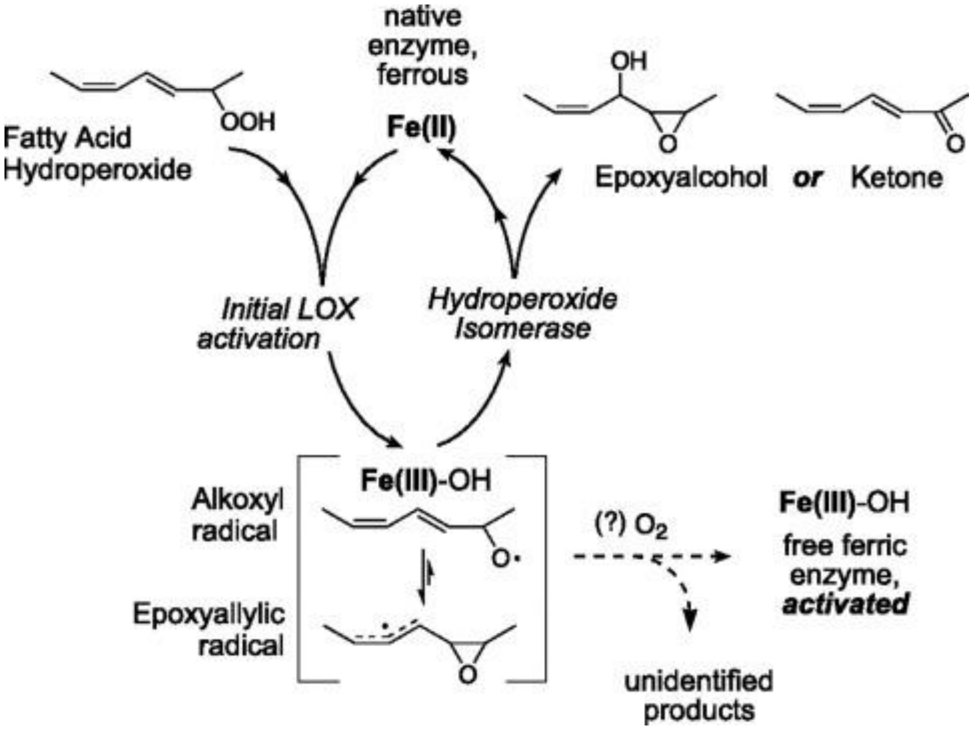


Figure 5: The activation of lipoxygenase and the formation of hydroperoxides by molecular oxygen (Hayward *et al.*, 2017)

LOX contains one iron atom in its structure which is located in the high spin state of Fe(II) (Baysal *et al.*, 2007). It is required to be oxidized to Fe(III) by the reaction product hydroperoxides and hydrogen peroxide before it can trigger an oxidation catalyst (Baysal *et al.*, 2007). This requirement leads to a lag period when the enzyme is utilized with fatty acid substrates. The action of the lipoxygenase starts by abstracting a hydrogen atom from a group of polyunsaturated fatty acids. It reduces the iron to Fe(II). The radical complex then undergoes oxidation by molecular oxygen under aerobic conditions. This process allows the transference of electrons from the ferrous atom into the peroxy group. Dissociation and protonation from the enzyme trigger the hydroperoxide's formation. In anaerobic conditions, the radical complex's alkyl radical components are separated and a mixture of products, including epoxides, is produced. In the presence of oxygen, the enzyme (LOX) transforms oleic acid into (E)-10-hydroperoxy-8-octadecenoic acid (HPOD), which then breaks down into the corresponding (E)-10-hydroxy-8-octadecenoic acid (HOD).(Busquets *et al.*, 2004).

1.8.1 Application of LOX

The hydroperoxides produced by LOX are utilized in various industrial processes, such as the production of resins, plastic products, and soap. In addition, they can be used in the whitening of bread (Hayward *et al.*, 2017). Lipoxygenases are an oxidative enhancing agent used in dough processing. Oxidising agents raise the molecular weight of dough proteins, which eventually improves the strength, workability, and development of the dough as well as the end product's repeatability. The byproducts of flour fatty acid lipoxygenase oxidation encourage flour protein cross-linking. In addition to enhancing dough rheology, lipoxygenase bleaches flour's carotenoid hues (Fernandes *et al.*, 2018).

In the fragrance industry, LOX enzymes are used to generate volatile aromatic compounds from polyunsaturated fatty acids, particularly linoleic and linolenic acids. Through LOX-mediated oxidation, these fatty acids are converted into hydroperoxides, which are further broken down into aldehydes, alcohols, and ketones—many of which have pleasant green, fruity, or floral aromas (Fernandes *et al.*, 2018). For example, compounds like hexanal and cis-3-hexenal, which have a characteristic "fresh-cut grass" or "green" scent, are formed through LOX activity. These naturally derived aroma compounds are used in perfumes, flavourings, and scented consumer products. LOX thus offers a bio-based method for producing high-value aromatic compounds used in both food and cosmetic industries (Fernandes *et al.*, 2018).

1.8.2 Extraction methods for LOX

The extraction of the LOX enzyme is usually carried out through water extractions, salt-induced precipitations and co-precipitations (Shi *et al.*, 2020). Water extractions are considered to be the easiest to many scientists because it does not damage the catalytic activity of the extracted LOX enzyme. The process usually starts by submerging soybean flour in water and stirring for a certain amount of time. Thereafter, centrifuging to obtain the crude LOX extracts. This method is simple and non-destructive, however, it forms impure proteins and further purification is needed to isolate the LOX enzyme (Shi *et al.*, 2020). The extraction of LOX from soybean can be achieved by using different methods such as homogenization and centrifugation, liquid extractions, precipitation and purification, and enzyme- assisted extraction.

The homogenization and centrifugation method involves homogenizing soybean samples with a suitable buffer or extraction solution to release the lipoxygenase enzyme. Various conventional methods were used to purify LOX, such as ammonium sulfate, acetone precipitation, gel filtration, and chromatography (Németh *et al.,* 2001). Various methods have been utilized to separate soybean proteins, such as lipoxygenase. However, they were not able to thoroughly purify the LOX from soybean. A two-phase extraction technique based on metal affinity was suggested for this process.

1.9 Biocatalytic conversion of Limonene to Carvone

The conversion of limonene to carvone using biocatalysis was first reported in 1966 by a research team in India. Dhavalikar et.al isolated a *Pseudomonas* strain to grow with (+)-limonene to form oxygenated moieties of limonene (Dhavalikar *et al.*, 1966). This study made use of a microbial biocatalyst and the authors discovered that the organism attacks the limonene molecule at different positions and that the major pathway begins with the hydroxylation at the seventh position which leads to perillyl alcohol (Dhavalikar *et al.*, 1966).

In a strain known as *Rhodococcus opacus* PWD4, which is a toluene-degrading organism, the researchers discovered that in the 6-position, (+)-limonene can be substituted with *trans*-carveol. They then studied the biotransformation of the cells using a chemostat culture platform (Duetz *et al.*, 2001). The researchers found that the *trans*-carveol formation rate was 14.7 U/g of cells. The yield was 94 to 97%. They noted that toluene was a strong inhibitor of the (+)-limonene conversion, and glucose-grown cells did not produce any *trans*-carveol. These results indicate that one of the enzymes that catalyses the degradation of toluene is responsible for monohydroxylation (Duetz *et al.*, 2001).

In 2006, a research team in China explored the conversion of limonene using marine bacteria (Li., 2006). Their findings revealed the distinctive biotransformation capabilities of *Vibrio cholerae*, *Listonella damsela*, and *Vibrio alginolyticus*. The biocatalytic processes included oxidation, such as hydroxylation and ketonization, occurring at various positions on (+)-limonene, as well as hydrogenation, hydrolysis, esterification, and cyclic cleavage. The biotransformation products included sesqui-, di-, and triterpenoids, highlighting the significant influence of (+)-limonene on the microbial metabolites. It offers a crucial hint that monoterpene substrates have the ability to control the synthesis of microbial metabolites, particularly terpenoids. The terpenoid compounds and the culture conditions of these three marine bacterial strains are discussed in this work.

In 2010, Hernandez *et al*. conducted an experiment using horseradish peroxidase (HRP) from *Armoracia rusticana*, which catalyses the oxidation of (+)-limonene into the oxidized products carveol and carvone. The study compared the effects of directly adding hydrogen peroxide versus its continuous electro-generation during the enzymatic oxidation of (+)-limonene. The experimental for this study consisted of HRP, (+)-limonene (as a substrate) and hydrogen peroxide (as an oxidising agent) which was directly added or electrogenerated in a 100 mM sodium potassium phosphate buffer of pH 7 at 25˚C. The enzymatic reaction took place for 3 hours, which lead to a 45% conversion of (+)-limonene into carveol and carvone (2:1). They discovered that electro-generation improved the half-life of the enzyme.

In 2021 Yoshida and team concentrated on utilizing *Escherichia coli* (*E. coli*) to optimize the manufacture of (−)-carvone, a spearmint-flavoured monoterpenoid. When cytochrome P450 limonene-6-hydroxylase (P450) and carveol dehydrogenase (CDH) were co-expressed in a single strain, low yields and the generation of byproducts (dihydrocarveol and dihydrocarvone) were detected. In order to address this issue, the Yoshida and team postulated that the issue could be caused by an imbalance in the amounts of enzyme expression. In order to test this, they expressed CDH and P450/CPR in different strains and mixed them in different ratios. Their research verified that modifying the ratio led to an increase in (−)-carvone yield and a decrease in byproducts; the highest output was attained when the CDH input was decreased. To further improve the sustainability and economics of (−)-carvone production, the authors intend to create a strain with a high limonene content and integrate the carvone conversion route.

A thorough investigation was done in 2023 into the best practices for bench-scale bioconversion of (*R*)-limonene into high-value fragrance compounds utilizing the *Aspergillus niger* LBM055 strain is presented in a paper by Velázquez *et al.*; who carefully examined how agitation, pH, and temperature affected the bioconversion of (*R)*-limonene. According to their research, bioconversion was most effective at 30.5°C, 100 rpm, and pH 6.2. However, slightly different conditions—28°C, 100 rpm, and pH 5.4—achieved the best yield of high molecular weight compounds such as pinene, citral and linalool. Following the application of these ideal circumstances to a bench-scale bioreactor, numerous important fragrance compounds, such as α-pinene, β-pinene, citral, linalool, carvone, carveol, and myrcene, were identified.

Interestingly, 3.1 g/L of a combination of carvone, carveol, and limonene oxide was found to be highly concentrated.

Velázquez *et al.* offers insightful information about the scaling-up and optimization of (*R*)-limonene bioconversion processes overall. Their finding opens the door to producing high-value fragrance compounds, which will broaden the citrus industry's value chain and further our understanding of bioconversion parameters. This work represents a significant advancement in the fields of bioconversion and industrial biotechnology of terpenoids because of the thorough experimental design and lucid findings presentation.

There are not many studies on the use of the lipoxgenase enzyme to convert limonene to carvone, thus our research aims to both validate the conversion's feasibility and optimize the process for optimal outcomes. The project will entail a number of biocatalytic reactions followed by a number of characterization techniques, such as column chromatography for product purification, gas chromatography (GC) for data analysis and reaction mechanism postulation, and nuclear magnetic resonance (NMR) for product characterization.

In 2022, a team from Germany published the first publication on the biocatalytic oxidation of limonene to carvone utilizing soybean lipoxygenase. The study by Nikolaiczyk and colleagues on hydrogen peroxide (HPOD)-induced co-oxidation of limonene enantiomers revealed that both limonene enantiomers formed the same product spectrum, which includes five main compounds. This shows that the reaction is not stereoselective; neither enantiomer is preferred over the other. As a result, it can be deduced that stereocontrol by a chiral template, such as an enzyme, is not involved, and that the oxidation is caused by a free peroxy radical mechanism. This finding is backed further by the discovery of both diastereomers of the epoxides and carveol produced during the process.

Additionally, they found that, in contrast to earlier reactions without oleic or linoleic acid, limonene turns out to be a more reactive substrate. Because cyclic compounds are typically more reactive toward allylic hydrogen abstractions than their acyclic counterparts, this greater reactivity is explained by their nature. The synthesis of carveol is particularly interesting because it implies that a more stable tertiary radical is not produced, possibly due to steric interference from the nearby iso-propenyl group. It is possible that this steric impact is impeding the development of the more stable radical. Furthermore, the generation of limonene epoxides was unexpected, considering that this oxidation pathway competes with allylic oxidation. The reaction can occur at either the endo- or exo-cyclic double bonds, producing

1,2-limonene epoxide and 8,9-limonene epoxide, respectively (Nikolaiczyk *et al.*, 2022). The oxygen function can be introduced from either the "re"- or "si"-faces, and both diastereomeric pairings were found in the final product spectrum.

A chiral hydrodex-β-6TBDM stationary phase GC study revealed that carvone was generated as a racemate as shown in **Figure** 5. This result clearly implies that throughout the process, carveol's four potential stereoisomers had to form. Overall, the work demonstrates how the limonene oxidation process is non-stereospecific and offers insight into product formation and reactivity, including the formation of racemic carvone and the impact of steric variables on radical stability (Nikolaiczyk *et al.*, 2022). This is the ideal starting place for the current work.

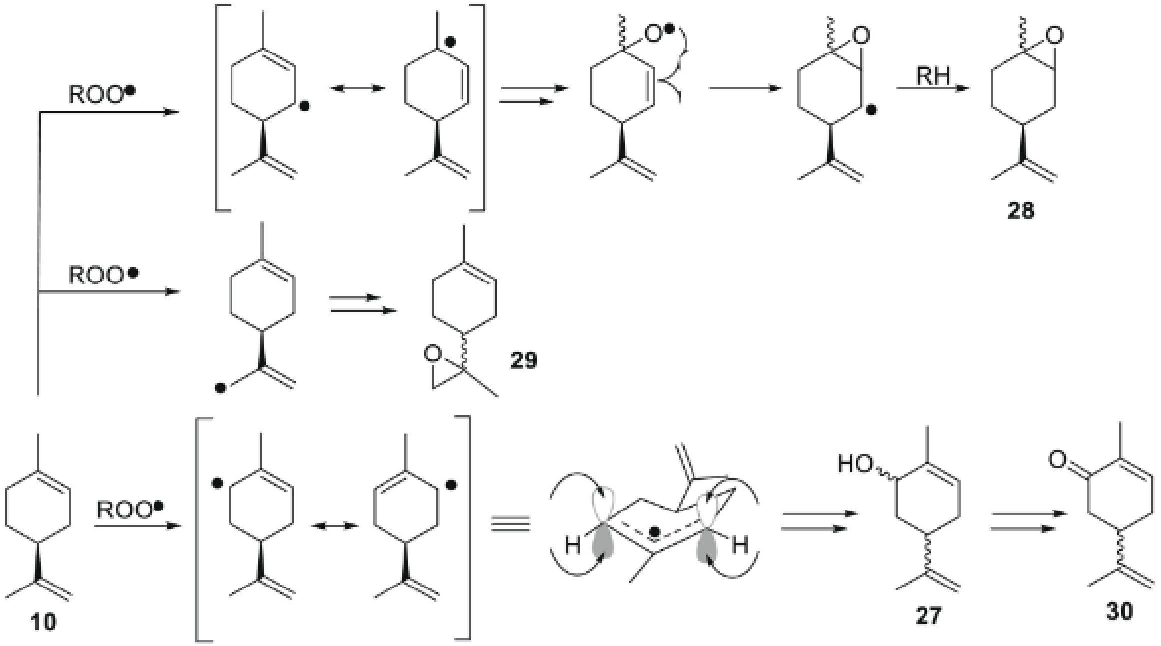


Figure 6: Proposed mechanism for the conversion of limonene to carvone (Nikolaiczyk *et al.*, 2022)

In this project, we envisaged to use different techniques to characterize intermediates or the final targeted compounds. In the next section, a general overview of the various techniques employed in the project is highlighted.

1.10 Chromatography

This section will examine three chromatography techniques: column chromatography, gas chromatography, and thin-layer chromatography.

1.10.1 Column Chromatography

In terms of separation and purification, column chromatography is the most straightforward and widely used technique. It can be applied to the separation and purification of solid and liquid materials. This method enables the adsorption and separation of chemicals by facilitating their passage through a stationary solid phase with the help of a liquid mobile phase. Elution results from the variable degrees of adsorption of various substances by the adsorbent, and compounds are adsorbed according to their chemical characteristics. A range of stationary phases, such as silica, alumina, calcium phosphate, calcium carbonate, starch, and magnesia, are used in column chromatography. Depending on the kinds of chemicals that must be isolated and separated, different solvent compositions are also used. The primary method we used in this study to separate the necessary components was column chromatography.

Normal column chromatography and flash column chromatography are the two types of column chromatography. Medium pressure column chromatography is another term for flash chromatography (MPCC). Here, the stationary phase is being covered by a high-rate, high-pressure solvent flow. Since plant materials consist of complex mixtures with many unknown components, column chromatography is an ideal method for isolating these substances. Solvents play a crucial role in this technique, with two main types of solvent systems used: isocratic and gradient. In the isocratic system, a single solvent is used to pass through the stationary phase. In the more advanced gradient system, two solvents are employed, with their ratios changing in a linear or non-linear fashion as they pass over the stationary phase. Because plant extracts contain a variety of compounds with different polarities, gradient systems are commonly preferred by natural product chemists for isolating these molecules from complex mixtures.

1.10.2 Gas Chromatography (GC)

Gas chromatography (GC) is a method of column chromatography that can be used to separate extremely complex mixtures, mostly based on polarity and changes in boiling point/vapour pressure (Stauffer *et al*., 2008).

An injection port, also known as an injector, is used to introduce a sample into the inlet of a GC. After passing through the intake, the sample is swept onto the column by the mobile phase, or carrier gas as shown in **Figure 6**. An oven with temperature control surrounds the column.

As the combination passes through the column, chromatographic separation occurs. Following their passage down the column, the separated components of the sample enter a detector, which generates an electrical signal according to the quantity of analytes that have been eluted (Stauffer *et al.*, 2008).

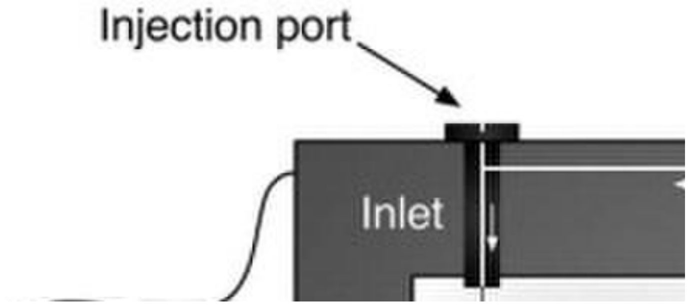


Figure 7: GC and its components (Stauffer *et al.*, 2008).

1.10.3 Thin Layer Chromatography (TLC)

The basis of thin-layer chromatography (TLC) is the idea that different components in a mixture have varied adsorption capabilities for the same adsorbent (Qin *et al*., 2021). A stationary phase is applied to a glass or plastic plate in thin-layer chromatography (TLC). Samples are spotted onto the stationary adsorbent of a plate and then placed in a liquid mobile phase chamber to facilitate their development. The mobile phase is usually a solvent or a mixture of solvents. The physical and chemical properties of the analytes dictate how the sample flows across the plate. The ability of these analytes to absorb ultraviolet light, their colour response to chromogenic or derivatization spray reagents, and their relative migration distance across the stationary phase are used to detect and identify them in combination to their relative mobility compared to standards (Wilson *et al.*, 2014).

1.11 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) spectroscopy is a versatile and effective technique for characterizing organic compounds (Kaliva *et al.*, 2020). Utilizing the nucleus's magnetic characteristics, NMR spectroscopy is a non-destructive and non-invasive method of sensing a nucleus' chemical surroundings within a molecular structure. The structure, content, purity, molecular weight, dynamics, and diffusion properties of nanomaterials can all be determined using NMR, which can be used in liquid and solid states, one-dimensional (1D), two-dimensional (2D), and multidimensional (nD) investigations (Kaliva *et al.*, 2020).

Since some nuclei have nuclear spin (I), which causes them to behave like magnets, NMR is based on electrically charged nuclei. However, this only holds true for nuclei with $I \neq 0$, such as 1H, 19F, and 31P; additional, less common nuclei include 13C, 15N, and 29Si. The 1H and 13C nuclei are frequently examined in polymer NMR spectroscopy (Rodríguez-Herrero *et al.*, 2023).

1.12 Aims and Objectives

1.12.1 Aims

The aim of this project is to utilize the Lipoxygenase (LOX) enzyme from soybean to facilitate the biocatalytic oxidation of Limonene to Carvone.

1.12.2 Objectives

- Extract crude LOX from soybean seeds.
- Determine the optimum enzyme conditions for high enzyme activity.
- Evaluate the biocatalytic conversion of limonene to carvone.
- Evaluate whether oxygen is essential in the transformation and postulate the transformation pathway from key intermediates identified.
- Isolate and confirm the presence of carvone using column chromatography and NMR spectroscopy.

Chapter 2

Results and Discussion

In this chapter we discuss the extraction of LOX; the optimization of the reaction by investigating the enzyme activity; the biocatalytic conversion of limonene to carvone; isolation of carvone by column chromatography; and structure elucidation using GC-MS and NMR .

2.1 Analysis of Limonene (starting material)

We started by looking into the starting material, limonene, which we obtained from Scattered Oils (Strydompark, Johannesburg, 2196, South Africa) at a reported purity of 98%. The chromatogram and accompanying table of results from the analysis of the limonene using gas chromatography (GC) are displayed in **Figure 8** The GC the chromatogram only showed one notable peak, supporting the 98% purity claim, but the analysis software indicated that limonene made up only 24% of the oil (**Table 2**). In light of this disparity, additional investigation was carried out utilizing nuclear magnetic resonance (NMR) spectroscopy to determine the presence of any additional chemicals identified by GC. The fact that the NMR spectra contained only limonene, and no other detectable chemicals suggests that the substance was extremely pure, supporting the 98% purity claim.

The potential issues with the GC separation on the particular column utilized could be the cause of the contradictory results between the GC and NMR analyses. Another possibility is that the GC was identifying limonene fragments and classifying them as multiple different chemicals according to similar masses in its reference library. This could account for the extra chemicals that appeared in the GC data but were not seen in the NMR investigation. As a result, the high purity of the limonene sample is confirmed with more reliability by the NMR data.

Table 2: GC area percentage of Limonene as determined by the LECO Chroma TOF software and NIST library.

Compounds	**Area/%**
α-Pinene	0.002616
Octane, 1-methoxy-	0.013414
o-Cymene	**24.975**
Limonene	**24.854**
Cyclohexane, 1-methylene-4-(1-methylethenyl)-	**24.919**
Eucalyptol	**24.808**
Decane, 3-methyl-	0.001146
Undecane	0.016356
2-Cyclohexen-1-ol,1-methyl-4-(1-methylethenyl)-, trans-	0.021152
cis-(-)-1,2-Epoxy-*p*-menth-8-ene	0.04073
Limonene oxide, trans-	0.15802
cis-p-Mentha-2,8-dien-1-ol	0.15802
Cyclohexanone, 5-methyl-2-(1-methylethyl)-, trans-	0.023223
Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1à,2á,5á)]	0.009203

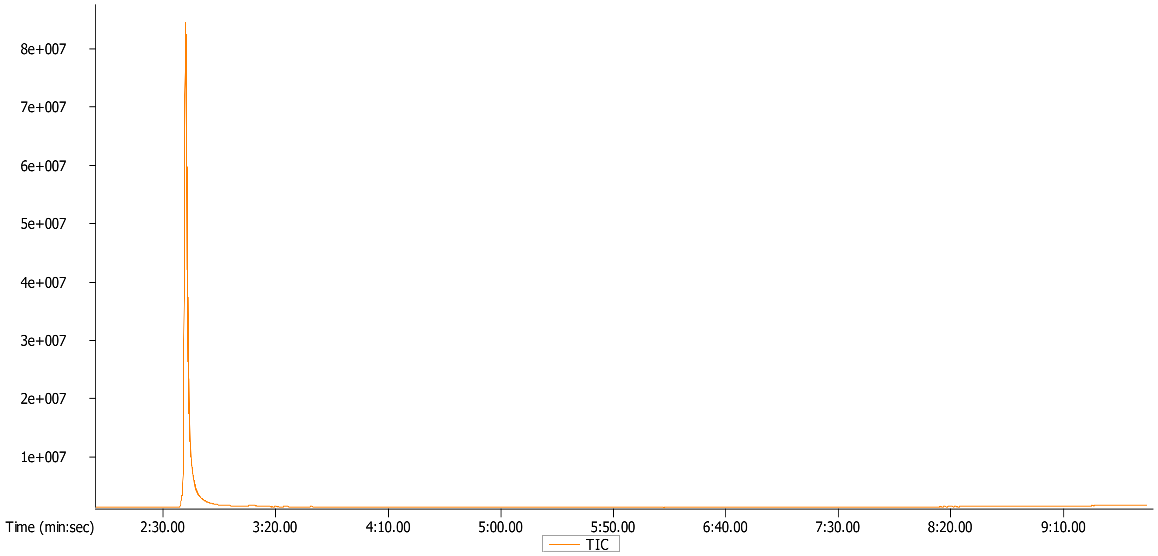
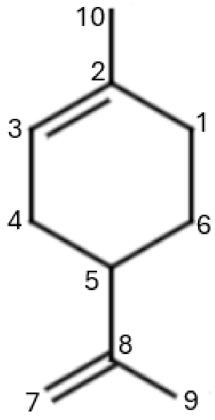


Figure 8: GC Chromatogram of limonene oil

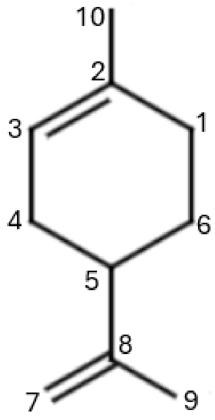
The NMR chemical shifts for limonene protons, as shown in the **Table 3**, reveal valuable information about the molecule's structure and the environment of each proton. Alkenes protons like H-3 (5.40 ppm, published data, 5.36 ppm, Fawzi *et al.*, 2024), are deshielded and resonate at higher ppm values due to the electron-withdrawing nature of the unsaturation. Similarly, protons on H-7 (4.70 ppm, published data, 4.72 ppm, Fawzi *et al.*, 2024) are in slightly deshielded environments, likely due to the anisotropic effect of the pi- electrons in the double bond. Protons on H-6 (2.10 ppm, published data, 2.12 ppm, Fawzi *et al.*, 2024) are attached to methylene carbon. Protons on H-5 (1.97 ppm, published data, 1.99 ppm, Fawzi et al., 2024) and H-4 (1.94 ppm, published data, 1.96 ppm, Fawzi *et al.*, 2024) are in relatively shielded environments. Methyl protons on H-10 (1.80 ppm, published data, 1.81 ppm, Fawzi et al., 2024) and H-9 (1.73 ppm, published data, 1.72 ppm, Fawzi *et al.*, 2024) are highly shielded and show typical shifts for methyl groups, around 1.7–1.8 ppm. Therefore, we can see that these chemical shifts are in correlation with the structure of limonene. It is also important to note that apart from confirming the structure of limonene, we were also able to determine its purity based on NMR as there were not any other signals in the spectrum which could signify the presence of any organic impurities.

Table 3: ^{1}H-NMR chemical shifts of Limonene

Protons 	**Limonene (ppm)**	**Limonene Published data (Fawzi *et al.*, 2024) (ppm)**
1H, m, H-3	5.40	5.36
2H, s, H-7	4.70	4.72
2H, m, H-6	2.10	2.12
2H, m, H-1	2.05	2.06
1H, m, H-5	1.97	1.99
1H, m, H-4	1.94	1.96
1H, m, H-4	1.92	1.90
3H, s, H-10	1.80	1.81
3H, s, H-9	1.73	1.72

The results from **Table 4** show the ^{13}C-NMR analysis of limonene also show excellent agreement with the published data by Oliveira *et al.* (1999), with chemical shifts varying by only a small margin (0.00 to 0.22 ppm). The observed chemical shifts for the methyl (C9, C10) and methylene (C6, C4, C1) in the limonene structure are consistent with expected values based on the molecule's functional groups and the electronic environment of each carbon. As expected, the alkenes carbons (C2, C3, C7 and C8) were observed between 100-150 ppm due to anisotropic effect of the pi electrons. The close correlation between the experimental data and the literature confirms the reliability of the NMR analysis and suggests that the limonene sample is of high purity.

Table 4: ^{13}C-NMR of limonene

Chemical Shift (ppm) 	**Limonene (ppm)**	**Limonene Published data (Oliveira *et al.*, 1999) (ppm)**
C-8	150.29	150.33
C-2	133.77	133.79
C-3	120.65	120.71
C-7	108.36	108.58
C-5	41.09	41.00
C-1	30.81	30.70
C-4	30.60	30.48
C-6	27.92	27.81
C-10	23.48	23.34
C-9	20.67	20.67

The DEPT 135 experiments (Appendix C, **Figure 31**) also showed the presence of 4 methylene carbons, one of which was very deshielded at 108 ppm, consistent with C7, and 4 signals consistent with the 2 methyl and 2 methine protons. The most deshielded signal of these was assigned to be the alkene carbon C-3 at 120 ppm. In addition, two quaternary carbons were confirmed (C2 and C8) as these signals were absent in the DEPT spectrum.

Overall, we unambiguously confirmed using NMR that the starting material was indeed Limone and further established that there were no other organic impurities in the sample based on the absence of any other signals in both the proton and carbon spectra.

The mass spectrometry (MS) analysis of the starting material, limonene, revealed a distinct fragmentation pattern that proved its identity, as evidenced by the high similarity score (957) to the reference library (**Figure 9**). The predicted fragments from the ionization of the limonene molecule are represented by the prominent peaks in the spectrum. A cyclohexyl cation $[C_5H_8]^+$, which is created when a methyl group from the six-membered ring of limonene is cleaved, is represented by the most noticeable peak at *m/z* 68. For cyclic terpenes, this fragmentation is common and signifies the disintegration of the ring structure. This further supports the structure of limonene as a cyclic hydrocarbon.

Propyne $[C_3H_3]^+$ and butenyl $[C_4H_5]^+$, two smaller hydrocarbon fragments, are responsible for the lesser peaks at *m/z* 39 and 53, respectively. Smaller alkyl groups from the primary ring cleave to produce these ions. Furthermore, the molecular backbone is further fragmented by peaks at *m/z* 79 and *m/z* 107, which correspond to bigger fragments and may result from the loss of isopropyl or other alkyl groups.

The molecular ion $[C_{10}H_{16}]^+$, represented by the peak at *m/z* 136, verifies the molecular mass of limonene. This peak offers clear proof of the presence of limonene. All things considered, the MS analysis's fragmentation pattern aligns with the structure of the cyclic monoterpene limonene and validates its use as the starting material for the biocatalytic conversion in this investigation. Before moving forward with other reactions, this analysis acts as a crucial verification step to guarantee the reactant's integrity.

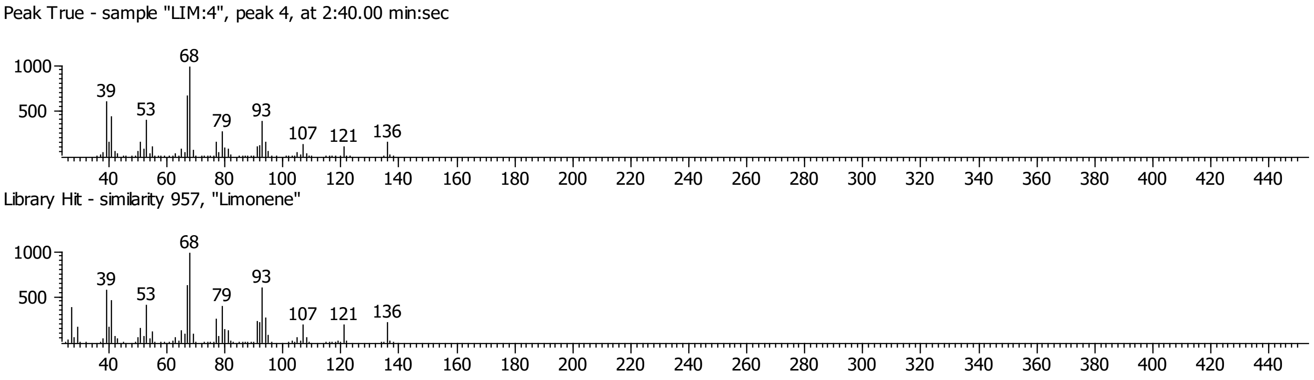


Figure 9: Mass Spectrometry data of limonene

2.2 Extraction of Crude LOX from Soybean

Soybean (*Glycine max* L. Merrill), native to East Asia, has taken on a critical role as a global crop, significantly contributing to food security, a diverse range of product lines, and highly demanding scientific research (Mishra *et al.*, 2024). Soybean seeds are a particularly rich source of the enzyme LOX, which is present in both plant and animal cells. LOX acts as a catalyst for the oxidation of polyunsaturated fatty acids (PUFAs), which produces hydroperoxides that have an impact on the food's flavour, colour, and general quality (Shi *et al.*, 2020). We suspect that LOX is the responsible enzyme for the conversion of limonene to carvone since it is the most abundant source found in soybeans. The aim of this project was to identify if LOX is the responsible enzyme for this conversion and, if possible, which isozyme of LOX is catalysing the conversion.

Two different kinds of soybeans that APBio supplied were used to extract LOX. LOX was extracted from two different soybean sources: milled defatted soybean (MDS) and ground soybean (GSB) seeds. According to the literature, the lipoxygenase (LOX) content in soybeans is approximately 2% of protein (Mandal et al., 2014). For defatted soybeans, which contain about 50% protein, the LOX activity is around 1%. For our project, we are working with 25 g of crude soybean extract, which contains 1% LOX activity.

To determine the amount of LOX in the 25 g of crude extract, we can calculate it as follows:

Amount of LOX in 25 g crude soybean extract:

$$\text{LOX content} = 25\ \text{g} \times \frac{1}{100}$$

$$= 0.25\ \text{g}$$

This means that in the 25 g of crude extract, we have 0.25 g of LOX responsible for the conversion reactions.

Thus, for every conversion taking place, we are using 0.25 g of LOX from the crude extract.

In earlier work by our group, ground soybeans were crushed into a coarse meal using a mortar and pestle. This method, however, was labour-intensive and time-consuming. To improve the extraction efficiency, a coffee grinder was introduced as a more effective alternative. The coffee grinder performed well, significantly reducing the effort required and making the grinding process much easier. Additionally, the coffee grinder was employed to reduce the particle size, increasing the surface area of contact. This helped optimize the extraction of the LOX enzyme from the soybeans. A pH study was conducted in the next section to determine the optimum level for enzyme extraction.

2.2.1 pH Variation using Borate Buffer

A study was undertaken, in which milled or ground soybean was extracted at pH levels ranging from 6.5 to 9.0 with a 0.1 M sodium borate buffer. Although sodium borate buffer is most effective between pH 8 and 10, we used it across different pH conditions to maintain consistency in the experiment. The buffer remained stable at pH 6.5. This pH range was chosen according to literature reviews in which Murphy and colleagues (2008) found that the ideal pH ranges for extracting soybean LOX-1, LOX-2, and LOX-3 are 8.0–9.0 and 6.5, respectively. Previous research conducted in our lab involved the extraction of lipoxygenase from milled defatted (MDS) and ground soybean (GSB) LOX using different pH and buffer solutions. The maximum enzyme activity for both MDS and GSB LOX was observed at pH 8.5 and 0.1 M buffer concentration. The project's goal was to validate that approach and determine whether extracting both MDS and GSB LOX at certain pH and buffer concentrations was feasible. We undertook these studies with the sodium borate buffer since it was more affordable when the reaction was upscaled and it as effective as other buffers (Tris buffer, sodium phosphate buffer, and the citrate buffer). The reactions were done in triplicates, and the average percentages were used.

Many studies have been conducted to determine how pH affects the kinetics and activity of soybean lipoxygenase (LOX). According to Asbi *et al.* (1989), LOX-1 has an active site histidine residue with a pK of 7.3. It is stable between pH 3.2 and pH 9.2 and follows Michaelis-Menten kinetics from pH 5.6 to pH 9.2. Different LOX isoenzymes perform best at specific pH levels: LOX-1 at 9.0, LOX-2 at 6.8, and LOX-3 at 7.1 (Chedea *et al.*, 2008). Changes in pH also have the ability to control the activity of LOX and prevent the production of off-flavours during the processing of soybeans (Chedea *et al.*, 2008).

As per our previous research, the buffer functions best at a pH of 8.5 at a concentration of 0.1 M (Makhubela, 2022). The pH-dependent enzyme activity for the biocatalytic conversion of limonene to carvone is shown in the graph below **(Figure 10,** also see Table A1, appendix). The reaction was conducted in a two-neck round-bottom flask containing limonene (40 g, 0.2935 mol), 25 g of LOX, and oleic acid (2.5 g, 0.0085 mol). For this experiment both MDS and GSB LOX were used. Enzyme activity is minimal at the acidic pH of 6.5, as the graph illustrates. Though the increment is slight, the activity begins to increase at pH values of 7.0 and 7.5.

However, at pH 8.0 to 8.5, there is a significant increase in enzyme activity compared to the lower pH values. The activity peaks at pH 8.5, which confirms the prior research. Beyond this, at pH 9.0, enzyme activity begins to decline, reinforcing that pH 8.5 is the optimal condition. The results obtained for MDS and GSB LOX are very similar because they show same trend albeit with, MDS LOX having a slightly better enzyme activity. The results of both types of soybean preparation are comparable and will be investigated in the next section. This also confirms our prior work on the pH variation of the LOX enzyme (Makhubela, 2022). Throughout the project, a pH of 8.5 and a buffer (sodium borate) concentration of 0.1 M was used for the reactions that follows.

Murphy (2008) reports that lipoxygenase (LOX) isozymes show notable pH selectivity, with each isozyme exhibiting optimal activity at various pH values. The conversion of limonene to carvone in our investigation showed an optimum pH of 8.5, which implies that the crude LOX extract that was used might have been plentiful in LOX-1. According to its best activity in a basic pH range, which is consistent with our observed results, this isozyme most likely catalyzed the conversion reaction.

However, further investigation is necessary to determine whether LOX-1 or another isozyme is responsible for the observed conversion of limonene to carvone. Additional studies, such as enzyme activity assays or isozyme characterization, will help clarify the specific contributions of different LOX isozymes in this reaction and provide a deeper understanding of their roles in the catalytic process.

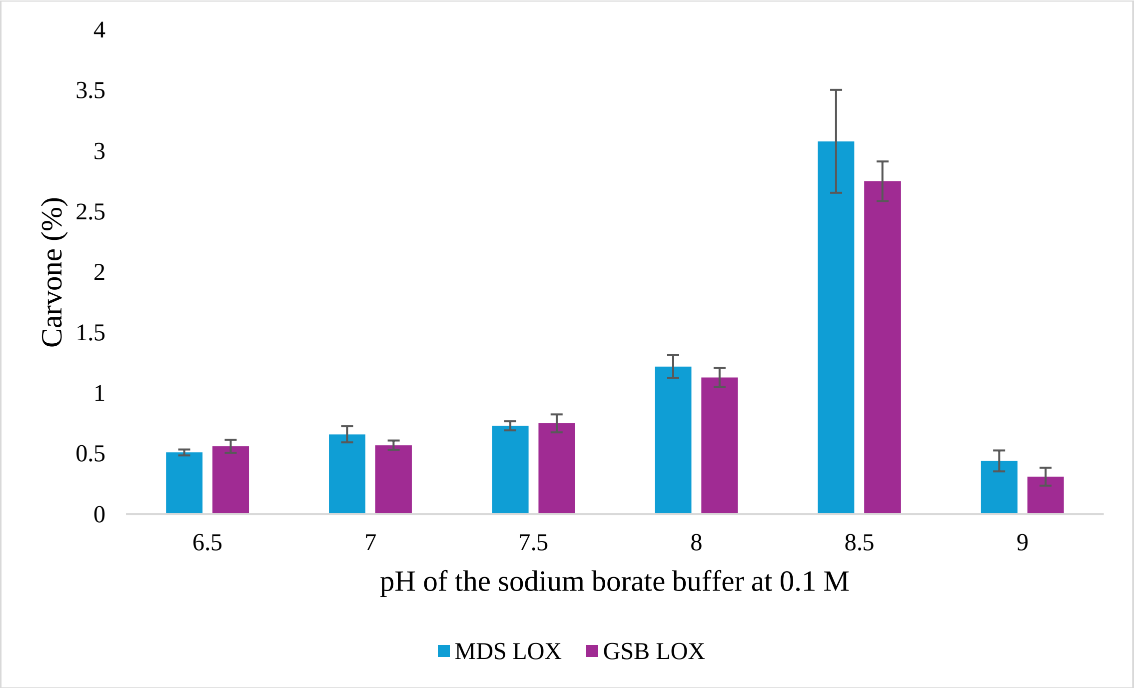


Figure 10: Effect of buffer pH on carvone synthesis using MDS and GSB LOX

2.3 Biocatalytic oxidation of Limonene to Carvone

The standard reaction for the biocatalytic conversion of limonene to carvone was adapted from a method developed by a collaborating company (Applied Protein Biotechnologies (APBio)). According to this method, lipoxygenase (LOX) exhibited optimal activity when pre-soaked in a sodium borate buffer at a pH of 8.5. For the reaction, a mixture of limonene, LOX, and oleic acid was subjected to reflux at temperatures ranging from 50 to 55°C. Building upon this method, we systematically investigated various parameters to optimize the reaction conditions. These parameters included temperature variations, enzyme-to-substrate ratios, the incorporation of metal salts as activators, and adjustments to pH. The effects of these parameters on the reaction efficiency and yield of carvone were thoroughly analysed, with results and discussions presented in subsequent sections. This approach aimed to enhance the understanding of the biocatalytic process and to identify optimal conditions for maximizing carvone production from limonene.

2.3.1 Extraction methods for MDS LOX vs GSB LOX

The phrase "ground soybean" refers to whole soybeans that have been ground very finely but still retain their oil and protein. On the other hand, defatted soybean flour is made from soybeans from which the majority of the oil has been chemically or mechanically drained. Soy products with bad flavours and low nutritional value are caused by the oxidation of polyunsaturated fatty acids, which is catalysed by the enzyme lipoxygenase (LOX. The difference in LOX activity between defatted and ground soybean is explained by the presence of oil. While defatted soybeans have had most of their oil removed, which results in lower levels of LOX activity, ground soybeans still retain oil and higher LOX levels.

A comparative study was conducted to investigate the enzymatic activity of lipoxygenase (LOX) extracted from two different soybean preparations: milled defatted soybean LOX and ground soybean LOX. The enzyme assays were performed under controlled conditions, with a constant temperature of 50°C and a continuous supply of oxygen. Oleic acid was employed as the substrate, and the reactions were allowed to proceed for a duration of five days. This experimental design enabled a thorough evaluation of the LOX activity in both soybean preparations, providing insights into the effects of soybean processing on enzyme functionality.

MDS LOX was substantially better in handling during the extraction process since the layers were distinct and the product was not trapped in the emulsion as compared to GSB LOX. After using MDS LOX, the layers would separate in a matter of minutes. The product would then be taken out of the organic layer and dried with magnesium sulphate to eliminate any remaining water. The emulsions in GSB LOX, on the other hand, made it difficult to differentiate the layers. The emulsions were broken with the use of 20 g of NaCl and 50 mL of ethyl acetate. The product extracted from the organic layer was then placed in a rotary evaporator to remove the ethyl acetate. **Figure 11A** depicts the GSB LOX sample as a single emulsion with no evident layers. In contrast, **Figure 11B** clearly reveals three distinct layers: organic, aqueous, and protein.

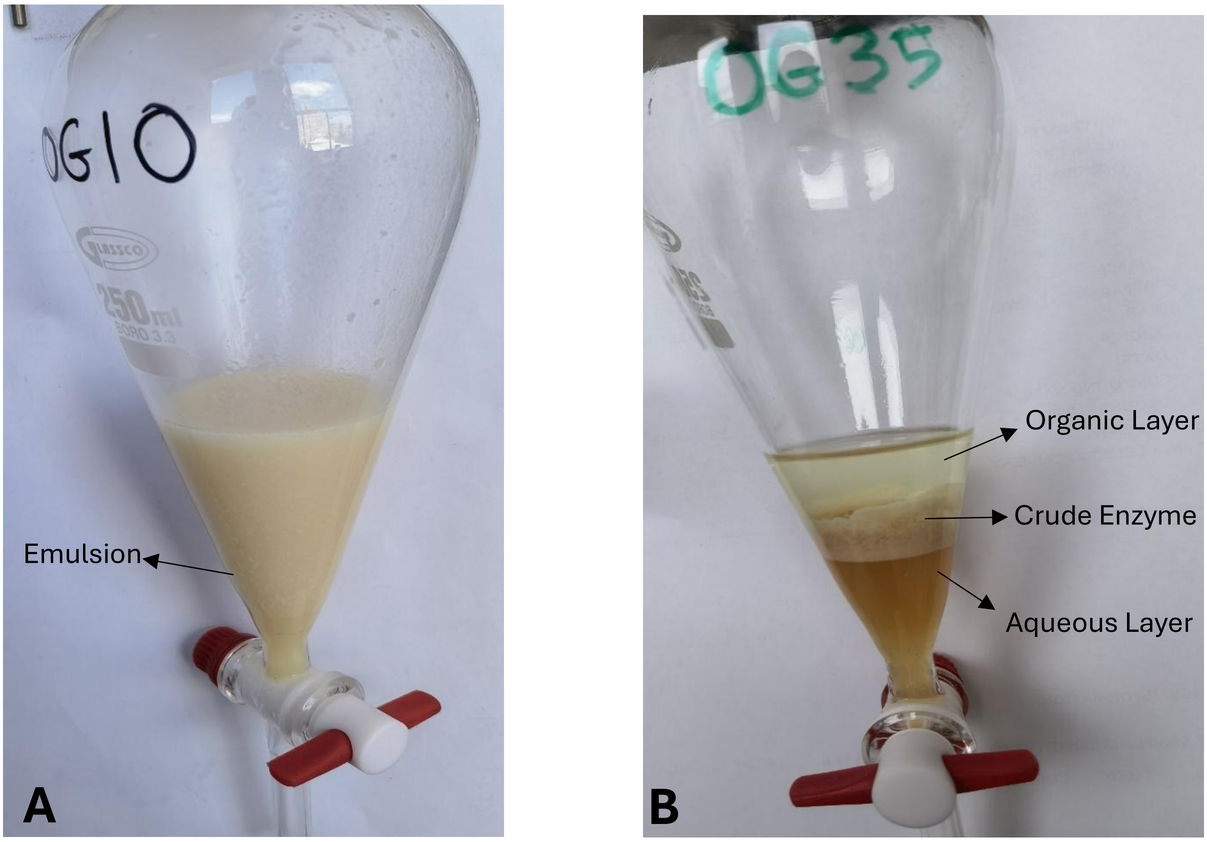
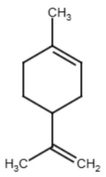
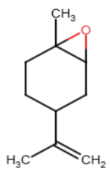
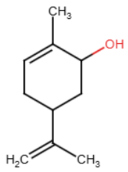
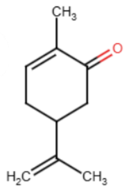
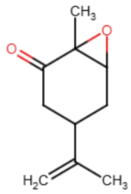


Figure 11: GSB LOX reactions (A) and MDS LOX reactions (B)

2.3.2 Fatty acid variation of MDS LOX VS GSB LOX

Several experiments were conducted to assess the impact of fatty acids and various LOX preparations on the conversion of limonene to carvone. Some experiments were carried out without oleic acid, while others included it. This was done to determine whether a fatty acid substrate is required and to observe how it influences the different types of LOX, specifically MDS and GSB. The reactions were done in duplicates, and the average percentage was used. It was observed that without oleic acid, the reaction produces carveol instead of limonene oxide, while in the presence of oleic acid, it produces both limonene oxide and carveol. The absence of oleic acid as a substrate allows the lipoxygenase enzyme to react with the limonene substrate directly, leading to the formation of carveol. This is because lipoxygenase can catalyze various reactions, including hydroperoxidation and epoxidation, depending on the substrate present. Without oleic acid, the enzyme's active site is more accessible to limonene, resulting in a different reaction pathway that favours carveol formation. **Table 5** presents the data obtained for both reactions, highlighting that the MDS reaction without oleic acid yields a higher amount of carvone compared to the reaction with oleic acid (OA). This indicates that the presence of oleic acid has a significant negative impact on the reaction and that the reaction performs more effectively in its absence.

Table 5: Conversion of Limonene to Carvone using GSB and MDS LOX

Reaction	**Limonene (%)** 	**Limonene Oxide (%)** 	**Carveol (%)** 	**Carvone (%)** 	**Carvone oxide (%)** 
GSB with OA	93.36	1.80	1.52	3.20	0.84
MSD with OA	79.56	0.72	1.26	5.99	2.18
GSB without OA	75.65	0	1.76	6.34	1.31
MDS without OA	72.86	0	2.18	11.64	3.71

The literature suggests that soybeans, also referred to as ground soybeans, typically possess a higher protein content, which would ostensibly translate to enhanced enzyme activity, including lipoxygenase (LOX). However, the findings of this study contradict this expectation, as the milled defatted soybean LOX exhibited higher activity compared to the ground soybean LOX. This disparity warrants further examination, considering factors such as processing methods, soybean cultivar variability, and the potential impact of defatting on enzyme activity. A critical evaluation of these factors is essential to reconcile the observed discrepancy and provide a nuanced understanding of the underlying mechanisms governing LOX activity in soybeans.

In contrast, the presence of oleic acid as a substrate directs the lipoxygenase enzyme to catalyse the specific reaction of limonene to limonene oxide via an epoxidation reaction. Oleic acid binds to the enzyme's active site, inducing a conformational change that enhances the enzyme's affinity for limonene and promotes the formation of limonene oxide. This substrate-specific interaction ensures that the reaction proceeds via the desired pathway, suppressing the formation of carveol (Nikolaiczyk *et al.*, 2022). This explanation highlights the importance of substrate specificity in enzyme-catalysed reactions and how the presence or absence of a substrate can influence the reaction outcome.

Depending on the substrate, crude soy lipoxygenase differs noticeably in activity from crude wheat lipoxygenase. In particular, soy lipoxygenase exhibits a considerable affinity for methyl linoleate and trilinolein, as evidenced by its ten-fold higher activity compared to wheat lipoxygenase (Ciabotti *et al.*, 2019). However, when linoleic acid is the substrate, soy and wheat lipoxygenase have comparable activity levels, indicating that this specific fatty acid is more widely accepted. This distinction emphasizes lipoxygenase's substrate specificity, which can change depending on the source and substrate, and has consequences for understanding the enzyme's function in diverse circumstances (Ciabotti *et al.*, 2019).

2.4 Substrate variation

LOXs exhibit substrate specificity based on their structural characteristics. Linoleic and linolenic acids serve as natural substrates for LOX in plant cells. Additionally, esters of fatty acids and unsaturated fatty acids with cis and trans-pentadiene structures are also compatible with LOX activity. Different LOX substrates and sources cause oxygenation at various sites (Singh *et al.*, 2022). In this section, the main focus is on the substrate variation which is linoleic acid, oleic acid and no substrate. **Figure 12** shows the conversion of limonene that was obtained using different substrates. The reactions were carried out in 250 mL round bottom flask. Approximately 40 g of limonene was used, 25 g of LOX and 2.5 g of the substrate. The substrates used were oleic acid, linoleic acid, and no fatty acid.

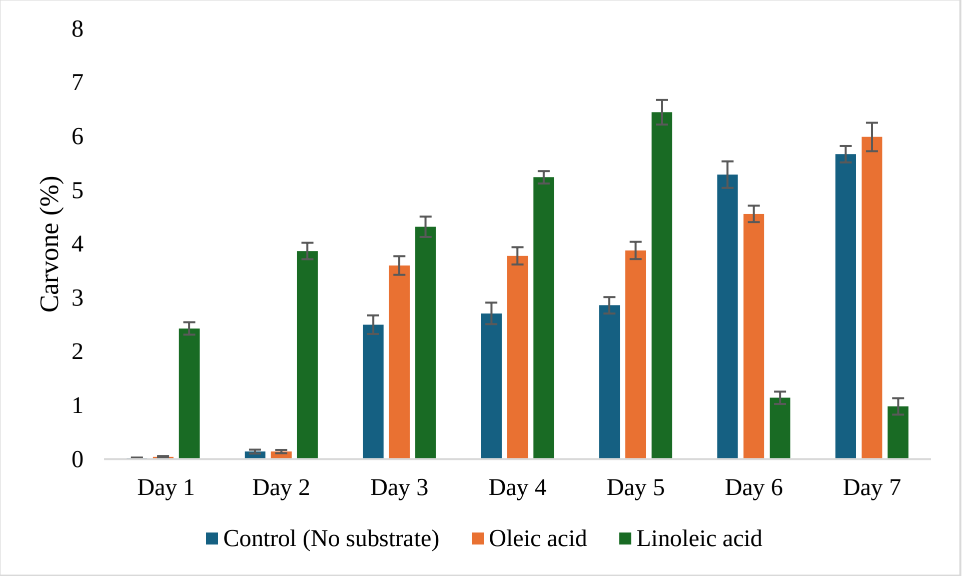


Figure 12: Carvone synthesis using MDS LOX in the presence of fatty acids and a control (no substrate).

The conversion for linoleic acid was much higher than oleic acid and no fatty acid substrate. On day one, the percentage was 2.43 compared to 0.04 and 0.02 for oleic acid and no fatty acid substrate respectively. However, after the 5th day, the percentage of carvone decreased due to over oxidation and the percentage of carvone oxide steadily increased as shown in **Figure 13**. Also, a change in colour was observed quicker with linoleic acid as compared to the other substrates. Usually, colour change happened around the 5th and 6th day for oleic acid and no fatty acid substrate, but with linoleic acid a change in colour was observed within the third day.

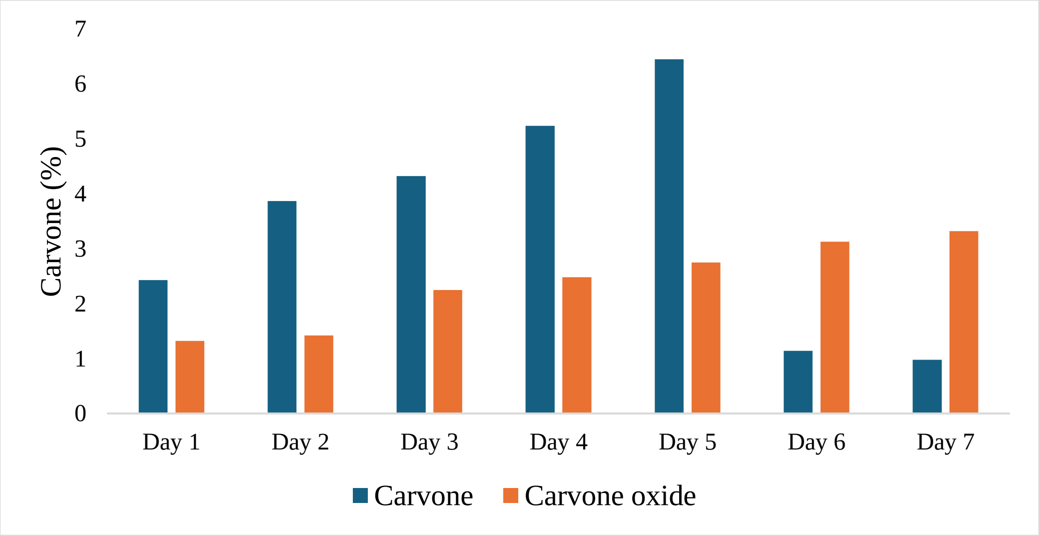


Figure 13: Percentage of carvone and carvone oxide produced using linoleic acid

As shown in **Figure 14** the colour change observed for oleic acid happens around the 5th day and the colour is a bright yellow whereas the colour change observed on the 3rd day for linoleic acid is a dark orange. The observed colour variations are a strong indication of the difference in enzyme activity between oleic acid and linoleic acid. The polyunsaturated fatty acid linoleic acid has two double bonds in its carbon chain as shown in **Figure 15**, which increases its reactivity and makes it a better substrate for the enzyme lipoxygenase (LOX) (Singh *et al.,* 2022).

Due to the rapid oxidation caused by this higher reactivity, the reaction turns vivid orange after three days. The presence of oxidation products, most likely hydroperoxides or other derivatives produced by LOX activity, is indicated by the intense orange colour. On the other hand, oleic acid is a monounsaturated fatty acid as shown in **Figure 15,** that is less likely to oxidize since it only has one double bond in its carbon chain (Singh *et al.,* 2022). Therefore, oxidation occurs much faster with linoleic acid as compared to the other substrates. The problem with linoleic acid is that the reaction cannot be left for long period of time because the carvone is further oxidised to carvone oxide which is depicted in **Scheme 1**.

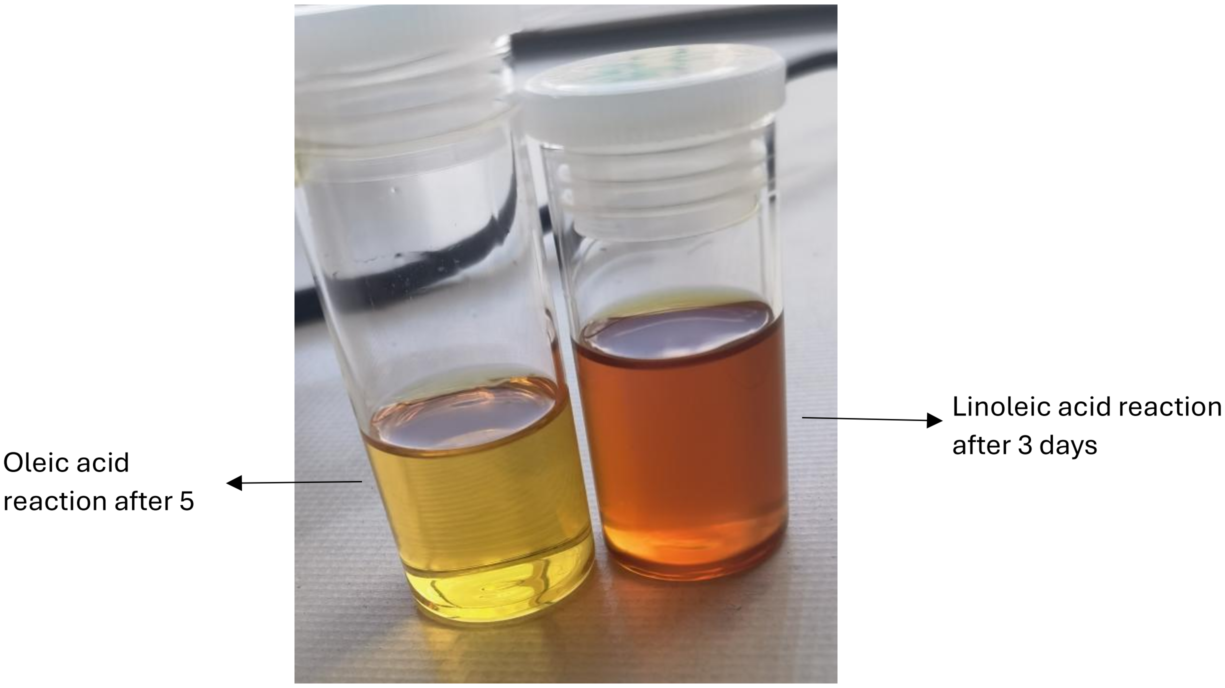
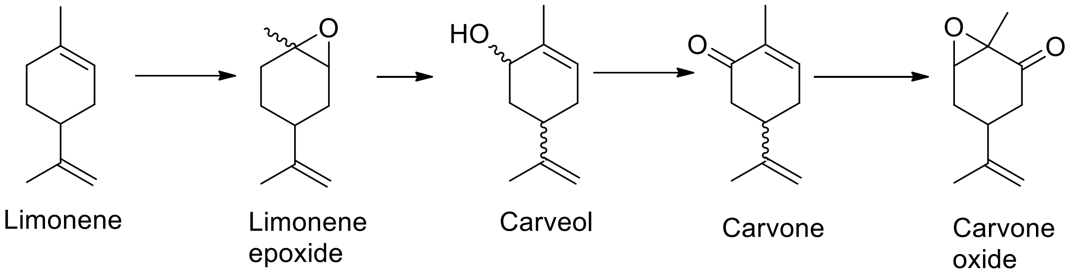


Figure 14: Colour changes observed when using oleic acid and linoleic acid as a substrate

For the reactions done with oleic acid and no fatty acid substrate the oxidation occurred steadily. During the first two days the reaction was very slow and the percentage of carvone was as low as 0.04%. According to literature there is a lag phase during which the resting ferrous enzyme is converted to the active ferric form by reaction with fatty acid hydroperoxide (Zheng *et al.*, 2010). The reaction starts to increase after the second day and it gradually increases as the days go by.



Scheme 1: Reaction pathway for linoleic acid

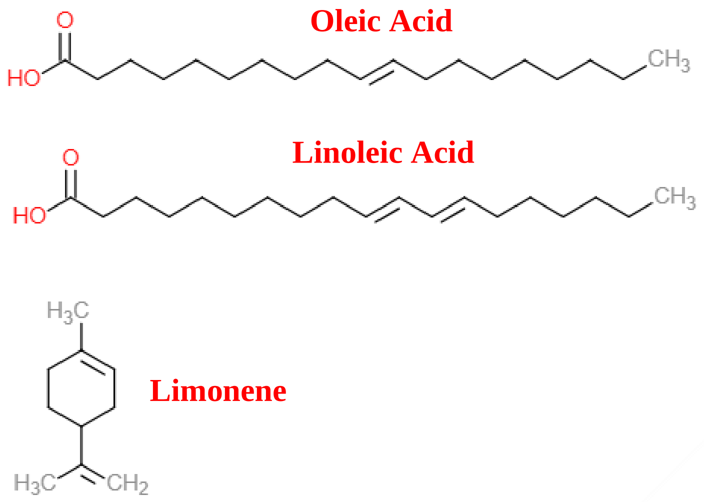


Figure 15: Substrate used for Lipoxygenase enzyme

2.5 Temperature Study

To find the ideal temperature for crude lipoxygenase (LOX) activity, an experiment was designed. The purpose of the study was to investigate the properties of crude LOX by performing the reaction in a two-neck reaction vessel containing limonene, oleic acid, and crude LOX (MDS LOX). A balloon system was used to supply oxygen. Throughout the experiment, temperature was the only variable that changed; all other factors remained constant. The first reaction was conducted at room temperature (25°C), and subsequently, the temperature was increased in increments of 10°C, ranging from 30°C to 90°C. The results were obtained in triplicates, and the average percentages were reported.

The ideal temperature for soybean lipoxygenase (LOX) activity depends on a number of variables, including the type of LOX enzyme present and the measurement conditions. Soybean LOX enzymes, however, are generally most active at a temperature range of about 25–40°C, which is typical of the conditions seen during food processing and storage.

At temperatures below this range, LOX activity may be reduced, while temperatures significantly above this range can denature the enzyme, leading to a loss of activity. Therefore, for food processing purposes involving soybeans, maintaining temperatures within this optimal range can help preserve the desired qualities of the soy-based products while minimizing undesirable off-flavours and other effects associated with excessive LOX activity.

The results shown in **Figure 16** shows that, up to 50 °C, there is a positive association between temperature and the percentage of carvone, suggesting that higher temperatures aid in the enzymatic conversion of limonene to carvone. Nevertheless, the percentage of carvone decreases significantly above this ideal temperature, especially between 80 and 90 °C. This quick decline indicates that the converting enzyme is denaturing and losing its functional integrity.

The ideal temperature of 50 °C is consistent with research in the literature showing that many biocatalysts, such as lipoxygenases, reach their maximal activity in a particular temperature range prior to thermal inactivation (Zheng *et al*., 2010; Mandal *et al.*, 2014). Notably, prior research has shown that different enzymes are stable under comparable settings, indicating that the crude enzyme extract has extraordinary robustness, continuing to function at temperatures above 35 °C (Marvian-Hosseini *et al.*, 2017; Ngin *et al.*, 2021).

A study done by Marvian-Hosseini, & Asoodeh, (2017) looked into the ideal temperature and thermal stability for the activity of the LOX enzyme. Marvian and team discovered that 94% of the enzyme's activity is retained at 35°C, which is the ideal temperature for activity. After that, the percentage of active enzyme drops dramatically, reaching 7.2% at 70°C. The enzyme gradually loses its ability to function above 35°C, according to stability study, with notable decreases occurring at higher temperatures (e.g., 12.8% at 65°C and 7.9% at 70°C after 10 minutes) (Marvian-Hosseini *et al.*, 2017). These findings highlight the temperature sensitivity of LOX and provide information on its potential uses.

Ngin, Cho, and Han (2021) have conducted a study that emphasizes the benefits of immobilizing soybean-derived lipoxygenase (LOX), with a particular focus on its thermal stability in comparison to free enzymes in solution. Because of their limited thermal mobility, immobilized enzymes—like those adsorbed on rice husk silica (RHS)—show increased resistance to heat denaturation and inactivation, according to the authors. The findings show that at 35 °C, soybean LOX, both free and immobilized, demonstrate their peak activity. However, there is a big difference when you evaluate their stability at high temperatures. The RHS-adsorbed soybean LOX maintains 70% of its activity at 45 °C, but the specific activity of free soybean LOX drops to 45%.

These results highlight the potential applications of crude enzyme extracts in biocatalytic processes, especially those that call for moderate temperatures. The enzyme's capacity to resist greater temperatures suggests that it could be used efficiently in industrial settings with frequent temperature variations. Furthermore, understanding the thermal stability of enzymes can aid in optimizing reaction conditions for enhanced yield and efficiency in biotransformation processes (Wang *et al.*, 2021). This demonstrates how crucial it is to characterize the features of enzymes because it helps develop application methods for them in different biochemical processes.

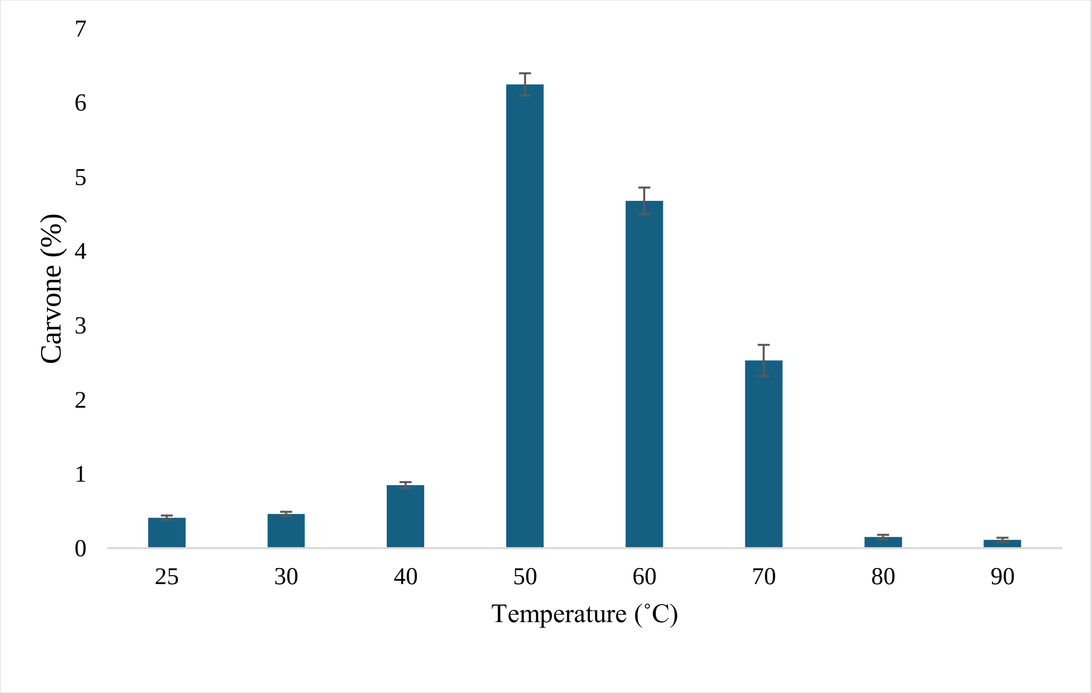


Figure 16: Effect of temperature on LOX synthesis of carvone.

2.6 Role of oxygen in Soybean Lipoxygenase

To investigate the study of the role of oxygen in soybean LOX an experiment was designed to test whether the reaction would proceed in the absence of oxygen. The oxygen free study was intended to narrow the source of activity down to an oxidative enzyme. A two-neck reaction vessel containing the starting material, limonene, and the substrate, oleic acid, was purged with nitrogen gas using the balloon system to remove atmospheric oxygen. The balloon was filled with nitrogen gas and attached to the vessel via a syringe, allowing for a slow and controlled release of the gas. As the nitrogen gas flowed into the vessel, it displaced any atmospheric oxygen present, creating an oxygen-free environment. The vessel was then sealed and subjected to reflux conditions at 50°C for five days to determine if the reaction would proceed in the absence of oxygen.

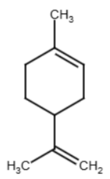
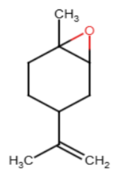
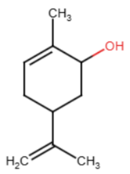
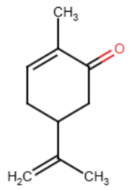
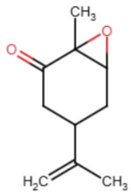
Consistent with literature reports, oxygen was found to be a crucial factor in facilitating the enzyme’s activity (Kong *et al.*, 2008). In a study published in 2017, Hayward and colleagues examined the effect of oxygen on LOX-catalysed reactions and found that the presence of oxygen is a pivotal in LOX-catalysed reactions. Oxygen serves as a terminal electron acceptor, facilitating the enzymatic conversion of the reaction. The oxygen molecule accepts electrons from the substrate, thereby enabling the oxidation reaction to proceed. The electron transfer process is crucial for the catalytic activity of LOX, and the absence of oxygen would significantly impede the reaction (Nikolaiczyk *et al.*, 2022).

In 2016 Callazo and team investigated the function of a well-defined O_2 route in controlling soybean lipoxygenase linoleic acid peroxidation (Callazo *et al.*, 2016). Strong evidence for a dominating delivery channel that transports molecular oxygen to a particular area of the active site and maintains the regio- and stereospecificity of the product comes from computational and mutagenesis experiments. The gas migration pathway has adaptability, as evidenced by the analysis of reaction kinetics and product distribution in channel mutants. The results demonstrate that a single site mutation (I553W) significantly increases the fraction of substrate that reacts with oxygen free in solution by limiting oxygen accessibility to the active site (Callazo *et al.*, 2016).

In 2003 Knapp and team investigated the oxygen reactivity of soybean lipoxygenase-1 (LOX-1), aiming to identify the catalytic cycle's rate-limiting phase. The rate-limiting step is reduced to either the combination of O_2 and the lipid radical (L•) or a subsequent conformational change in the enzyme. Kinetic analyses rule out a number of potential limiting factors, including the diffusional encounter of O_2 with the enzyme, outer-sphere electron transfer, and proton transfer. The Ile(553) → Phe mutant, which narrows the O_2 binding channel, is studied.

The rate-limiting step does not alter even when $k(cat)/K_m(O_2)$ is reduced by more than 20 times when compared to the wild-type enzyme. This implies that the reaction rate is influenced by proximal protein dynamics and that the slow step includes the combination of O_2 and the L• radical. The results show that the enzyme is able to control O_2 reactivity without the need for metal cofactors. Rather, the enzyme-bound radical is mostly produced by the Fe^{3+} cofactor, and the surrounding protein structure is crucial in regulating the stereochemical and regiochemical interactions between O_2 and the radical. This study sheds light on how O_2 reactivity can be controlled by enzyme dynamics and structure without requiring metal coordination.

Table 6: Reactions without the presence of oxygen

Reaction	**Limonene (%)** 	**Limonene Oxide (%)** 	**Carveol (%)** 	**Carvone (%)** 	**Carvone oxide (%)** 
MDS LOX/OA	98.1	0	0.09	0.02	0
MDS LOX/LA	98.8	0	0.06	0.07	0
GSB LOX/OA	97.8	0	0.02	0.01	0
GSB/LA	98.2	0	0.01	0.03	0

It is evident from the **Table 6** data that the process cannot continue in the absence of oxygen. When identical reactions were conducted previously, with the use of oxygen under similar conditions, a significantly greater conversion was noted a comparison is shown below in **Table 7**. The reaction, however, does not continue in the absence of oxygen. For lipoxygenase (LOX) reactions to occur, oxygen (O_2) must be present since the enzyme needs oxygen to catalyze the addition of O_2 to polyunsaturated fatty acids (PUFAs), resulting in the formation of hydroperoxides. In order to complete the catalytic cycle, O_2 must react with a lipid radical that the enzyme has produced. The regio- and stereochemistry of the products, reaction efficiency, and enzyme activation are all influenced by oxygen. This is because oxygen changes LOX from its inert ferrous state to the active ferric form. O_2 is necessary for LOX enzymes to perform their vital dioxygenation tasks.

Table 7: Effect of oxygen on carvone formation

	Carvone (%)	
Reaction Type	**Oxygen**	**Control (No Oxygen)**
MDS LOX/OA	3.88	0.02
MDS LOX/LA	6.45	0.07
GSB LOX/OA	3.22	0.02
GSB LOX/LA	4.25	0.04

2.7 Effect of Metal Salts on LOX

Metal salts such as iron and calcium can affect the activity of the lipoxygenase enzyme, or it can impede the activity of the enzyme. Our investigation was to see the impact these salts will have on our crude LOX and the findings are reported in the next section below.

2.7.1 Iron Salt (Fe^{2+})

In this study, we explored the impact of varying iron concentrations on the activation of lipoxygenase (LOX), specifically examining how different mole ratios of iron to LOX influence enzyme activity. Iron is crucial for the oxidation process that converts it from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) form, which is essential for LOX's functionality. The oxidation of iron into its ferric state is a critical step for the enzyme's operation. In our experiment, we tested iron-to-LOX ratios ranging from 1.3 to 26.4 moles of iron per mole of LOX to investigate how higher iron concentrations may enhance the conversion of iron to its ferric form, thereby increasing enzyme activity.

Our results, presented in **Figure 17**, confirm that increasing the amount of iron increases LOX activity, resulting in increased carvone production. This is consistent with de Groot's (1975) theory that iron stimulates LOX. Interestingly, we found that enhanced LOX activity is correlated with higher iron concentrations, indicating that iron functions as an activator rather than a direct catalyst in the reaction. A control experiment was done in the absence of iron. To clarify this point, we relied on literature evidence indicating that iron stabilizes the ferric centre of the enzyme and promotes its activity, rather than acting as a direct oxidizing agent. Since the conversion cannot occur without the enzyme itself, this emphasizes how crucial both LOX and iron are in accelerating the reaction.

According to GC-MS analysis, we saw a notable rise in the reaction rate at the maximum iron-to-LOX ratio of 26.4:1, with the conversion of limonene to carvone reaching 58.02% (Figure 17). For this reaction the limonene percentage observed is 32.67, the limonene oxide is 6.65%, carveol is 1.52% and carvone oxide is 0.6%. Therefore, we can see that as the limonene percentage decreases, we can see the reaction pathway forming which is similar to **Scheme 1**. Our highest conversion was 58.02%, resulting in 232 g of carvone per liter, starting with 400 g of limonene per liter (i.e., 40 g in 100 mL). In comparison, according to the Nikolaiczyk paper, their best conversion was 20%, yielding only 11 g of carvone per liter from 400 μmol/L of limonene.

Previous research has established the importance of iron in LOX activation, including the seminal study on soybean lipoxygenase-1 (LOX-1) conducted by de Groot et al. (1975). In this work, de Groot examined the iron centre of the enzyme and its interactions with substrates and products using electron paramagnetic resonance (EPR) spectroscopy. Since LOX's action involves the production of free radicals, EPR makes it possible to monitor radicals and paramagnetic intermediates produced during the catalytic cycle of the enzyme in real time. According to De Groot's research, the addition of 13-L-hydroperoxylinoleic acid changed the EPR signals, suggesting that the substance affects the iron environment of the enzyme and maintains the ferric (Fe^{3+}) state, which is essential for enzyme activation (de Groot *et al.*, 1975).

This is consistent with our own findings, which showed that increased iron levels seem to maintain the enzyme's ferric form and increase its activity. The significance of iron in LOX's catalytic action is highlighted by De Groot's 1975 work, which demonstrated that the enzyme alternates between the ferric and ferrous (Fe^{2+}) states in a dynamic redox cycle. A higher percentage of the enzyme stays in the active ferric state when the iron content is raised, as it was in our investigation, which may increase the enzyme's catalytic efficiency.

Our study expands on these discoveries by demonstrating that iron is essential for maximizing LOX functionality at different iron-to-enzyme ratios in addition to supporting the enzyme's activity. The results of our experiment and de Groot's previous research demonstrate the significance of the iron centre in controlling LOX activity and imply that raising iron levels may be a viable tactic to enhance enzyme function in many contexts.

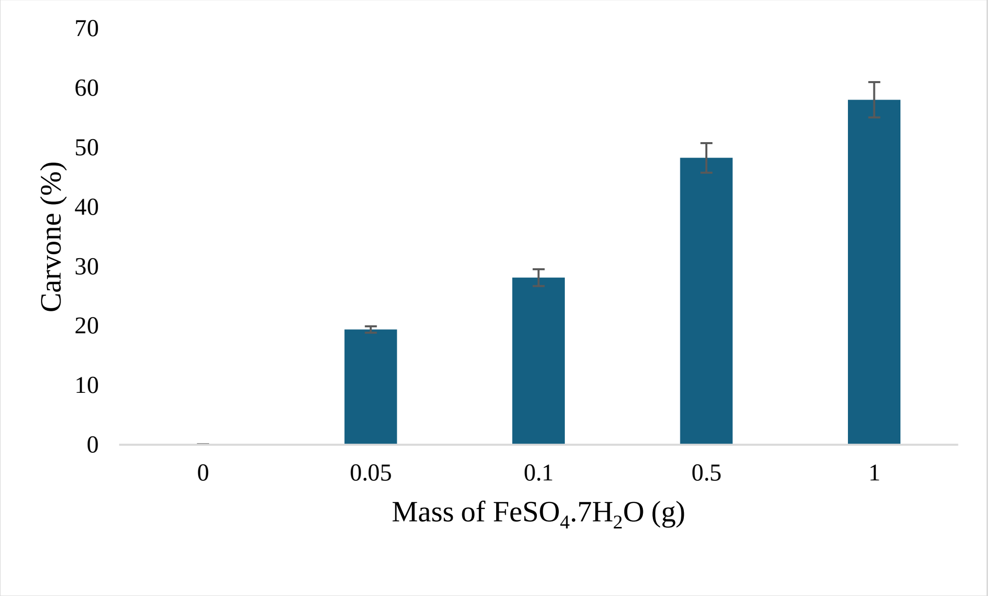


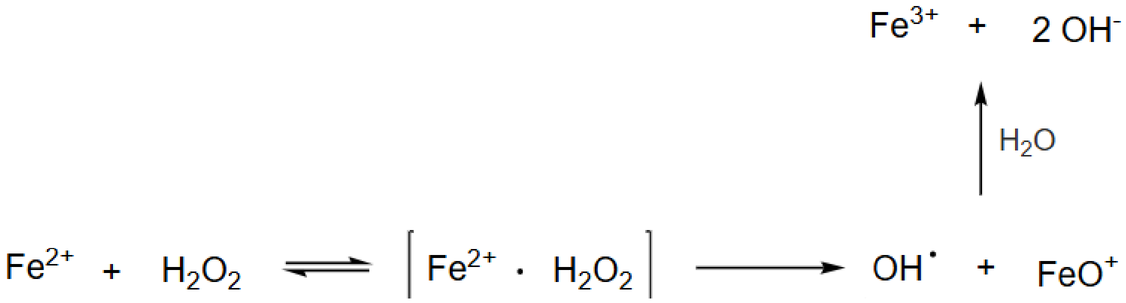
Figure 17: Effect of iron salt on the production of carvone

In order to better understand how iron salts and lipoxygenase (LOX) interact and how LOX catalyses the conversion of limonene to carvone, we conducted an experiment that focused on the function that iron plays in this process. Although the results showed that iron does interact with LOX, it was not obvious how exactly this interaction aided the reaction. We reasoned that oleic acid might be essential for the reaction to continue because LOX activity appeared to be dependent on the presence of a substrate.

In our earlier work, we found that limonene and LOX reacted without the need for substrates like oleic or linoleic acid. We conducted a reaction with iron salts without oleic acid to determine if the reaction would still occur in order to further investigate the role of the substrate. The fact that the reaction could not occur without oleic acid, as indicated in **Table 8**, demonstrated that the substrate was necessary for both the catalytic process and enzyme activity. This discovery made us think about other possible explanations, namely the Fenton reaction's role.

A plausible explanation for how iron could aid the reaction was provided by the Fenton reaction, which involves ferrous ions (Fe^{2+}) reacting with hydrogen peroxide (H_2O_2) to create ferric ions (Fe^{3+}) and hydroxyl radicals (•OH) as shown in **Scheme 2** (Li *et al.*, 2024). Literature claims that LOX catalyzes the formation of hydroperoxides from fatty acid substrates like oleic acid. Ferric ions, which are essential for enzyme activity, are created during this process from ferrous ions found in LOX. Moreover, hydrogen peroxide is a byproduct of processes that are accelerated by LOX. We postulated that the hydrogen peroxide generated by LOX during the oxidation of oleic acid would react with the ferrous ions that were added to the reaction mixture in conjunction with this information. Through the Fenton reaction, this process would produce hydroxyl radicals and ferric ions.

Due to their high reactivity, these hydroxyl radicals have the potential to attack limonene and produce limonene oxide. According to the suggested chemical pathway, more oxidation would then take place, resulting in the production of carvone, carveol, and carvone oxide. The literature data on LOX activation, which is displayed in **Figure 5**, confirmed this hypothesis by indicating that the catalytic conversion of limonene required the presence of both iron ions and oleic acid. Therefore, we can postulate that our reaction mechanism is similar to that of the Fenton reaction. Our work provides new insights into the role of iron in LOX-catalysed reactions and lays the groundwork for further investigation into the specific isozyme involvement and the detailed mechanisms behind the oxidation of limonene.



Scheme 2: Fenton Reaction Pathway

Table 8: Comparison of reactions with and without oleic acid

Reaction Type	**Carvone (%)**	
	Oleic acid	**Control (No Oleic acid)**
0.05 g $FeSO_4.7H_2O$	19.38	0.25
0.1 g $FeSO_4.7H_2O$	28.10	0.53
0.50 g $FeSO_4.7H_2O$	48.24	0.68
1 g $FeSO_4.7H_2O$	58.02	0.81

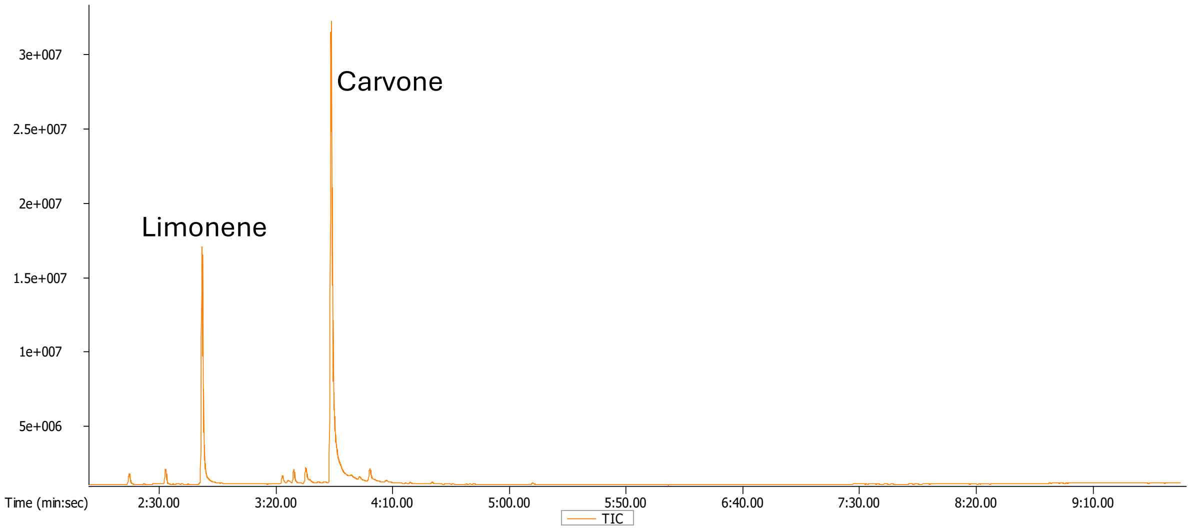


Figure 18: GC chromatogram of the highest conversion (58%) of limonene to carvone

2.7.2 Calcium (Ca^{2+})

A study was carried out to investigate the impact of calcium salts on the enzymatic conversion of limonene to carvone by lipoxygenase (LOX). Calcium chloride was introduced to a two-neck reaction vessel containing limonene, crude LOX, and oleic acid. After that, the reaction was refluxed at 50°C for five days. The purpose of this study was to examine how calcium salts affect LOX activity. According to existing research, calcium salts increase the activity of LOX-1 and LOX-2 while inhibiting the activity of LOX-3. Our goal was to identify the specific LOX isozyme that might be responsible for converting limonene to carvone.

The data presented in **Table 7** sheds important light on how various salt compositions affect limonene's enzymatic conversion. The presence of limonene at 35.97% and its conversion to carvone at 5.30% in the sample that contained only calcium salts suggests that calcium alone promotes a moderate amount of LOX activity. This finding lends credence to the theory that calcium can activate and stabilize LOX, improving its capacity to convert limonene to carvone. Carvone content increased slightly to 5.99% and carveol emerged at 0.60% when iron salts were added in addition to calcium. This suggests that iron and calcium may work in conjunction to increase LOX activity, which would probably improve the stability or catalytic efficiency of the enzyme.

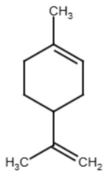
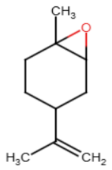
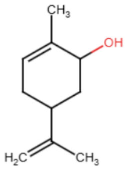
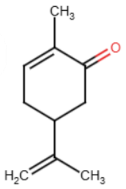
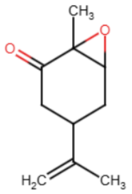
We can infer from these data that LOX-3 is absent from the crude enzyme preparation as it is clear that the reaction takes place when calcium is present. Given that calcium is known to impede LOX-3 activity, its presence would have stopped the process. This suggests that only LOX-1 and LOX-2 are probably present in the crude extract, which is in line with our previous pH tests (described in Section 2.2.1), in which we postulated that LOX-1 was prevalent, and LOX-3 was not.

The regulating function of calcium in soybean lipoxygenases has been clarified by literature. The earlier belief that plant lipoxygenases function independently of calcium ions (Ca^{2+}) and membrane contacts was contested by Tatulian and associates in 1998. Their spectroscopic data demonstrated that LOX-1 is calcium-regulated, and that calcium is necessary for its membrane binding (Tatulian *et al.,* 1998). This discovery points to a more intricate regulation structure for LOX-1 than was previously thought. LOX-1 exhibits restricted accessibility to polar solvents and mostly transforms linoleic acid into hydroperoxides when in solution. Conjugated ketodienes are produced as a result of LOX-1's enhanced solvent accessibility and enzymatic activity upon membrane attachment. This demonstrates how important membrane contacts are for increasing the catalytic efficiency of the enzyme (Zheng *et al.,* 2010; Murphy *et al.,* 2008).

Additional research has confirmed the significance of calcium in regulating the activity of lipoxygenase. For example, it has been demonstrated that at pH 7.5, calcium ions activate lipoxygenases in navy bean and soybean extracts, supporting the idea that calcium has a regulatory role (Hayward *et al.,* 2017). In particular, it has been discovered that calcium preferentially inhibits LOX-3 while increasing the activity of LOX-1 and LOX-2 (Koch *et al.,* 1968; Mandal *et al.,* 2014). These results are consistent with our discovery that calcium salts block LOX-3 and increase enzymatic activity, most likely via their effects on LOX-1 and LOX-2.

Furthermore, it has been demonstrated that calcium affects LOX-1's structural and functional characteristics, which in turn affects how quickly it binds to membranes. A unique regulatory mechanism, this calcium-induced membrane connection promotes the synthesis of conjugated ketodienes and increases the enzyme's accessibility to solvents (Yamamoto *et al.*, 1970; Tatulian *et al.*, 1998). These discoveries highlight the significance of calcium in controlling the activity of lipoxygenase and its possible involvement in plant signalling pathways. Our results, which show that LOX-3 is absent from the crude extract and that calcium increases LOX activity, are consistent with earlier investigations, confirming the crucial function of calcium in controlling soybean lipoxygenase.

Table 9: Effect of calcium and iron salts on limonene conversion

Reaction	**Limonene/%** 	**Limonene Oxide/%** 	**Carveol/%** 	**Carvone/%** 	**Carvone oxide/%** 
Control – no salts/ MDS LOX & OA	42.53	1.71	0	3.88	1.36
0.05 g $CaCl_2$ Salt & OA	35.97	5.88	0	5.30	0.68
0.05 g $CaCl_2$ Salt & 0.05 g $FeSO_4.7H_2O$ Salt & OA	31.13	3.42	0.60	5.99	0

2.7.3 Manganese (Mn^{2+})

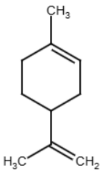
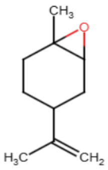
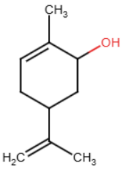
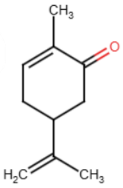
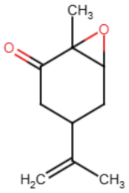
An investigation on manganese's impact on enzyme activity was carried out. According to literature iron and manganese lipoxygenases (LOX) both catalyse the oxidation of polyunsaturated fatty acids, but they differ in their metal centres, catalytic mechanisms, and reactivity. Iron LOX (Fe-LOX) uses a redox-active iron centre (Fe^{2+}/Fe^{3+}), allowing for greater flexibility in electron transfer and enabling a broader range of reactions. This makes FeLOX more reactive and versatile in oxygenating substrates (Oliw *et al.*, 2022). In contrast, manganese LOX (Mn-LOX) relies on a less redox-flexible manganese centre (Mn^{2+}/Mn^{3+}), resulting in a more stable and selective catalytic cycle. While both enzymes share similar structural features, the differences in their metal centres lead to distinct mechanisms, with FeLOX being more dynamic and MnLOX exhibiting more selective reactivity and oxygenation patterns (Wennman *et al.*, 2016).

The goal of our investigation was to determine whether manganese would inhibit or enhance the reaction. If manganese enhances the reaction, it could suggest the presence of manganese LOX in our system. However, if manganese inhibits the reaction, it would indicate that iron LOX is the only enzyme responsible for the reaction.

The data obtained from **Table 10** demonstrates that manganese exhibits an inhibitory effect on the reaction. In the control reaction, which lacked manganese, the carvone percentage was 3.88%. However, upon the addition of 0.05 g of $MnSO_4 \cdot 4H_2O$, the carvone percentage significantly dropped to 1.58%. Further increases in manganese concentration resulted in minimal changes to the reaction outcome, indicating a plateau in its inhibitory effect. Interestingly, when both 0.1 g of $MnSO_4 \cdot 4H_2O$ and 0.1 g of $FeSO_4 \cdot 7H_2O$ were included in the reaction, the carvone percentage increased to 3.15%, comparable to the control. The addition of $CaCl_2$ further enhanced the carvone percentage to 4.44%, suggesting that calcium, in combination with iron, may be promoting the reaction.

The observed inhibitory effect of manganese, coupled with the restorative effect of iron and calcium, suggests that the manganese itself is not playing a catalytic role in this system. Instead, the data supports the hypothesis that iron is the primary metal responsible for the catalytic activity of LOX in this reaction, with manganese potentially interfering with its function. The absence of a significant reaction enhancement with increasing manganese concentrations further reinforces the conclusion that manganese LOX is not present in our crude LOX preparation.

Table 10: Effect of manganese on limonene conversion

Reaction	**Limonene/%** 	**Limonene Oxide/%** 	**Carveol/%** 	**Carvone/%** 	**Carvone oxide/%** 
Control (no salts) MDS LOX & OA	42.53	1.71	0	3.88	1.36
0.05 g $MnSO_4.4H_2O$ Salt & OA	58.97	0.10	0	1.58	0.68
0.1 g $MnSO_4.4H_2O$ Salt & OA	47.30	0.33	0.09	2.20	1.25
0.1 g $MnSO_4.4H_2O$ Salt & 0.1g $FeSO_4.7H_2O$ & OA	45.62	0.57	0.12	3.15	1.50
0.05 g $MnSO_4.4H_2O$ salt 0.05 g $FeSO_4.7H_2O$ salt & 0.05 g $CaCl_2$ salt	40.94	1.13	4.63	4.44	0

2.8 Enzyme-to-Substrate Ratio

The purpose of this study was to determine how changing the substrate-to-enzyme ratio affected lipoxygenase (LOX) activity when oleic acid was used as the substrate. The enzyme source was milled defatted soybean rather than pure LOX. As stated in section 2.2, the LOX content in milled defatted soybean is 1%. In our experiment, we used 25 g of crude LOX extract, which corresponds to 0.25 g of LOX. Oleic acid was chosen for this experiment because it has a greater enzymatic activity than other fatty acids, such as linoleic acid, and it is less expensive, making it an appealing alternative for large-scale industrial applications.

Independent studies conducted by our commercial partner tested lower substrate-to-enzyme ratios, such as 1:1, 2:1, and 3:1, among others. Their findings indicated that the highest enzyme activity was achieved at a 10:1 ratio. Based on this, we decided to test higher enzyme-to-substrate ratios to assess whether they would have any impact on enzyme activity. According to the experimental results, which are shown in **Figure 19**, the crude enzyme-to-substrate ratio of 10:1 produced the most carvone (5.99%). This implies that a key factor in raising conversion efficiency is a larger substrate concentration in relation to the enzyme. This ratio maximized the conversion of oleic acid to carvone because there was sufficient lipoxygenase to conduct the reaction efficiently. This result is consistent with the general idea that, especially in situations where substrates are abundant, enzyme concentration is essential for effective catalytic reactions. At enzyme-to-substrate ratios higher than 10:1, no activity was observed.

The intricacy of maximizing enzyme catalysis in a reaction system is shown by these findings. In some industrial applications, raising the substrate concentration could be more economical, but making sure there is enough enzyme present is just as important for obtaining high conversion rates and optimizing product output. In our investigation, we found that a reduced yield was the consequence of having too much substrate and not enough enzyme to catalyse the reaction. The sudden decrease in conversion with a doubling of the fatty acid content was unexpected. There are three possible contributing factors. Firstly, we have effectively reduced our aqueous component, thereby decreasing the buffering capacity with respect to fatty acid addition. Secondly, addition of fatty acids may lead to the formation of micelles, which could trap reagents, reducing the reaction rate. Thirdly, the decrease in product yield at high substrate concentrations is likely due to active site saturation and/or substrate inhibition, where excess substrate interferes with the enzyme’s catalytic efficiency

An important observation regarding the enzyme-to-substrate ratio was that as the amount of oleic acid (substrate) increased, the pH of the reactions gradually decreased from 8.5 to 6. This drop in pH can be attributed to the dissociation of oleic acid into its protonated form (H^+ + oleate ion) under the reaction conditions. This decrease in pH likely contributed to the lower enzyme activity observed at higher oleic acid concentrations. Specifically, at an acidic pH, LOX (lipoxygenase) activity was notably reduced, which explains why the enzyme activity at a 10:1 enzyme-to-substrate ratio was higher, as the pH remained more favourable for LOX activity.

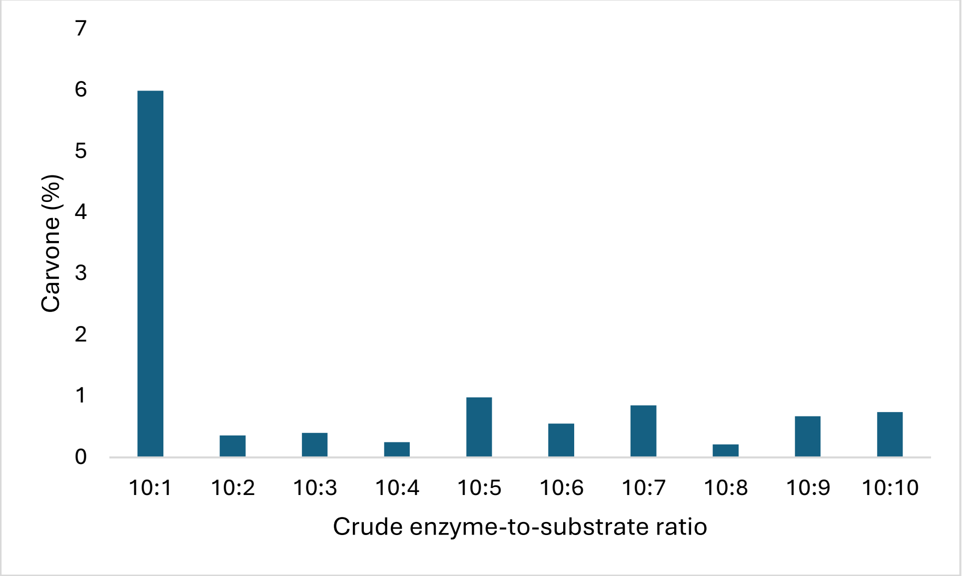
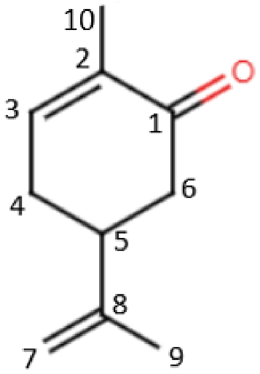


Figure 19: Effect of crude enzyme-to-substrate ratio on the reaction.

2.9 Purification, Isolation and Characterization of Carvone

Carvone was isolated using column chromatography, which used silica gel as a stationary phase. A combination of 10% distilled ethyl acetate and 90% hexane was utilized as the mobile phase. The same mobile phase (10% ethyl acetate/90% hexane) was used to monitor fractions in a systematic manner using silica thin layer chromatography (TLC). Using an Ultraviolet (UV) lamp set at 254 nm, the spots were visible on the TLC plate. The pure isolated carvone was subsequently characterized using NMR (proton, **Table 9** and **Figure 20**; and carbon, **Table 10** and **Figure 21**) and GC-MS techniques (**Figure 22** and **Figure 23**).

Table 11: Proton NMR signals of purified carvone as compared to published data

Protons  	**Purified carvone (ppm)**	**Carvone Published data (Mak *et al.*, 2006) (ppm)**
1H, m, H-3	6.76	6.76
1H, s, H-7	4.83	4.81
1H, s, H-7	4.75	4.76
2H, m, H-6	2.72	2.69
2H, m, H-6	2.61	2.56
1H, m, H-5	2.30	2.43
2H, m, H-4	2.29	2.40
2H, m, H-4	2.24	2.20
3H, s, H-10	1.79	1.75
3H, s, H-9	1.76	1.73

The numbering of carbons from 1 to 10 in the carvone structure is followed by the IUPAC name which is 2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-one. According to literature, a study done by Mak *et al.* (2006), carvone is known for having three vinyl protons. As shown in **Table 11,** their signals are at 4.76 and 4.81 ppm, which are assigned to two geminal vinyl protons of the isopropenyl group. The proton more downfield with a signal of 6.76 ppm is assigned to the β-proton of the enone moiety (Mak *et al.*, 2006). The proton NMR analysis of carvone done herein showed distinct splitting patterns that corresponded to the protons chemical surroundings.

A multiplet was detected at 6.76 ppm, corresponding to the β-olefinic proton, while singlets at 4.83 ppm and 4.75 ppm were ascribed to geminal vinylic protons. These three protons are in line with literature values and are comparable to Mak *et al.* (2006). Protons next to the ketone group appeared as multiplets at 2.72 ppm and 2.61 ppm due to being in axial and equatorial positions and their interactions with nearby methine proton. Multiplet signals were observed at 2.29 ppm and 2.24 ppm consistent with the methylene γ-protons and at 2.30 ppm assigned to the methine proton. Furthermore, singlets at 1.79 ppm and 1.76 ppm correlated to methyl groups in isolated settings. The ^{1}H-NMR signals obtained is very comparable to Mak *et al.* (2006) and this validates carvone's structure and ensures its successful purification.

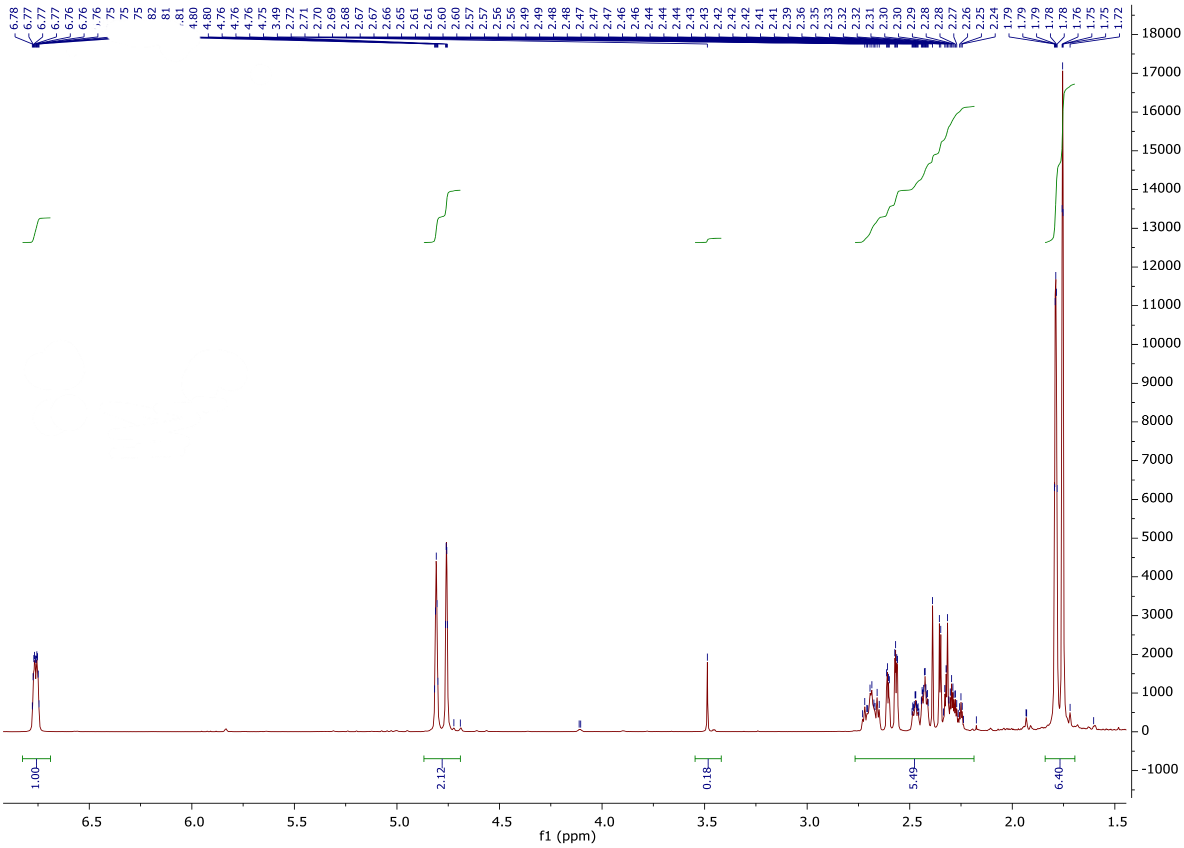
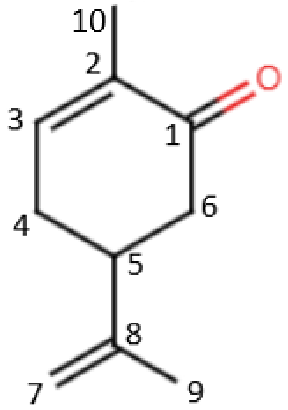


Figure 20: ^{1}H-NMR spectra for carvone.

Table 12: Carbon NMR signals of purified carvone as compared to published data

Chemical Shift (ppm) 	**Purified Carvone (ppm)**	**Carvone Published data (Riester *et al.*, 2022) (ppm)**
C1	199.78	198.30
C8	146.68	148.62
C3	144.62	145.30
C2	135.41	134.11
C7	110.44	109.21
C6	43.12	44.52
C5	42.44	41.24
C4	31.21	30.92
C9	20.51	20.50
C10	15.69	15.90

The carbon (^{13}C) NMR spectrum as shown in **Figure 21** further supports the identification of carvone. The chemical shifts at 15.69 ppm and 20.51 ppm are indicative of alkyl carbons, likely representing methyl and methylene groups, respectively. These shielded carbons align with carvone's saturated alkyl regions. The signal at 31.21 ppm corresponds to methylene carbons adjacent to an unsaturated group, such as the alkene or the ketone, which causes slight deshielding. Carbons appearing at 42.44 and 43.12 ppm suggest quaternary carbons in highly substituted environments, such as near the carbonyl group, which is consistent with carvone's structure.

A key region of the carbon NMR spectrum lies between 110.44 and 146.68 ppm, which identifies unsaturated carbons. The peak at 110.44 ppm corresponds to an alkene carbon, while the peaks at 135.41 ppm and 144.62 ppm suggest carbons involved in a conjugated system, such as the double bonds in carvone's structure. These peaks are critical in confirming the presence of a conjugated alkene system within the molecule. The most downfield signal at 199.78 ppm confirms the presence of a carbonyl group, characteristic of the α,β-unsaturated ketone, which is a key functional group in carvone. The ^{13}C-NMR data signals obtained are comparable to (Riester *et al.*, 2022), therefore this confirms that validity of the purified carvone structure.

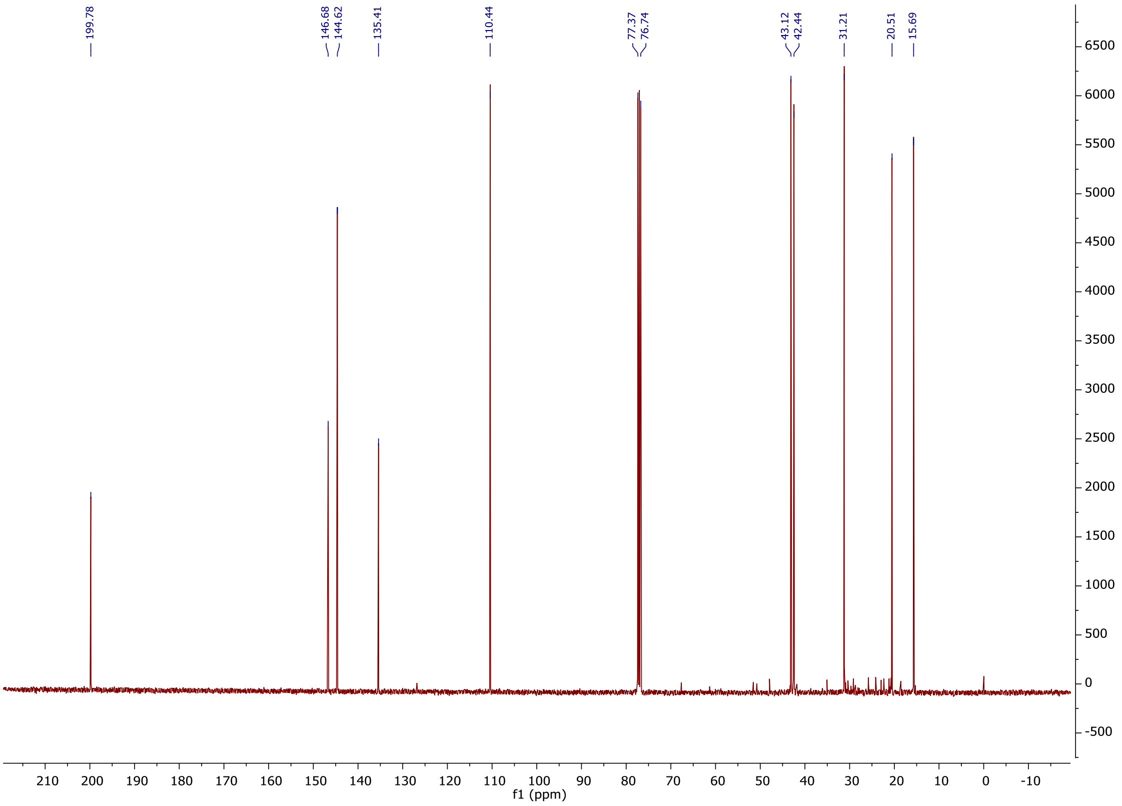


Figure 21: ^{13}C-NMR Spectrum of Carvone

The NMR data, in combination with the splitting patterns observed in the proton NMR and the chemical shifts in both the proton and carbon NMR spectra, strongly support the conclusion that carvone has been isolated. The presence of characteristic signals—such as the alkene protons around 6.40 ppm and 5.49 ppm in the proton NMR and the conjugated alkene and carbonyl carbons in the carbon NMR—match the known structure of carvone, confirming its identity. This demonstrates how NMR spectroscopy is a powerful tool for the structural elucidation of natural compounds like carvone, allowing us to confirm that the desired compound has been successfully isolated through its distinct chemical shifts and splitting patterns.

Proof that carvone was successfully isolated was also supported by the GC-MS analysis, which chromatographically identified carvone elution at a retention time of 3 minutes and 44 seconds according to the reference library. A peak with an area percentage of 99.99% was visible on the chromatogram, confirming the high purity of the product. This shows that the mobile phase selection, TLC monitoring, and column chromatography techniques were all very effective and low in contaminants.

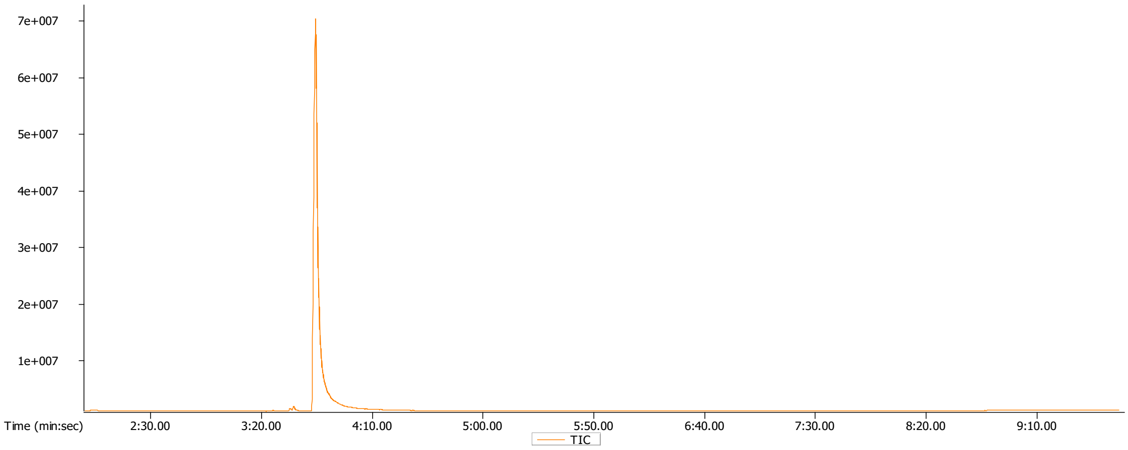


Figure 22: GC Chromatogram of Carvone

The isolated carvone sample's effective identification and purification are strongly supported by the mass spectrometry data that was collected for it. The high signal strength and distinct, sharp peaks indicate that the isolated compound is highly pure, with little interference from other compounds or contaminants. The sample produced a spectrum with a high similarity score of 953 when it was evaluated at a retention time of 3:44.40 minutes. This spectrum closely resembles the reference spectrum for (-)-carvone. The existence of distinctive fragment ions at *m/z* 39, 54, 67, 82, 93, 108, and 135, consistent with the known fragmentation pattern of carvone, provides confirmation of this almost perfect match. The compound's identity is further confirmed by the peak at *m/z* 82, which is particularly noteworthy as it represents one of the distinctive fragments of carvone.

The strong alignment of the reference and sample spectra highlights the effectiveness of the purification procedure. The efficient separation of carvone from a complicated mixture is evidence of the potency of the selected chromatographic and biocatalytic conversion methods.

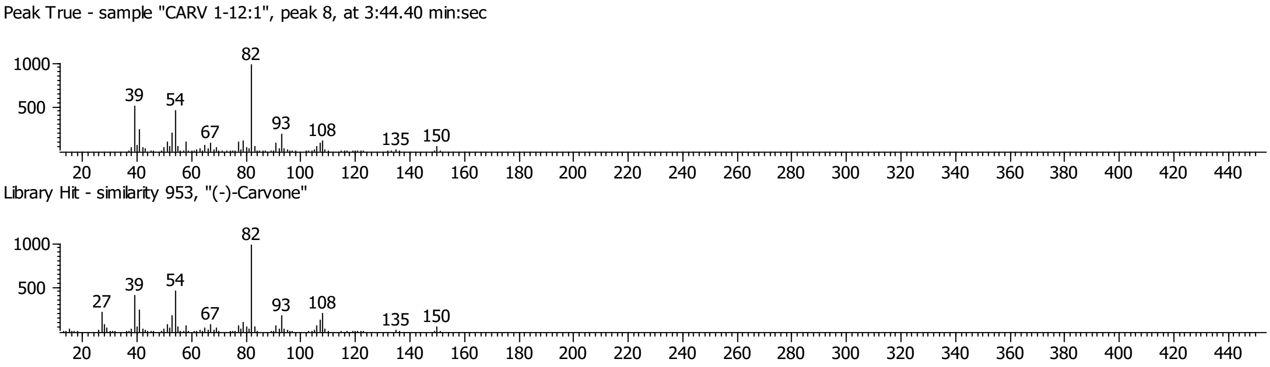
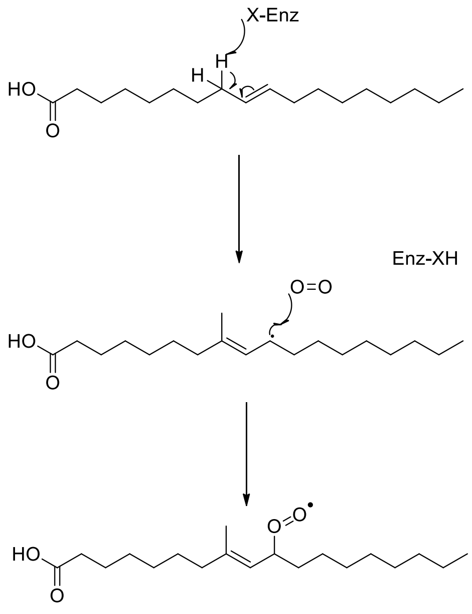


Figure 23: Mass Spectrum of Carvone

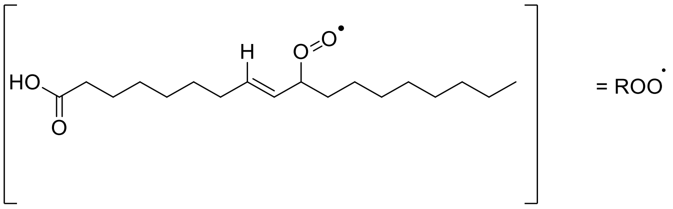
2.10 Postulation of the reaction mechanism

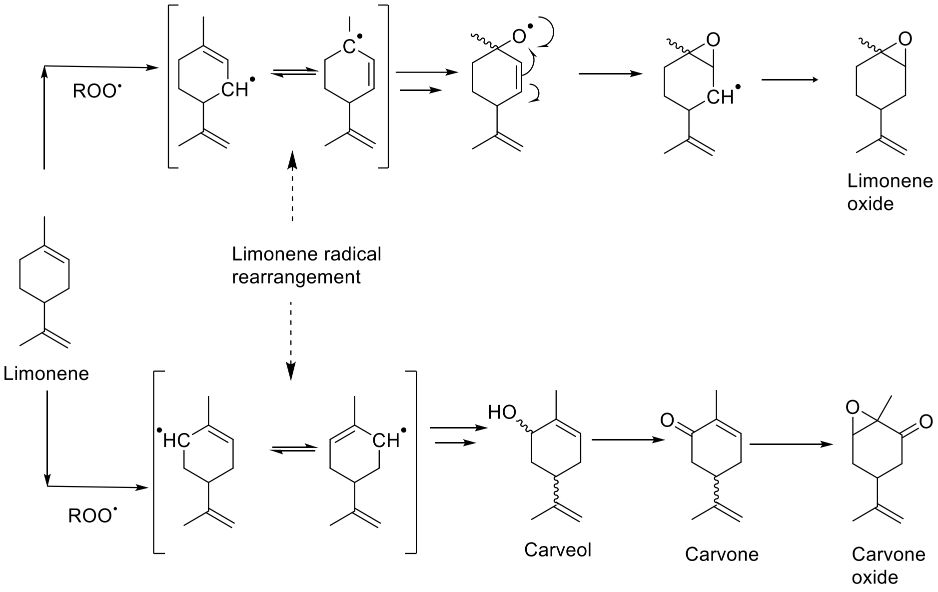
Based on our investigation's reaction mechanism can be proposed. As shown in **Scheme 3,** we believe that the reaction initiates with the enzyme (X-Enz) abstracting a hydrogen atom from the substrate's C11 carbon, resulting in a carbon-centred radical and reducing the enzyme to Enz-XH. The radical is resonance-stabilized within the monosaturated fatty acid system, allowing molecular oxygen to insert at the C13 site and produce a peroxy radical intermediate. This intermediate is then reduced, yielding the hydroperoxide product, and the enzyme is spontaneously regenerated for additional catalytic cycles.



Scheme 3: Reaction mechanism for the formation of hydroperoxide

Scheme 3 illustrates the formation of the hydroperoxide intermediate, which is an important step in the conversion of limonene into other compounds such as limonene oxide, carveol, and carvone. This intermediate supports the oxidative transformations required for various product pathways, emphasizing its critical involvement in the entire reaction mechanism.





Scheme 4: Reaction mechanism for the conversion of Limonene to Carvone using LOX

The mechanism as shown in **scheme 4** shows the oxidative transformation of limonene into important compounds such limonene oxide, carveol, and carvone via the action of a hydroperoxide radical (ROO•). The process starts with the production of a carbon-centred radical on limonene, which interacts with molecular oxygen to form the hydroperoxide radical. This radical then attacks limonene, generating a variety of reactive intermediates depending on where the assault occurs. In one process, the hydroperoxide radical epoxidizes the double bond in limonene, producing limonene oxide. In another, hydroxylation occurs, resulting in the creation of carveol, which can then oxidize to carvone by dehydrogenation and rearrangement.

The reaction pathway in our system primarily followed the formation of 1,2-limonene epoxide and the subsequent conversion to carveol, which were the primary products detected. This is the main difference between our reaction mechanism and the one proposed by Nikolaiczyk and colleagues. Additionally, we did not observe the formation of 8,9-limonene epoxide, a key intermediate in their pathway, which suggests that our enzyme does not generate the specific radicals necessary for the formation of this epoxide, which is crucial for moving through their proposed mechanism.

The existence of carvone oxide in our system and the absence of 8,9-limonene epoxide could be signs of minute variations in the catalytic efficiency or substrate selectivity of the enzyme. Variations in the soybean kinds employed in our experiment could be one of the reasons for these discrepancies, since various soybean varieties may manufacture lipoxygenase (LOX) enzymes with slightly different characteristics. Furthermore, the enzyme's activity and the products that are produced may be impacted by the particular biocatalytic circumstances, including temperature, pH, and substrate concentrations. Therefore, these intrinsic variations in the enzyme supply and experimental settings may be the cause of the divergence in reaction pathways between our work and that of Nikolaiczyk *et al.*

Chapter 3

Conclusions and Recommendations

In 2020 our collaborators at APBio discovered that limonene was converted to carvone in the presence soyabean meal, a result that was confirmed by the subsequent publication by Nikolaiczyk *et al*. As we had been using LOX active soyabean meal to generate nootkatone from valencene in our laboratories we wanted to determine if the same enzyme was involved in this terpene oxidation, and what the optimal conditions were for this biocatalytic reaction.

The biocatalytic reaction was conducted in a 100 mL two-neck round-bottom flask, containing 0.294 mol of limonene (40 g), 25 g of crude lipoxygenase (LOX), and 0.00885 mol of either oleic acid (2.5 g) or linoleic acid (2.5 g). The reaction was carried out at 50°C under reflux for seven days, with a continuous supply of oxygen provided by a balloon reservoir system. Finally, using a variety of analytical methods, the isolated substance was clearly identified as carvone. A highly pure product was shown by the GC-MS analysis, which showed a retention time of 3 minutes and 44 seconds and an area percentage of 99.99%. Carvone's identity was further verified using NMR spectroscopy, which compared the distinctive chemical shifts and coupling patterns with those predicted by the compound's molecular structure. All these findings support the effectiveness of the column chromatography purification procedure and provide strong evidence that the separated product is, in fact, carvone.

The experiments and research conducted confirmed that lipoxygenase (LOX) can be successfully used as a soybeans meal, but with specific requirements for buffer composition and pH. For this investigation, it was established that the LOX enzyme performs best in a 0.1 M sodium borate buffer at pH 8.5. This conclusion is consistent with previous enzyme characterisation research demonstrating that LOX-1 activity is highest under these conditions. To support the idea that LOX-1 is the major isozyme driving the catalytic conversion, additional experiments were carried out with calcium salts, which are known to inhibit LOX-3 activity while increasing LOX-1 and LOX-2 activity. The ongoing activity of the reaction in the presence of calcium salts supports the conclusion that the enzyme mixture is primarily composed of LOX-1 and possibly LOX-2. Furthermore, the ideal reaction pH supports the conclusion that LOX-1 is the primary isozyme responsible for the catalytic activity detected in this work.

The results of this research demonstrated that lipoxygenase (LOX) exhibits substrate specificity, with a much faster reaction rate when using linoleic acid as the substrate. However, oleic acid was found to serve the same purpose as linoleic acid but is more cost-effective. Given the economic advantage and practical feasibility, especially for large-scale industrial reactions, oleic acid was selected as the preferred substrate for the process. This choice enhances the practicality and efficiency of the reaction for industrial applications. Interestingly, the reaction occurred, albeit at a low level, without the presence of added fatty acid, although this could be due to residual fatty acids that may be present in the soya meal defatted soya meal. When oleic acid was utilized, the reaction produced both limonene oxide and carveol; without a fatty acid substrate, the products were only carveol and carvone. Furthermore, oxygen was confirmed to be necessary for the reaction, supporting the assumption that these are biocatalytic oxidations probably catalysed by LOX. Finally, the ideal enzyme active meal-to-substrate ratio was found to be 10:1, with variations resulting in lower enzyme activity.

Metal salts, particularly iron salts, played an important role in accelerating the process. The maximum conversion rate of 58% was attained by adding 26 moles of iron salts. These salts were found to enhance the enzyme's activity rather than function independently, as they required the presence of LOX to enable the process. This demonstrates that iron salts operate as reactants rather than catalysts in this process, participating in a Fenton reaction involving the fatty acid peroxide generated by the LOX.

Recommendations:

In our study, we focused primarily on MDS LOX due to the simplicity of the reaction workup. However, future studies could explore different soybean preparations and cultivars to compare their LOX activity, as well as purified LOX. Investigating the impact of varying the enzyme-to-substrate ratio, particularly by increasing the substrate amount, could provide valuable insights. Exploring a broader range of substrates may also be of interest. While our research centred on LOX, it would be worthwhile to explore other oxidative enzymes, such as laccase and cytochrome P450. Further studies could utilize a chiral GC column to identify the carvone isomers produced during the reaction.

Chapter 4

Methods and Materials

4.1 Materials

All chemicals were used as received and were purchased from Sigma-Aldrich or Merck (South Africa), unless otherwise stated. Solvents used were HPLC grade methanol and deuterated chloroform (for NMR analysis). Both limonene enantiomers (+) and (-) (98% purity) were purchased from Scatter Oils (Johannesburg, South Africa). Soybean seeds were purchased from Seeds for Africa (Cape Town, South Africa) and the milled defatted soybean was provided by APBio. Oleic acid (98% purity) and linoleic acid (98% purity) were the fatty acid substrates used. The salts and metal salts used were magnesium sulphate anhydrous, sodium hydroxide, iron(II) sulphate heptahydrate, manganese(II) chloride tetrahydrate and calcium chloride. Boric acid was used for the preparation of the buffer.

4.2 Instrumentation

For compound characterisation Gas Chromatography Mass Spectroscopy (GC-MS) and Nuclear Magnetic Resonance (NMR) were applied.

4.2.1 Gas Chromatography Mass Spectroscopy

An Agilent 7890B system with a GERSTEL MPS-2 auto-sampler connected to a Leco® Pegasus 4D time-of-flight mass spectrometer (ToF/MS) was used to conduct gas chromatography. A 29.687 m × 0.25 mm × 0.25 µm Rxi-5Sil (Restek) primary column and a 1 m × 0.25 mm × 0.25 µm Rxi-17Sil (Restek) secondary column, both made of 5% diphenyl/dimethyl polysiloxane, were used to achieve the separation. To improve chemical separation and detection, the temperature program, flow rate, and spectra acquisition rate were all modified as the method followed below:

Helium was used as the carrier gas for a 1 µL splitless injection, with a steady flow rate of 1.4 mL/min. The primary GC oven was initially set to 50°C for 0.2 minutes, then increased by 20°C/min to 280°C and held that temperature for one minute. The secondary oven was adjusted to be +5°C cooler than the main oven. Temperatures of 200°C for the front inlet and 280°C for the transfer line were maintained. This method took 9 minutes and 29 seconds to complete. With the modulator off, the mass spectrometer's ion source temperature was 200°C. With a mass range of 25–450 *m/z*, the mass spectrometer acquisition rate was set to 10 spectra per second. Leco ChromaTOF software was used for data processing and peak identification, using references to the NIST library.

4.2.2 Preparative Column Chromatography

Column chromatography was used for sample purification. The stationary phase consisted of Macherey-Nagel silica gel (particle size: 0.063-0.200 mm), whereas the mobile phase was distilled ethyl acetate and hexane. The mobile phase consisted of 10% EtOAc/Hexane. Each fraction was monitored by TLC and the solvent system used for TLC monitoring was 10% EtOAc/Hexane. The spots were checked under a UV lamp.

4.2.3 NMR

Bruker III 500 MHz or AVANCE 300 MHz spectrometers were used to acquire nuclear magnetic resonance (NMR) spectra. The solvent for each measurement was chloroform-d. The internal standard, trimethylsilane (TMS), has a chemical shift of zero parts per million (ppm). Chemical shift results are presented in ppm. Coupling constants (*J* values) are reported in Hertz.

4.3 Sample preparation for analysis

Sample preparation was done for the GC-MS and NMR instruments. Sample preparation is an important step and below are the detailed sample preparations for GC-MS and NMR.

4.3.1 GC Sample Preparations

For the GC sample preparation extra precaution was taken to avoid contaminating the sample since GC-MS is highly sensitive when it comes to analysing samples. Approximately 10 mg of the product obtained was dissolved in 10 mL of HPLC-grade methanol. A syringe was used to measure the amount of methanol used since it is more accurate. The prepared GC sample was then mixed thoroughly using a pipette. The mixed solution of approximately 1 mL was then transferred into a GC vial and analysed using the instrument.

4.3.2 NMR Sample preparations

NMR sample preparation involved weighing 20 mg of the obtained product and dissolving it in 0.7 mL of deuterated chloroform in an NMR tube. The prepared NMR sample was then tested and analysed using the instrument.

4.4 Enzyme Extraction of Crude LOX

There were two types of soybean preparations that were used in this project, which were Milled Defatted Soybean (MDS) and Ground Soybean (GSB). Using a coffee grinder, soybeans were ground into a coarse grain to create ground soybean (GSB). Seeds for Africa provided milled defatted soybean (MDS) as a defatted coarse meal. The coarse meal was suspended 20% (w/v) by combining the sample with sodium borate buffer (pH 8.5, 100 mM). To guarantee adequate mixing, the suspension was agitated using a magnetic stirrer at room temperature (22 ± 3°C). The sample was stirred and then kept at 4°C in a refrigerator without being stirred again. The sample was kept in the refrigerator for about eighteen hours. The crude LOX enzyme was then extracted using a cheesecloth. The liquid containing the crude LOX enzyme was then separated and the biomass was discarded in a container. The crude LOX enzyme was then ready to use for the oxidative biocatalytic reactions of limonene.

4.4.1 Extraction of LOX at various pH

The procedure was carried out as described in Section 4.4, with the following modification: after grinding the Milled Defatted Soybean (MDS) and Ground Soybean (GSB) into a fine powder, the MDS and GSB crude LOX powders were suspended in borate buffer solutions adjusted to varying pH levels, ranging from 6.5 to 8.5. Each sample was soaked for 12-14 hours at 4°C to investigate the effect of pH on enzyme activity.

4.5 Biocatalytic Oxidative Reactions of Limonene

The reaction was carried out in a 100 mL two-neck round-bottom flask, which contained 0.294 mol of limonene (40 g), 25 g of crude lipoxygenase (LOX), and 0.00885 mol of the fatty acid substrate, either oleic acid (2.5 g, 0.00885 mol) or linoleic acid (2.5 g, 0.00891 mol). The reaction mixture was refluxed at 50°C for seven days under a continuous supply of oxygen using a balloon system. Approximately 5 mL of the reaction mixture was sampled daily over the seven-day period. The collected samples were centrifuged to separate the liquid phase (crude product) from the biomass (enzyme). The centrifuged liquid was then analysed using gas chromatography (GC). There were several reaction conditions that were varied throughout the reactions and subsequent reactions such as pH of the buffer, temperature, amount of enzyme to substrate ratio, the use of oxygen the reaction, and the use of metal salts such as iron and magnesium.

4.6 Comparison of reactions using Ground Soybean (GSB) and Milled Defatted Soybean (MDS)

Two types of soybeans—Ground Soybean (GSB) and Milled Defatted Soybean (MDS)—were utilized to investigate their respective enzyme activities. Each soybean type was soaked in a 0.1 M sodium borate buffer at pH 8.0 to enhance the extraction efficiency of lipoxygenase (LOX). The soaking process lasted for 12 to 14 hours at 4°C with continuous stirring, ensuring optimal hydration and activation of the enzyme. Following the soaking period, the enzyme was extracted from both soybean types using the procedure outlined in Section 4.4.

This involved grinding the soybeans into a fine powder, and suspending the material in the borate buffer, and filtering the mixture through cheesecloth to obtain the crude LOX enzyme. For the biocatalytic reaction, a 100 mL two-neck round-bottom flask was prepared, containing 40 g of limonene, 25 mL of the crude LOX extract (derived from either GSB or MDS), and 2.5 g of oleic acid. The reaction setup was designed to compare the enzymatic activity of LOX from the two soybean types, with the mixture refluxed at a temperature of 50–55°C under a continuous supply of oxygen to facilitate the oxidative conversion of limonene to carvone.

4.7 Substrate Variation

The biocatalytic reactions were conducted as described in Section 4.5, with the primary objective of evaluating the effects of different substrates on the enzymatic activity of lipoxygenase (LOX) derived from Milled Defatted Soybean (MDS). In this variation, three different substrates were tested: linoleic acid, oleic acid, and a control reaction with no substrate. For each reaction, the crude LOX was extracted from MDS according to the procedure outlined in Section 4.4. The reaction setup included the addition of either 2.5 g of linoleic acid, 2.5 g of oleic acid, or no substrate at all, along with 40 g of limonene and 25 g of the crude LOX enzyme. The reaction conditions, including temperature (50–55°C) and a continuous supply of oxygen, were maintained as per the original protocol to ensure consistency across all trials. This substrate variation aimed to elucidate the influence of different fatty acids on the oxidative conversion of limonene to carvone and to identify which substrate may enhance the enzymatic activity of MDS LOX.

4.8 Enzyme to substrate ratio

This procedure was carried out according to the parameters stated in Section 4.5, with the exception that the substrate concentration was systematically adjusted. The ratio of substrate to enzyme was changed from 1:10 to 10:10, and the substrate concentration was increased by 10% at a time to evaluate the impact on the activity of the enzyme. The precise ratios of each substrate were achieved by meticulously measuring and combining the corresponding quantities of substrate and enzyme. The enzyme extraction was carried out as described in Section 4.4, with the Milled Defatted Soybean (MDS) and Ground Soybean (GSB) powders suspended in a borate buffer solution. The enzyme was allowed to incubate for 12-14 hours at 4°C before being filtered through cheesecloth to extract the crude LOX enzyme.

4.9 Temperature Variations

The reaction was carried out as described in Section 4.5, using a 100 mL two-neck round-bottom flask containing 0.294 mol of limonene (40 g), 25 g of crude lipoxygenase (LOX), and 0.00885 mol of the fatty acid substrate oleic acid (2.5 g, 0.00885 mol). The temperature was the only variable altered, while all other parameters were kept constant. The reaction temperature was varied between 25°C and 90°C, with reactions conducted at each temperature to assess the effect of temperature on the reaction.

4.10 Absence of oxygen

To prepare the reaction vessel for the subsequent reactions, a nitrogen purging procedure was employed. The vessel was initially loaded with the reactants: 40 g of limonene, 25 g of crude lipoxygenase (LOX), and 2.5 g of oleic acid. A balloon and syringe system was utilized to purge the reaction vessel with nitrogen gas. The purging process involved connecting the syringe to the balloon, which was filled with nitrogen. The nitrogen was introduced into the reaction vessel to displace any residual air, thereby creating an inert atmosphere. This step was crucial to prevent unwanted oxidative reactions that could compromise the integrity of the reactants. The purging continued until the reaction vessel was thoroughly saturated with nitrogen, ensuring optimal conditions for the subsequent reaction.

4.11 Fe^{2+} Salts

For this procedure, the reaction method described in Section 4.5 was used, but different concentrations of iron(II) salts were added to see how they affected enzyme activity. The ratios of iron(II) salts to LOX were investigated systematically, with concentrations ranging from 1.3 moles of iron per mole of LOX to 2.6, 13.3, and 26.4 moles of iron per mole of LOX. To guarantee precise iron and enzyme concentrations, each reaction mixture was meticulously produced, and the effects of the different iron ratios on the enzymatic reactions were regularly observed. Iron(II) salts were added in an effort to clarify their possible involvement as cofactors in increasing LOX's catalytic activity during the reactions.

4.12 Ca^{2+} Salts

For this procedure, the reaction method described in Section 4.5 was used, but 0.05 g of $CaCl_2$ was added to the reaction to see how it affects the activity of LOX.

4.13 Mn^{2+} Salts

For this procedure, the reaction method described in Section 4.5 was used, but 0.05 g of $MnSO_4$. $4H_2O$ was added to the reaction to see how it affects the activity of LOX.

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APPENDICES

APPENDIX A: TABLES

Table 13: Carvone production at various pH levels for MDS LOX

MDS LOX	Carvone/%			
pH	**Run 1**	**Run 2**	**Run 3**	**Average**
6.5	0.54	0.51	0.48	0.51
7	0.62	0.6	0.75	0.66
7.5	0.78	0.72	0.69	0.73
8	1.3	1.28	1.09	1.22
8.5	3.05	3.62	2.58	3.08
9	0.32	0.48	0.52	0.44

Table 14: Carvone production at various pH levels for GSB LOX

GSB LOX	Carvone/%			
pH	**Run 1**	**Run 2**	**Run 3**	**Average**
6.5	0.58	0.49	0.62	0.56
7	0.52	0.59	0.61	0.57
7.5	0.79	0.82	0.65	0.75
8	1.02	1.15	1.21	1.13
8.5	2.9	2.52	2.82	2.75
9	0.23	0.41	0.3	0.31

Table 15: Substrate variation data for the conversion of limonene to carvone

Substrate	Limonene/%	Limonene oxide/%	Carveol/%	Carvone/%	Carvone oxide/%
Oleic acid – Day 1	49.75	1.22	0.00	0.04	0.00
Day 2	48.69	0.80	0.00	0.14	0.00
Day 3	47.62	1.52	0.00	3.60	0.77
Day 4	47.04	1.02	0.00	3.78	0.83
Day 5	42.53	1.71	0.00	3.88	1.36
Day 6	40.93	1.18	0.00	4.56	1.47
Day 7	39.11	0.72	0.00	5.99	1.58
No substrate – Day 1	49.72	0.26	0.00	0.02	0.00
Day 2	46.55	0.37	0.00	0.14	0.00
Day 3	46.45	0.77	0.00	2.50	0.75
Day 4	46.43	0.83	0.00	2.71	0.92
Day 5	46.05	1.17	0.00	2.86	1.18
Day 6	45.96	0.46	0.00	5.29	2.21
Day 7	45.70	0.43	0.00	5.67	2.53
Linoleic acid- Day 1	49.09	2.06	0.00	2.43	1.32
Day 2	48.46	1.18	0.00	3.87	1.42
Day 3	47.51	0.81	0.00	4.32	2.25
Day 4	43.83	0.52	0.00	5.24	2.48
Day 5	42.16	0.49	0.00	6.45	2.75
Day 6	41.43	0.00	0.00	1.14	3.13
Day 7	41.24	0.00	0.00	0.98	3.32

Table 16: Effect of temperature on the reaction

Temperature/°C	Limonene/%	Limonene oxide/%	Carveol/%	Carvone/%	Carvone oxide/%
25	48.54	1.58	0.16	0.41	0.00
30	48.42	1.50	0.25	0.46	0.00
40	47.43	2.46	0.34	0.85	0.04
50	37.06	0.50	1.51	6.25	1.02
60	40.30	2.95	1.43	4.68	0.72
70	48.91	1.45	1.98	2.53	0.15
80	49.09	0.39	0.00	0.15	0.00
90	49.64	0.51	0.06	0.11	0.00

Table 17: Effect of iron salt on carvone production

Reaction Type	Carvone/%
Control	0.06
0.05 g $FeSO_4.7H_2O$	19.38
0.1 g $FeSO_4.7H_2O$	28.10
0.5 g $FeSO_4.7H_2O$	48.24
1 g $FeSO_4.7H_2O$	58.02

Appendix B: GC spectra

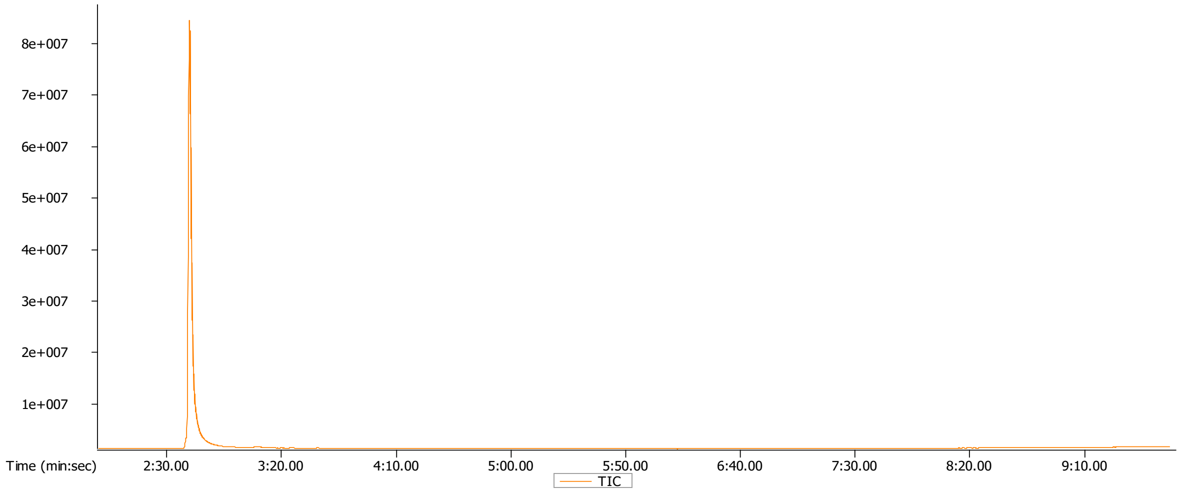

Figure 24: Limonene Oil spectra

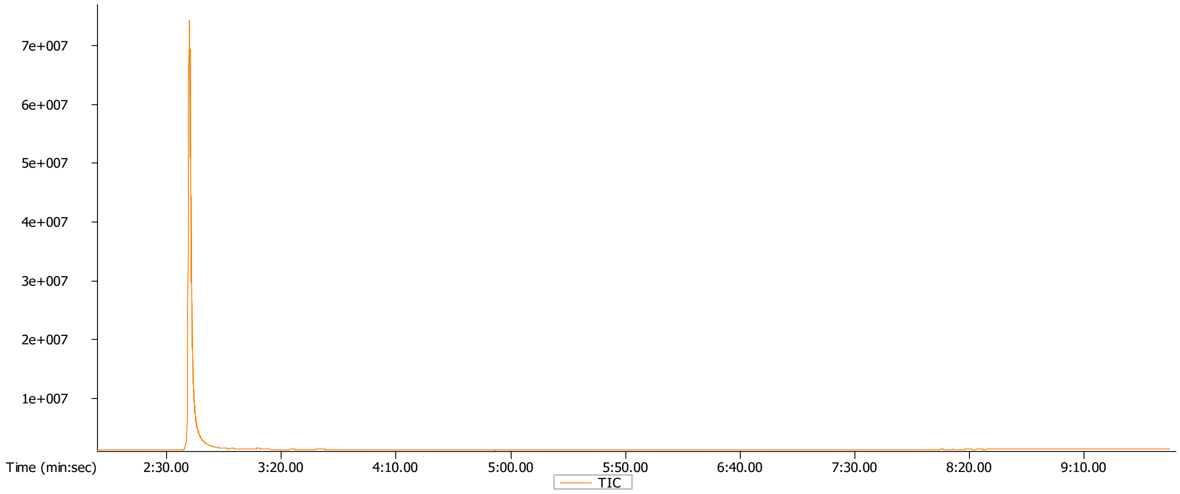

Figure 25: Control reaction without LOX

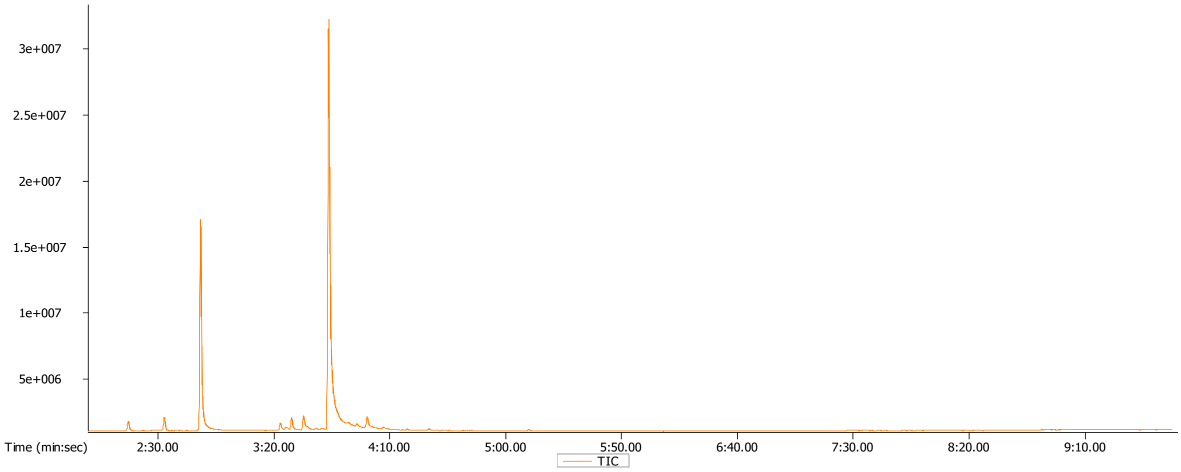

Figure 26: GC spectra of the highest conversion of limonene to carvone

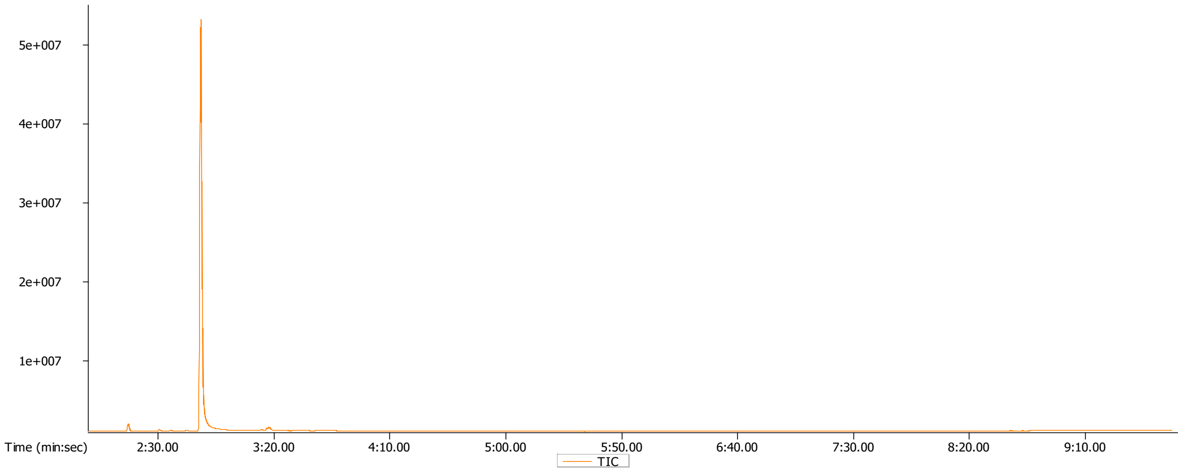

Figure 27: GC spectrum of the reaction without oxygen

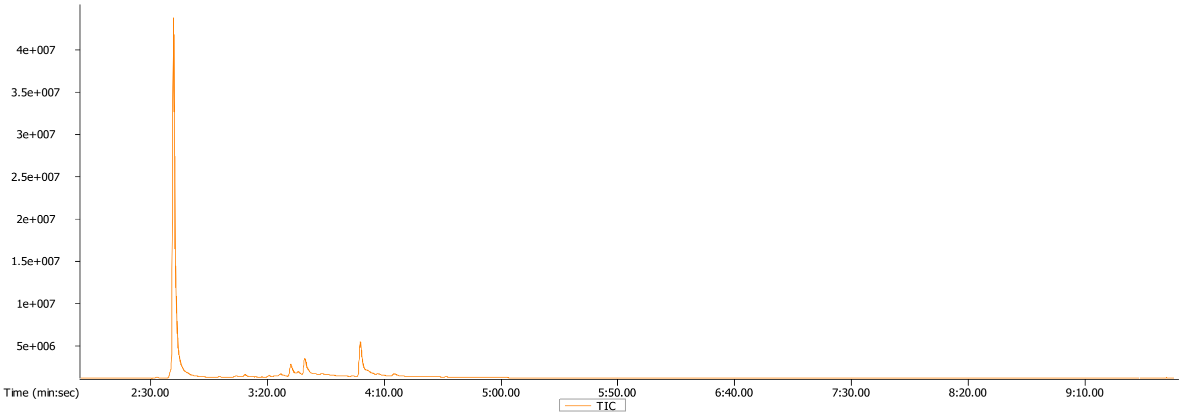

Figure 28: GC spectrum of the reaction at 50̊C

Appendix C: NMR Spectra

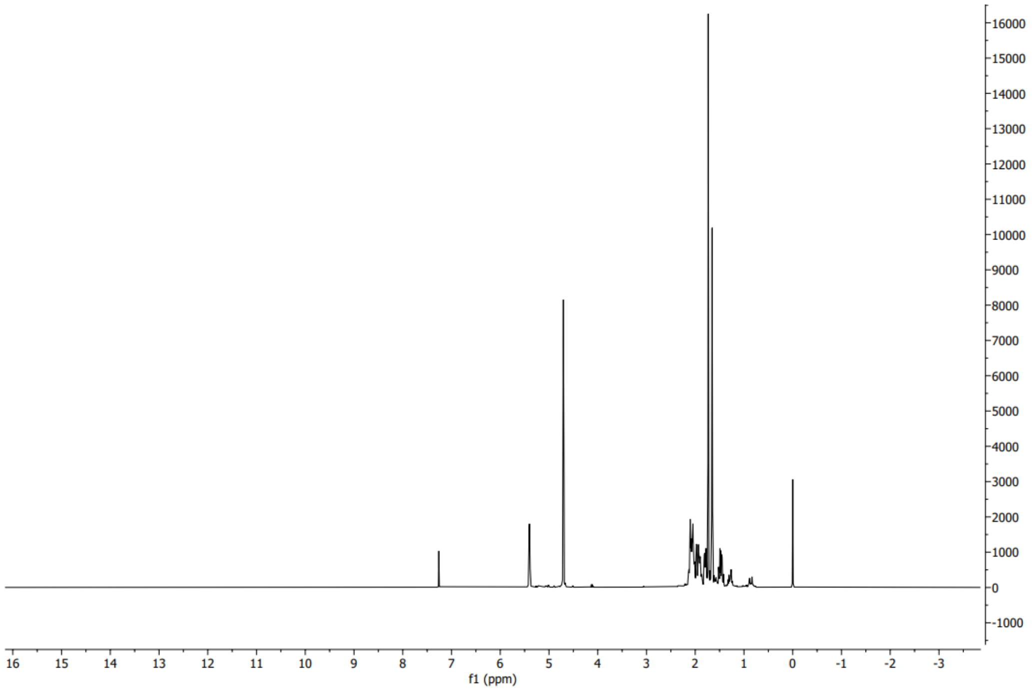

Figure 29: ^{1}H-NMR of Limonene

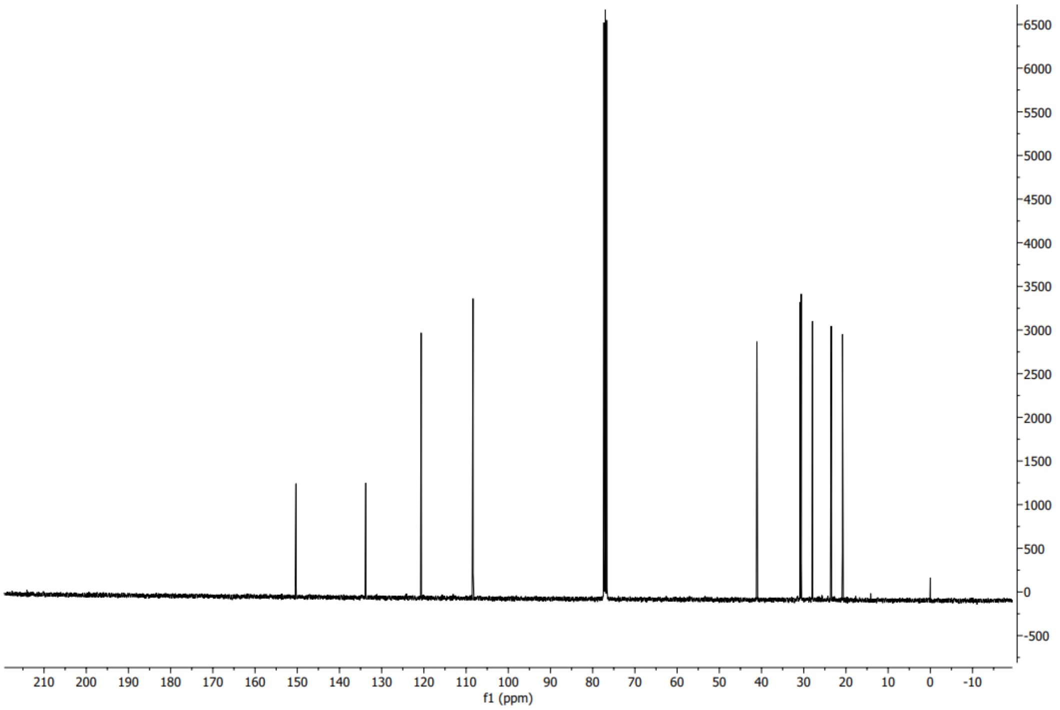

Figure 30: ^{13}C-NMR of Limonene

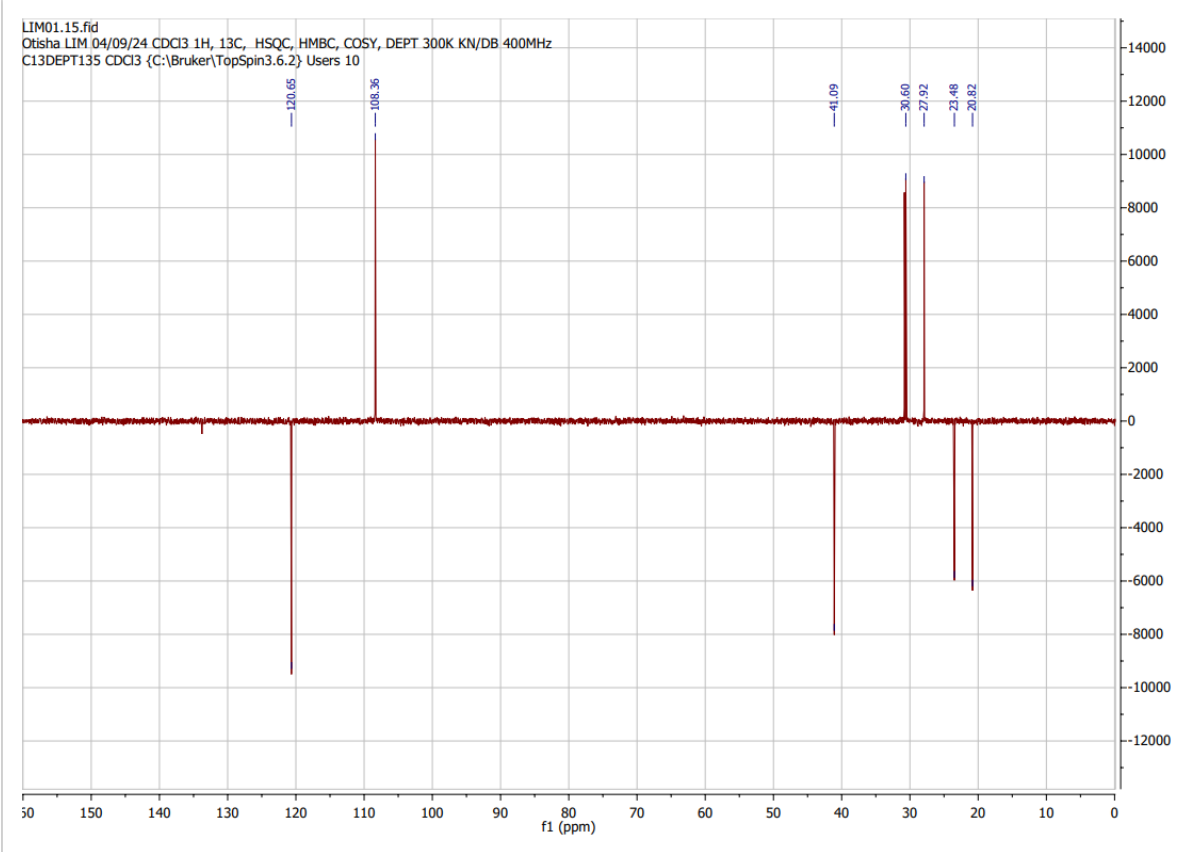

Figure 31: DEPT 135 spectrum of limonene

Appendix D: Calibration Curve

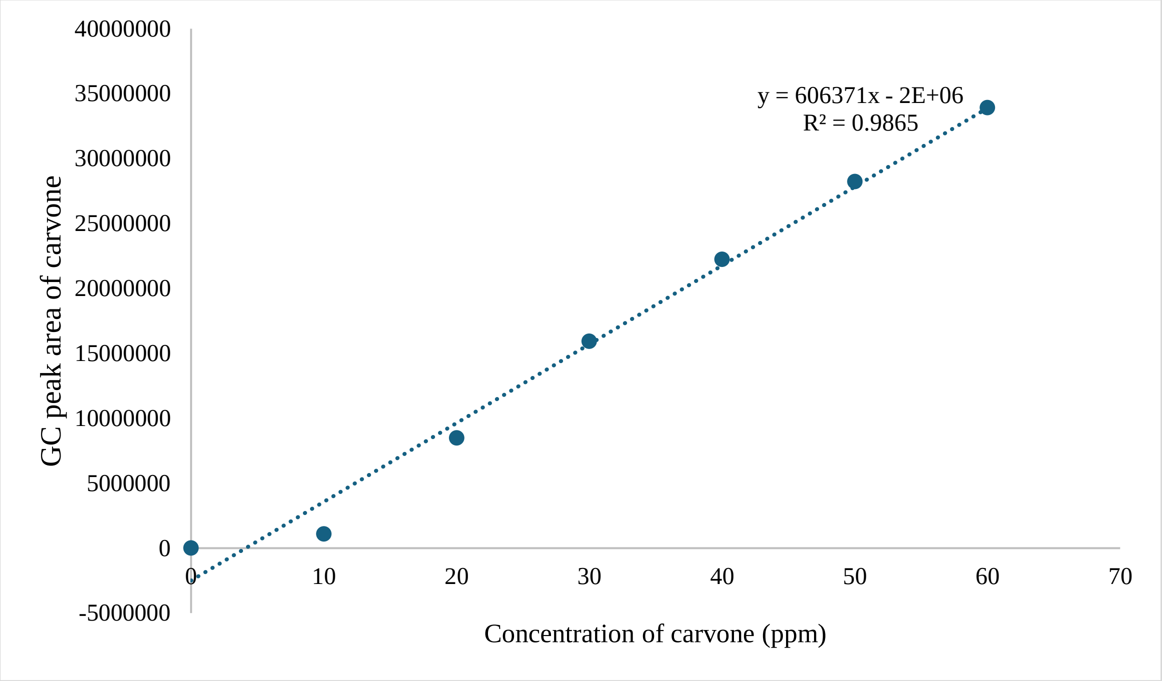

Figure 32: Calibration curve of carvone standard