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The Conversion of Various Terpenes by Oxidative Biocatalysis

by

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(1828933)

A dissertation submitted to the Faculty of Science at the University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

Supervisor: Prof. Dean Brady

Co-supervisor: Dr Daniel Pienaar

Date: March 2022

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination.



_____

Ida Mamolemo Makhubela

__11_ day of June _2022__ at Johannesburg_ .

ABSTRACT

Nootkatone (NK) is a sesquiterpenoid that is highly sought after in the flavour, fragrance, pharmaceutical and agricultural industries. The challenge is that it is extracted from plant species that are limited, seasonal and are not able to meet the demand. Scientists have found ways to chemically synthesize this compound, but due to the toxicity of the reagents and procedures, the resulting product does not meet the requirements to be classified as a natural product. Therefore, biocatalysis has become vital in the production of NK because it focuses on the use of renewable resources, non-toxic reagents, mild reaction conditions and cost effective methods.

The production of NK by biocatalytic methods has been faced with challenges of low yield, expensive biocatalyst, low selectivity, enzyme recovery, long and tedious enzyme purification procedures. Therefore, the purpose of this research was to firstly develop an easy yet cheap method of extracting lipoxygenase (LOX) enzyme from soybeans. Secondly, the crude LOX enzyme was applied in synthesis to convert valencene to NK, before and after, purification and immobilization. Thirdly, the crude LOX enzyme was precipitated with ammonium sulfate (50%-80%) and cross-liked with glutaraldehyde to form CLEAs.

The use of soybean LOX to convert VL to NK was a success with more than 90 mol% VL converted, and the highest concentration of NK was 74 mol%. This was especially significant because the reaction conditions were at pH 3-6, this has expanded the range that LOX can convert VL to NK. The emulsion challenges we encountered propelled us to developing an effective method that utilizes inorganic salts (NaCl, $NaHCO_3$, K_2CO_3) to make product separation easy.

There is great potential for the huge demand of natural NK to be met through this process because of it is effective, green, and sustainable. There is also an opportunity to explore the biocatalytic activity of soybean LOX in converting other terpenes or organic compounds to important oxidized compounds.

DEDICATION

To my amazing, loving, and supportive husband, *Calvin Themba Makhubela*

To the best mom in the universe, *Maleshoane Thoahlane*

To my loving & caring brothers, *Celi & Cyril Maitin*

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LIST OF ABBREVIATIONS

ADH	Alcohol Dehydrogenase
BHT	Butylated Hydroxytoluene
$CDCl_3$	Deuterated chloroform
CLEA	Cross-Linking Enzyme Aggregates
CP450	Cytochrome 450
EtOAc	Ethyl Acetate
$FeSO_4.7H_2O$	Iron (II) Sulfate Heptahydrate
FID	Flame Ionization Detector
FOX	Ferrous Oxidation-Xylenol Orange
GA	Glutaraldehyde
GC	Gas Chromatography
GCP	Green Chemistry Principles
HBT	1-hydroxybenzotriazole
H_2SO_4	Sulfuric acid
HPLC	High Pressure Liquid Chromatography
LOD	Limit of detection
LOQ	Limit of quantification
LOX	Lipoxygenase
MB	Methylene Blue
$MgSO_4$	Magnesium Sulfate
mM	Millimolar
$MnCl_2.4H_2O$	Manganese (II) Chloride Tetrahydrate
mol%	Mole percentage

MS	Mass Spectrometer
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide hydrogen
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium Hydroxide
NaOL	Sodium Oleate
NK	Nootkatone
NMR	Nuclear Magnetic Resonance
NP-HPLC	Normal Phase HPLC
OA	Oleic Acid
ppb	Parts per billion
ppm	Parts per million
Rf	Retention factor
SDG	Sustainable Development Goals
TLC	Thin Layer Chromatography
TOF	Time-of-flight
Tris	Tris-hydroxymethyl aminomethane
UV-VIS	Ultraviolet-visible
VL	Valencene
wt%	Weight percentage

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CHAPTER 1

INTRODUCTION

1.1 Background

The world we live in is full of fragrances and flavours that mankind has been enjoying as early as the 1500s [1]. Egypt, Persia, and India are the main pioneers in the industrial use of essential oils. The Egyptians used perfumed balms largely for religious ceremonies [1]. The scents from essential oils are all unique and are extracted from different parts of a plant, including leaves, peels, bark, flowers, buds, and seeds [2]. Scents also a play a vital role in influencing the livelihoods of both animals and humans. For instance, flowers emit chemicals such as geranylacetate and methyleugenol that are enjoyably pleasant to humans. However to bees it is a signal to find food [3,4].

These essential oils are a mixture of compounds called terpenes. Due to their extensive uses, terpenes have become highly sought after. Rubber is the most used (poly)terpene in the world, when Charles Goodyear added sulfur to it, it yielded vulcanized rubber. This type of rubber is used world-wide in motor vehicle tyres [5]. Terpenes play a vital role as building blocks for various industries such as agriculture [6-8], cosmetics [9] , food and flavour fragrance [10,11], perfumes [12,13] and pharmaceuticals [14,15].

The industrial production of most of these terpenes, however, has faced a few challenges. Some of the challenges encountered in the commercialization of terpenes are their instability, the substrate and product toxicity and the presence of multiple biotransformation pathways in microorganisms [16]. The traditional synthetic chemistry has been unsuccessful in solving these problems and it is not the best for all life on earth. An advantage of producing these compounds by biotechnological methods is that the final products can be considered ‘natural’. Being natural extends

their uses for human consumption with much less suspicion arising over product toxicity [11]

A very well-studied biotechnological method that has been explored for this project is biocatalysis. It is the transformation of natural and non-natural compounds by enzymes or whole cells [17] Biocatalysts are (i) compatible, biodegradable and non-hazardous; (ii) They are highly enantio-, chemo-, and regio-selective; (iii) The reactions don't require harsh conditions, they can be performed in aqueous conditions, at ambient temperature and atmospheric pressure; (iv) Biocatalysts afford routes that are more step economical, thereby generating less waste compared to traditional synthesis; (v) The methods are not only environmentally attractive and sustainable but also cost-effective [18-20].

The advantage of using enzymes in the production of terpenes is that the availability of these compounds no longer depends on the harvest or the quality of the plant material. The use of enzymes is an essential part of Green Chemistry, because of their non-hazardous characteristics, safe for both humans and the environment. In this chapter the significance of Green Chemistry (**1.2**), Terpenes (**1.3**), Biocatalysis (**1.4**), Catalytic synthesis of NK (**1.5**) and Non-conventional biocatalysis of LOX (**1.6**) will be explored. Together with the importance of LOX Purification and Assay (**1.7**), Immobilization (**1.8**), followed by the Nootkatone identification and separation methods (**1.9**). The Aims and objectives (**1.10**), and Proposed mechanism (**1.11**) will also be outlined.

1.2 Green Chemistry

Many chemicals, such as heavy metals, solvents, and detergents, contribute to a great extent to global mortality and disability. Solvents lead to unintentional poisoning that causes death annually from preventable chemical exposures [21,22]. Asthma, through second-hand smoke and air pollution, has led to the development of and increased mortality. Aquatic environments have been greatly affected by the contamination of hazardous chemicals. Additionally, organic pollutants and heavy

metals are transported and processed globally, thereby bioaccumulating in rain forests and deep oceans, and causing toxic effects in wildlife and humans [23].

As the world is moves towards a ‘greener’ environment through reducing, reuse and recycling of waste, the modern practice of Green Chemistry focuses on the elimination of waste, the utilization of renewable raw materials and the avoidance of the use of toxic reagents [24]. This approach does not only protect the environment against hazardous chemicals, but sustainably preserves it. With natural and renewable raw materials, there will be fewer fatalities caused by harmful chemicals.

1.2.1 Green chemistry metrics

In the past decade, the use of metrics was proposed to drive businesses, governments, and communities towards more sustainable practices. This brought great awareness to chemists to change how chemicals are synthesized so that they are less wasteful [25]. Some of the metrics include:

1.2.1.1 Effective mass yield

It is defined as the percentage of the desired product relative to the mass of all the non-benign starting materials used. This metrics eliminates the risk of human toxicity and ecotoxicity before the reaction begins. Therefore, ensuring that the toxic reagents are replaced with safe ones [26]. It is expressed as,

$$\text{Effective mass yield (\%)} = \frac{mass\ of\ product}{mass\ of\ non{-}benign\ reagents} \times 100$$

1.2.1.2 E-Factor

A metric used to quantify the amount of waste generated per kilogram of product. Introduced by Roger Sheldon in 1992, this factor exposes the relative wastefulness in different parts of a chemical process [26]. It is stated mathematically as,

$$\text{E factor} = \frac{total\ waste\ (kg)}{product\ (kg)}$$

1.2.1.3 Atom economy

Independently introduced by Barry Trost and Roger Sheldon in 1990, its purpose is to maximize the use of raw materials (*A&B*) so that the final product (*C*) contains the maximum number of atoms from the reactants [26,27]. It can also be stated as,

$$\text{Atom economy} = \left[\frac{m.w.\ of\ C}{m.w.\ of\ A + m.w.\ of\ B}\right] \times 100$$

1.2.2 How Green Chemistry Principles are in line with the Sustainable Development Goals

The approaches to Green Chemistry were outlined by Paul Anastas and John Warner, in 1998 as the Twelve Green Chemistry Principles (GCPs) [28]. These principles were developed to help reduce the use of hazardous chemicals that have the potential to harm humans and the environment [29]. Green chemistry is also a guide to 'safer' chemistry; it includes atom economy, use of safe solvents, and the prevention of waste and pollution.

Therefore, a process is considered greener and more sustainable if it follows most of these guidelines. For example, a process involving an addition reaction (GCP 2) of two starting materials originating from renewable feedstock (GCP 7) taking place in an ideal stereoselective way (GCP 1) at room temperature (GCP 6) in water (GCPs 3 & 4) and using recyclable catalyst (GCP 9), would be considered "green" [30].

In 2015, the United Nations introduced the 17 Sustainable Development Goals (SDGs) and 169 targets to be upheld by all countries around the globe. The 15-year plan has been set to make the livelihood of all mankind and the conservation of the planet better than what it is [31].

The twelve GCPs can be divided into 4 main groups: toxicity reduction, waste minimization, the use of renewable resources and energy efficiency [30]. There is correlation between the SDGs and the GCPs (**Table 1.1**). They both aim for the use

of safe chemicals to reduce mortality rate and illnesses caused by hazardous chemicals [22]. To achieve an environmentally sound management of chemicals and wastes throughout their life cycle and to reduce their release into air, water, and soil [22].

Table 1.1 The correlation between the Green Chemistry Principles and the Sustainable Development Goals [28, 30].

Green chemistry principles (GCPs)	**Sustainable development goals (SDGs)**
Toxicity reduction	
3 - Less hazardous chemical synthesis	3 - Good healthy & well-being
4 - Designing safer chemicals	6 - Clean water for all
5 - Safer solvents & auxiliaries	14 - Conservation of marine life
	15 - Protection of terrestrial ecosystems
Waste minimization	
1 - Prevention of waste	13 - Combat climate change
2 - Atom economy	
8 - Catalysis	
11 - Real-time pollution prevention	
12 - Safer chemistry for accident prevention	
Use renewable resources	
7 - Use of renewable feedstock	12 - Sustainable consumption and production patterns
10 - Design for degradation	
Energy efficiency	
6 - Design for energy efficiency	7 - Affordable & Clean energy

Green chemistry approach strives to achieve sustainability at a molecular level and has been applied successfully in all sectors, including pharmaceuticals, automobile, cosmetics, agriculture, and energy [32,33]. It is a goal of most chemical or pharmaceutical companies globally to have chemical processes that are in line with

these principles. These include Dow Agrosciences (now part of Corteva Agriscience), Merck and Pfizer. In implementing these processes to their production scale, these companies have reduced the amount of waste produced, thereby making their processes more cost effective and environmentally more friendly [32].

1.3 Terpenes

Essential oils are odorous and volatile liquids that are responsible for the aromas and scents in plants. These oils are extracted from plant material by various methods that include solvent extraction, aqueous infusion, cold or hot pressing, effleurage, supercritical fluid extraction and phytonic process. The most common methods, however, are hydrodistillation, steam, and water/steam distillation [34].

Essential oils have gained their importance in fragrances and flavour, aromatherapy, cosmetics and phytomedicine. All over the world there is a strong demand for pure natural oils. Citrus essential oils are the most popular, abundant, and cheaper, being produced in large proportions. Other essential oils are produced in smaller quantities and are more expensive because of their rarity [12].

1.3.1 Terpene classification

Terpenes and terpenoids (usually oxygen-containing terpenes) are the most abundant class of compounds in essential oils [35]. The building blocks of terpenes are made up of two or more isoprene units **(Figure 1)** that differ in their degrees of saturation and/or the variety of functional groups incorporated within their structures.

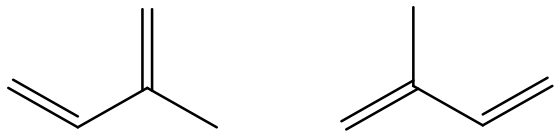


Figure 1: Two molecules of isoprene (2-methyl-1,3-butadiene), the simplest 5-carbon terpene building block. [36].

The classification of terpenes is dependent on the number of isoprene units (C_5) it contains. Monoterpenes ($C_{10}H_{16}$) are widely spread in essential oils, and sesquiterpenes ($C_{15}H_{24}$) are unsaturated compounds and the most the diverse among the volatile terpenoids. The heavier terpenes, diterpenes ($C_{20}H_{32}$) and triterpenes ($C_{30}H_{50}$) [36], are generally not in essential oils. **Figure 2** shows the structures of various terpene compounds, all of which can be seen to be built from isoprene units; carvone is a monoterpene; farnesol, a sesquiterpene; p-camphorene, a diterpene and ganoderic acid, a triterpene [37,38].

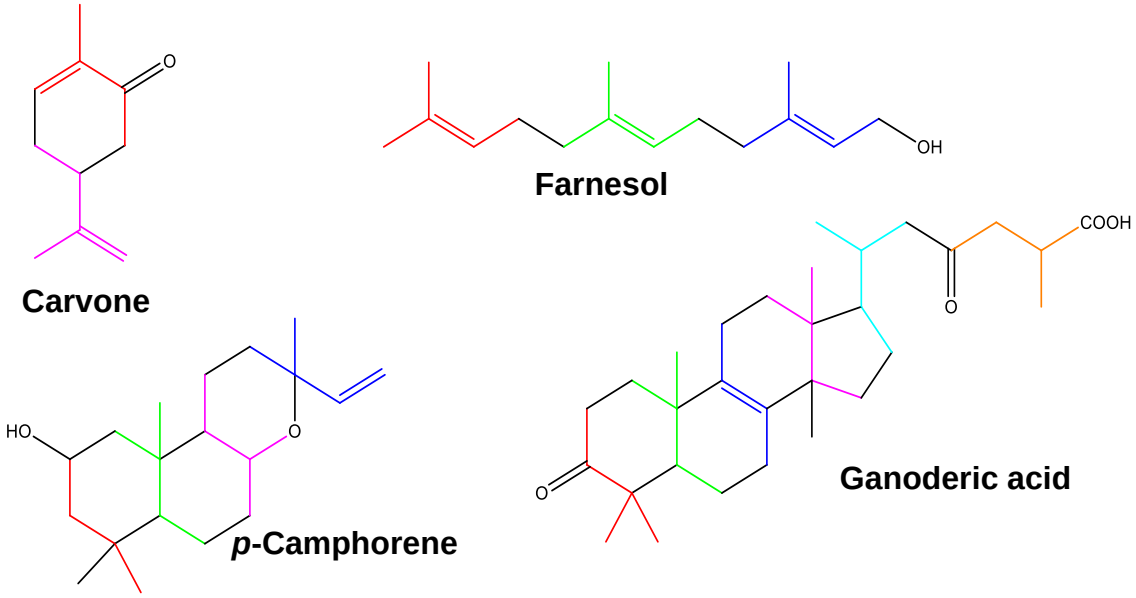


Figure 2: Structures of various terpene compounds and their built isoprene units.

1.3.2 Terpenoids and their application

Monoterpenes in plants have mainly an ecological role, while other forms of terpenes in mammals function as regulators of enzymatic reactions, in stabilizing cell membranes and metabolic pathways. Plants containing terpenoids (oxygenated terpenes) and their derivatives have been used as fragrances and flavours for centuries. Terpenes have grabbed the attention of more industries with 22 000 terpenoids currently having been discovered, thus making them the largest group of natural products [39].

Besides their high demand in the fragrance and flavour industry, terpenoids are also sought after for their extraordinary biological activity and therapeutic uses (**Table**

1.2). Eugenol ($C_{10}H_{12}O_2$) (**Figure 3**) is a monoterpenoid phenol from clove oil (*Syzygium aromaticum*) and is the major constituent, the oil being composed of 70-90% eugenol [40]. It has been widely used for its anaesthetic and analgesic activity in dentistry [41], and also possess anti-fungal, anti-platelet, antioxidant, anti-carcinogenic effects [42]. Tea tree oil (*Melaleuca alternifolia*) contains a monocyclic terpene alcohol, terpinene-4-ol ($C_{10}H_{18}O$), which comprises 30-48% of the oil [43]. This compound is responsible for the treatment of genitourinary and skin diseases such as acne, warts, and recurring herpes [44]. Its biological activity extends to antimicrobial, anti-inflammatory, anti-tumoral and insecticidal [45].

Eucalyptol also referred to as 1,8-cineole ($C_{10}H_{18}O$), is a monoterpene cyclic ether isolated from Eucalyptus oil (*Eucalyptus globulus*) [46] and it comprises 60-80% of the oil [47]. The compound exhibits antinociceptive properties thereby acting as a calmative and depressant of the central nervous system [48]. Thymol ($C_{10}H_{14}O$), the major compound in thyme (*Thymus vulgaris L*), comprises 10-64% of the oil and imparts a herbaceous odour [49]. It treats disorders affecting the respiratory, nervous, and cardiovascular system, as well as oral infections [50]. It also exhibits antioxidant, antiseptic, and antimicrobial properties [51].

Linalool ($C_{10}H_{18}O$) has a lavender-like aroma with a very high composition, comprising up to 100% of rosewood oil (*Aniba rosaeodara*) [52]. It is a noncyclic monoterpenoid that is best used for relief of dermatitis and eczema, also has wound healing properties [53] and anti-influenza activity [54]. Linalool has shown insecticidal effects for the control of ticks, fleas, and flies [55]. Peppermint (*Mentha piperita*) oil is known for the minty, cooling effect of menthol ($C_{10}H_{20}O$) that can comprise up to 80% of the oil [56]. As a monoterpene alcohol, it exhibits biological activity including analgesic, antipruritic, and antimicrobial effects [57].

Nearly 70-80% of spearmint oil (*Mentha spicata*) is carvone ($C_{10}H_{14}O$), a ketone monoterpenoid with a minty and sweer odour [58,59]. It has been used to treat children's maladies due to its mild nature, and it displays excellent stimulant, carminative, and antispasmodic properties [60]. Nootkatone ($C_{15}H_{22}O$), a central subject in our work, is one of the compounds found in yellowwood cedar (*Chamaecyparis nootkatensis*), comprising less than 20% of the oil [61].

Nootkatone (NK) is an effective insect repellent. In 2009, the Centers for Disease Control and Prevention (CDC) reported close to 30 000 cases of Lyme disease caused by ticks. Most people have resorted to the use of harmful and hazardous chemicals to control ticks [62,63]. NK has not only shown effectiveness as a repellent and insecticide but has also been approved by the EPA (Environmental Protection Agency) as of August 2020 [64]. It was reported to be safe on sensitive skin at its highest concentration (>98%), unlike the current repellent, DEET (*N,N*-Diethyl-m-toluamide). Which was found to cause rashes, swelling and irritation of the skin [65]. NK is highly volatile and therefore does not persist in the environment [66].

Additionally, NK is a biocidal and repellent activity against several insects, fleas, ticks, mosquitoes [67] and cockroaches [68]. Also, it inhibits the activity of acetylcholinesterases [69]. The natural oil of NK has become increasingly important and in demand, because of its excellent attributes. No amount of trees can meet this need, therefore, the biocatalysis of NK has become a more attractive research.

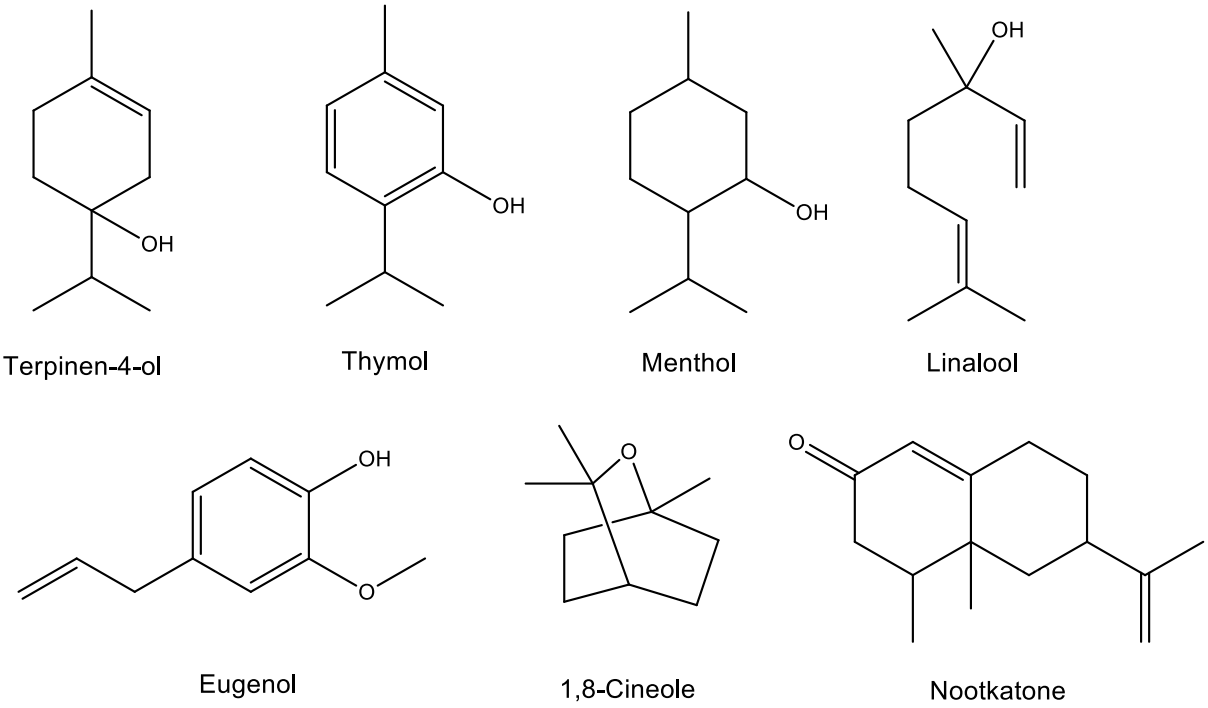


Figure 3: Structures of some biologically active terpenoids

Table 1.2: Biological activity of essential oils that contain terpenoids used in various industries.

Essential oil (Origin)	**Benefits**	**Major compound**	**Odour description**	**References**
Clove (*Syzygium aromaticum*)	Antioxidant, antiplatelet effect, anti-carcinogenic, anti-fungal, analgesic, and antimicrobial activity	Eugenol	Spicy pungent aroma	[40-42,70-72]
Tea tree (*Melaleuca alternifolia*)	Treatment for genitourinary and skin disease, insecticidal, anti-inflammatory, anti-tumoral effects	1-Terpinen-4-ol	Metallic, musty, green	[43-45,72-74]
Eucalyptus (*Eucalyptus globulus*)	Herbicidal, antinociceptive, and anti-herpes properties	1,8-Cineole	Camphor-like	[46-48,72,73,75]
Thyme (*Thymus vulgaris L*)	Antifungal, antioxidant activity, oral infections, and disorders of respiratory, nervous & cardiovascular system	Thymol	Spicy aroma	[49-51,72,76,77]
Rosewood (*Aniba rosaeodora*)	Relief for dermatitis, eczema, wound healing, insecticidal and anti-influenza virus activity,	Linalool	Floral, sweet, fruity	[52-55,72]
Peppermint (*Mentha piperita*)	Analgesic, anti-pruritic, and antimicrobial activity	(-)-Menthol	Minty, fresh, cool	[56,57,72,78-80]
Spearmint (*Mentha spicata*)	Antimicrobial, antioxidant, treats digestive problems, children's maladies, antispasmodic, stimulant, and carminative properties	(-)-Carvone	Minty & sweet	[58-60,72]
Alaska cedar (*Callitropsis nootkatensis*)	Biocidal, repellent, inhibits acetylcholinesterases	Nootkatone	Grapefruit-like	[61,67-69]

1.3.3 Challenges in Terpene production

In nature the majority of terpenes/flavour compounds are racemic, which is a great challenge, because only one of the enantiomers may display the desired activity or scent. In the food additives and pharmaceutical compounds market, pure enantiomers are required to have *ca.* 98% of one enantiomer in excess [39].

This makes chirality an important factor in these industries because the perception of odour and flavour depends on the absolute conformation of the isomers. De Carvolho and da Fonseca (2006) gave a summative table of different isomers of the same compound with different scents. The (*R*)-carvone emits the odour of spearmint, while the (*S*)-isomer that of caraway. Also, the (*R*)-limonene has a scent of oranges and the (*S*)-isomer of turpentine [39]. In turn, (+)-nootkatone that has a grapefruit-like scent, (-)-nootkatone has a spicy and woody flavour which is less useful. The high demand of this compound cannot be met by the natural plant extraction [81].

1.4 Significance of biocatalysis

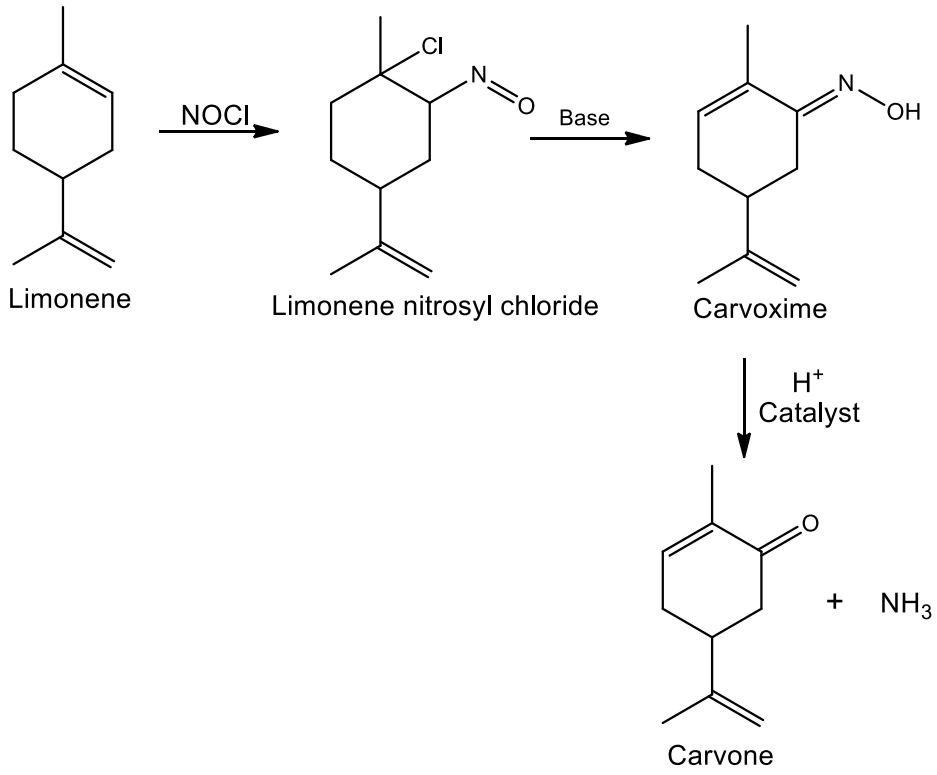
Traditional catalysis comprises heterogeneous and homogeneous catalytic methods. In heterogeneous catalysis, the solid phase catalyst provides a surface for temporary adsorption of reactants. The industrial application extends to syngas conversion, hydrogenation, and oxidation processes [30]. The benefits of this type of catalysis include easy recovery, recycling, and stability of the catalyst. While in homogeneous catalysis, a dissolved organometallic catalyst [82] serves as tool for synthesis of optically active molecules such as Active Pharmaceutical Ingredients. The drawback of homogeneous catalysis is the difficulty in separation and reuse of the catalyst, however, it offers high activity and selectivity. Approximately 75% of catalytic processes are heterogeneous [82].

The whole world needs to answer a very urgent and important call to save our planet. This places chemists at the centre stage due to the very harmful and toxic waste produced annually in the chemical industry. One of the ways this is done is by

moving away from the traditional catalysis to more sustainable alternatives, such as biocatalysis.

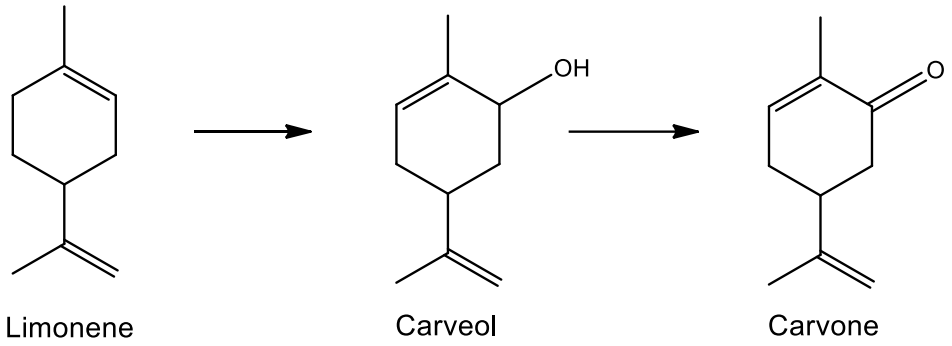
Davey and others (2000) published a patent on the synthetic preparation of carvone [83]. The invention comprises of the hydrogenation of carvoxime in the presence of a catalyst (e.g. palladium on calcium carbonate) that is selectively poisoned, e.g. by a lead salt. **(Scheme 1)** The carvoxime is prepared by reacting limonene with nitrosyl chloride to give a chloronitrosylated product, which then goes through dehydrochlorination and tautomerization.

This method is not environmentally friendly due to the hazardous metals and solvents used. Also, there are toxic side products that form including iron oxide and ammonia. The method has low enantioselectivity and purification is done with organometallic compounds, which also adds more steps to the procedure. In addition, the temperatures of the reactions can be as high as 155°C and pressure at 4-6 MPa [83].



Scheme 1: The synthetic conversion of limonene to carvone [83].

The use of enzymes, whole cells and microbial cells have been studied in the conversion of limonene to carvone. *Rhodococcus erythropolis* DCL14 was used to produce (-)-carvone from cis/trans-carveol, **Scheme 2**, at a diastereomeric excess of more than 98% [84,85]. The formation of carvone begins with the hydroxylation of limonene at position 6 to form carveol. Then followed by the oxidation of the alcohol group of carveol to form carvone, and the reaction conditions were mild, at 30°C and atmospheric pressure [84] .



Scheme 2: Biotransformation of limonene to carvone by *Rhodococcus erythropolis* DCL14 [84].

The biotransformation of limonene to carvone is in line with the GCPs; (1) reduced toxicity as no toxic chemicals were used, making the reaction conditions safer compared to the synthetic procedure. Not only does the biotransformation reduce toxicity, but it (2) promotes energy efficiency by reducing the energy used for the reaction to 30°C, instead of temperatures as high as 155°C of the synthetic procedure. The reaction did not generate any side products because both isomers were converted to carvone, therefore this method also (3) minimizes waste.

1.5 Catalytic synthesis of Nootkatone

1.5.1 Chemical synthesis

Several approaches to produce (+)-nootkatone from the sesquiterpene valencene continue to be explored. This includes chemical synthesis and biocatalytic methods. Most chemical synthesis make use of hazardous oxidizing agents such as, *tert*-butyl peracetate [86] **(Scheme 3a)** and *tert*-butyl hydroperoxide [87] on supported silica metal catalysts [88]. The reaction first forms α- and β-acetate as major products, followed by the α- and β-nootkatol intermediates which both react with chromic acid to form nootkatone.

The disadvantages of the chemical synthesis are lengthy reaction procedures, environmentally hazardous reagents, tedious work-up, strict reaction conditions and an overall low yield make chemical synthesis not the most suitable approach [81]. Also, the resultant (+)-nootkatone does not satisfy the requirements in order for the chemically produced compound to be marketed as “natural” [89]. Therefore, biocatalytic methods have been explored to overcome the chemical synthesis challenges and are more promising especially because of their safety towards humans and the environment. Nootkatone produced through these methods is also considered “natural” since all the starting materials are from natural sources.

1.5.2 Microbial transformations

The use of plant cell cultures and microorganisms as biocatalysts have extended to esterification, glycosylation, hydroxylation reactions, and oxidation-reduction of alcohols and ketones [90,91]. Recently, cultured cells of *Gynostemma pentaphyllum* were used to transform VL to NK and the highest conversion of NK was 72% yield according to GLC peak area after 20 days. The intermediates, α- nootkatol and β-nootkatol were obtained in yields of 11% and 5%, respectively [90].

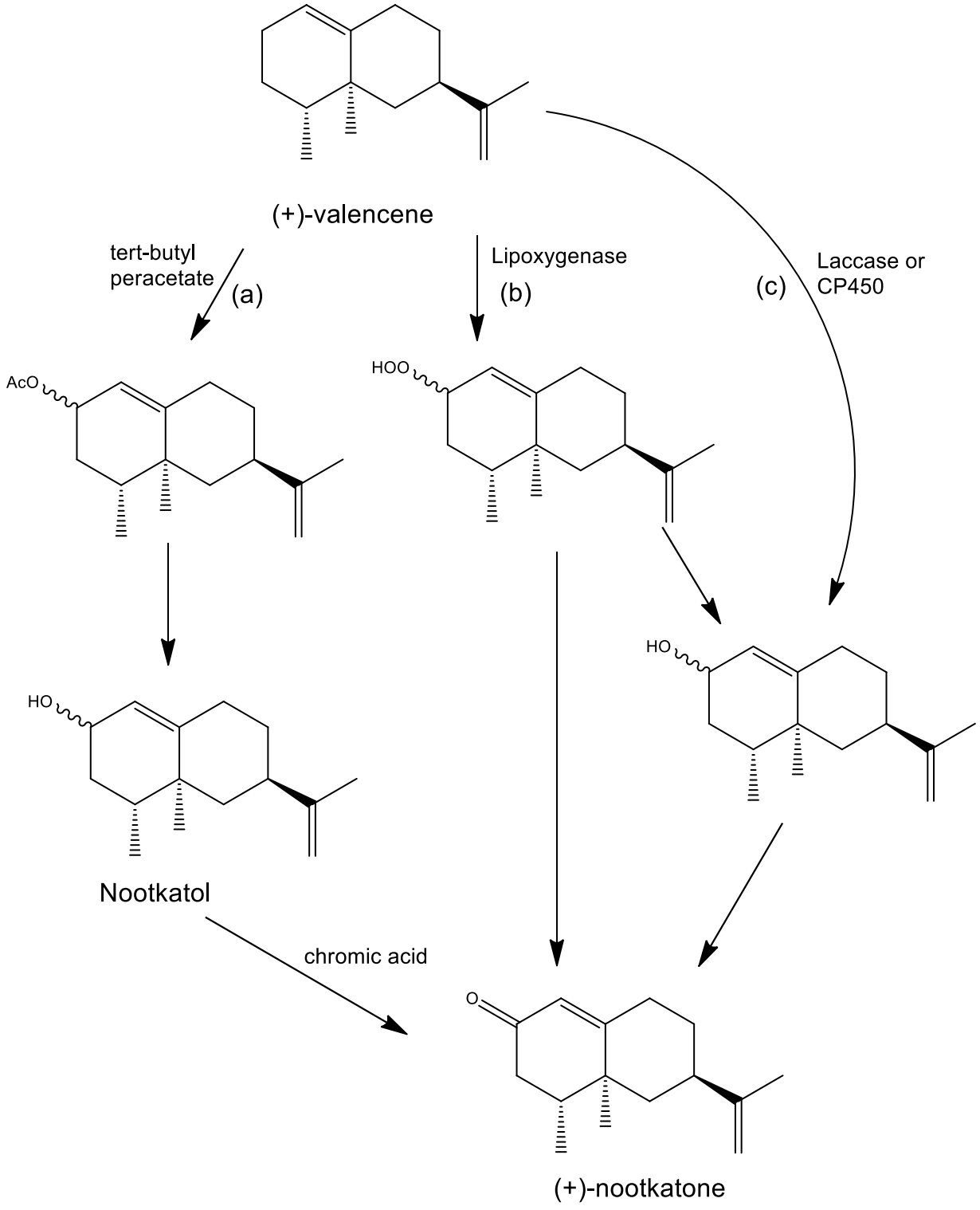
Green algae species, *Chlorella*, were used for the biotransformation of VL to NK. The algae was first inoculated and cultivated while stationary in Noro medium at pH

8.0, 25°C for 7 days. *Chlorella fusca* produced 89% (GC-MS peak area) NK after 18 days, while *C.pyrenoidosa* reached a high 93% NK after 20 days. There was formation of α/β-nootkatol but with time both were converted to NK. *C.vulgaris* produced 100% NK, the highest and best after 14 days [91,92].

1.5.3 Laccase

Laccase is a copper-containing polyphenol oxidase that catalyzes the oxidation of phenolic compounds and the reduction of one dioxygen molecule to two molecules of water [93]. Laccase is also an extracellular glycoprotein containing 4 atoms of copper, which are situated at the active site found at three different places of the enzyme [94]. These extracellular enzymes occur widely in fungi and less frequently in plants [95].

Huang *et al.* and his team invented a process for the conversion of VL to NK by laccase from *Botrytis cinerea*. The reaction formed nootkatol at 55°C and pH between 3-7, and within 24 h nootkatol degraded to NK **(Scheme 3c)**. Chemicals such as 1-hydroxybenzotriazole (HBT) were used as mediators which improved the yields to up to 59% NK GC peak area [96]. Another type of laccase from *Funalia trogii* was used for the conversion together with a dye decolourizing peroxidase (Ftr-DyP). The highest NK concentration reached was 1100 mg/L within 48 h, and the reactions were carried out at 40°C, pH 4.0 [97].



Scheme 3: (a) chemical synthesis of nootkatone, (b) and (c) the biotechnological conversion of (+)-VL to (+)-NK

1.5.4 Cytochrome P450 (CP450)

CP450 is a heme monooxygenase enzyme that uses molecular oxygen to oxidize, by inserting one of its atoms into a substrate and reduces the second oxygen to a water molecule. The enzyme consists of an iron (III) protoporphyrin IX covalently linked to a protein by a sulfur atom of a proximal cysteine ligand. These enzymes are effective oxidants that catalyze: (i) the hydroxylation of saturated carbon hydrogen bonds, (ii) the epoxidation of double bonds, (iii) the oxidation of heteroatoms and (iv) the oxidation of aromatic compounds [98].

In a two-enzyme reaction for the synthesis of NK, cytochrome P450 monooxygenase regioselectively catalysed the allylic C-2 hydroxylation of VL to yield α and β-nootkatol. The alcohols were further oxidized to nootkatone **(Scheme 3c)** by alcohol dehydrogenase (ADH) and NADH as the cofactor. The concentration of NK increased from 221 mg/L to 336 mg/L was caused by the addition of methyl-β-cyclodextrin. The reaction conditions were typically 30°C, with a pH of 7.5 and for a duration of 20-21 h [99].

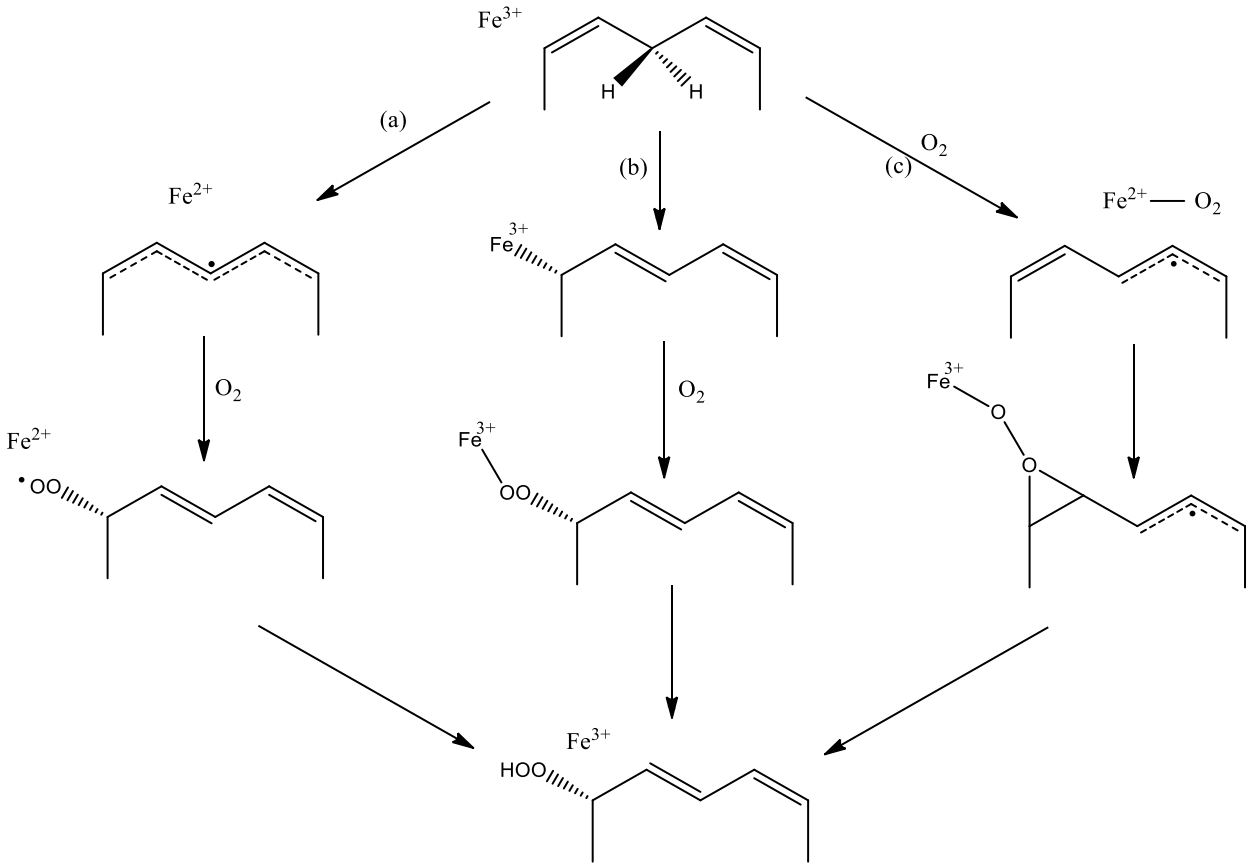
1.5.5 Lipoxygenase

Lipoxygenase (LOX) is a non-heme, iron-containing dioxygenase that catalyzes the regio- and enantio- selective dioxygenation of unsaturated fatty acids such as oleic, linoleic, and linolenic acid that contain one or more 1Z,4Z-pentadienoic moieties. These are most abundant in grain legume seeds (beans and peas) and potato tubers [100]. Plant LOX enzymes are multifunctional enzymes that catalyze reactions by: (i) deoxygenation of lipid substrates, (ii) secondary conversion of hydroperoxy lipids, and (iii) formation of epoxy leukotrienes [101].

LOX enzymes are not capable of direct oxidation of VL to NK. The LOX-catalyzed reaction involves the co-oxidation of fatty acids to hydroperoxides and the subsequent oxidation of VL to NK **(Scheme 3b)**. Side products such as α and β-

nootkatol are converted to NK [101,102]. There are three LOX catalytic mechanisms proposed in literature as shown in **Scheme 4**. The radical mechanism **(Scheme: 4a)** involves an abstraction of the hydrogen atom and the reduction of the active site iron. This is followed by the reaction of molecular oxygen with a pentadiene radical to form a peroxy radical. The peroxy radical is then reduced by the ferrous form of the active site resulting in the regeneration of the ferric active site and hydroperoxide formation [102,103].

The organo-iron mechanism **(Scheme: 4b)** suggests that there is a proton shift between the substrate to the proton accepting site of the iron, thereby forming a carbanion. The organo-iron then reacts with molecular oxygen to form a peroxy radical-iron site complex. The hydroperoxide is formed by reaction between the complex and acid [103,104]. The ene-radical mechanism **(Scheme: 4c)** assumes that the hydrogen atom abstraction by the ferric site results in the formation of a vinyl-allyl radical. The molecular oxygen first binds to the ferrous site, causing the oxygen to be activated. Subsequent attack of the vinyl radical produces an epoxy-like complex. This complex rearranges to form the hydroperoxide [103].



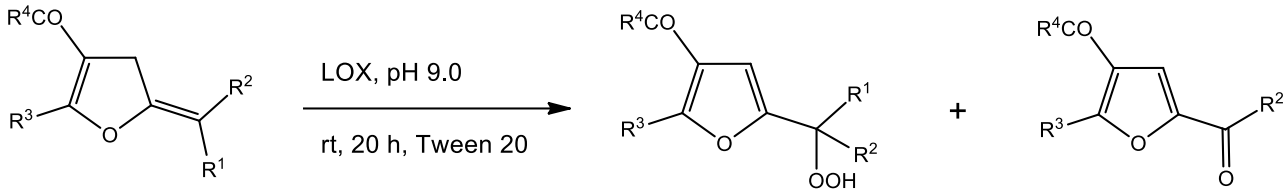
Scheme 4: Proposed LOX catalytic mechanisms (a) radical, (b) organo-iron and (c) ene-radical mechanism [103].

Muller and others (1995) used purified LOX-1 for the biocatalytic conversion of VL to NK [105]. The source of fatty acids was sunflower oil which contained 65% linoleic acid, under pure oxygen, pH 8.0 and temperatures as high as 60°C. The reaction pH was maintained by the constant addition of NaOH solution. A good concentration of 41.7 wt% NK and 9.9 wt% nootkatol was isolated within 24 h [105].

1.6 Non-conventional biocatalysis of LOX

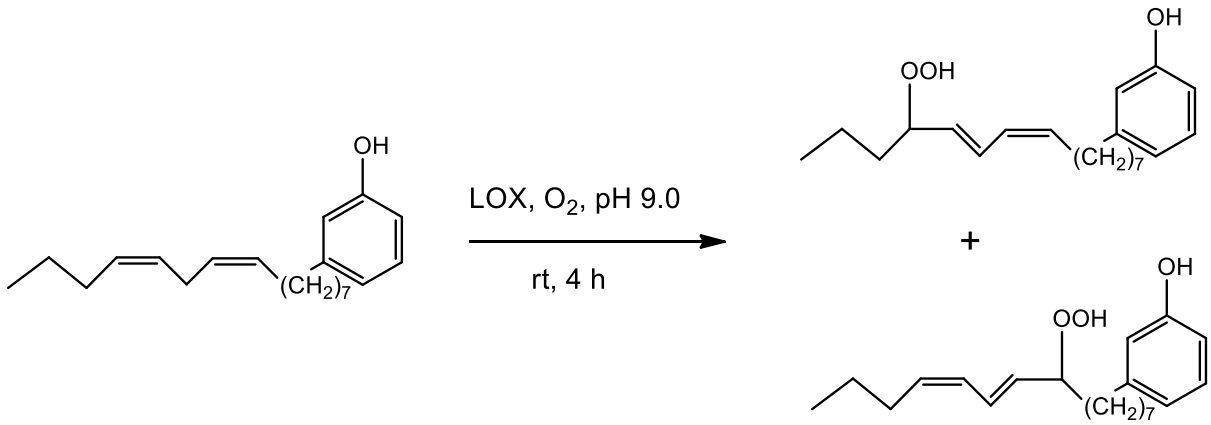
Scettri *et al.* (1997) reported on the conversion of 5-alkylidene-4,5-dihydrofurans to furylketone **(Scheme 5)** by LOX without the use of fatty acids [106]. The furylhydroperoxides were formed by using borate buffer at pH 9.0, under oxygen

atmosphere within 20 h. Tween-20 was added as a solubilizing agent, and it improved product yields significantly [106].



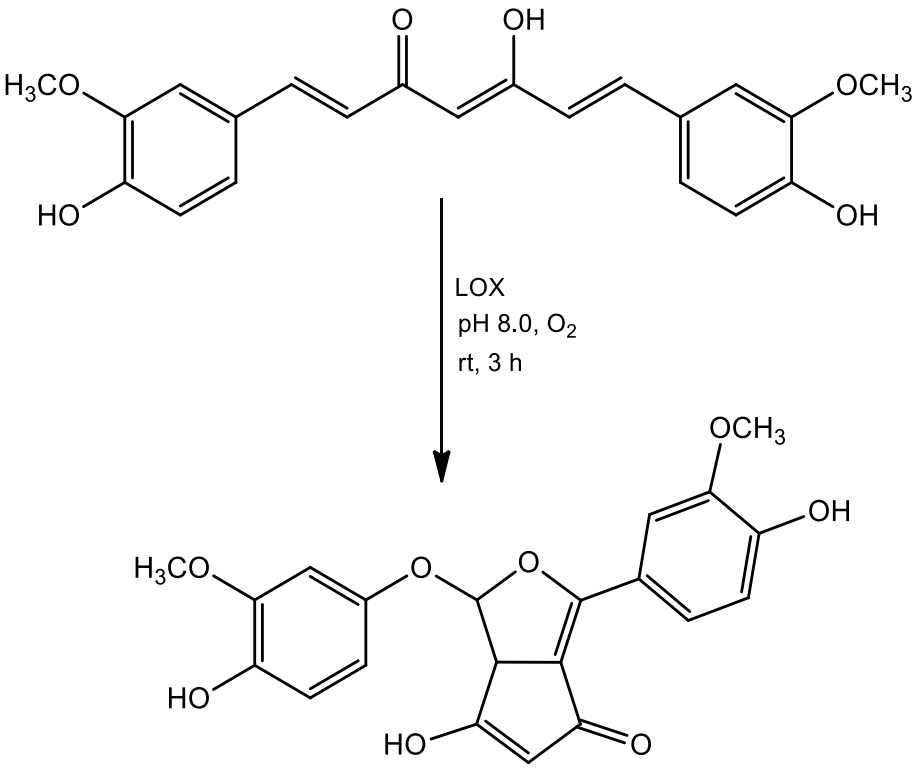
Scheme 5: Oxidation of 5-alkylidene-4,5-dihydrofurans by LOX [106].

Roth *et al.* (1998) performed a LOX dioxygenation of long chain alkadien(trien)ylphenols such as cardanol, cardol, and anacardic acids as well as acetylated and methylated derivatives [107]. **Scheme 6** shows how 3-[(Z,Z)-8',11'-pentadecadienyl]phenol was selectively dioxygenated by LOX to form the phenolic hydroxy products. LOX was in borate buffer (pH 9.0), and the reaction was under oxygen atmosphere for 4 h [107].



Scheme 6: Dioxygenation of 3-[(Z,Z)-8',11'-pentadecadienyl]phenol by LOX [107].

Schneider *et al*. (1998) also presented the oxygenation of curcumin to form a furanone and a five-membered ring product **(Scheme 7)** [108]. Further studies are required to understand the mechanism leading to this product.



Scheme 7: Oxygenation of curcumin by LOX [108].

1.7 LOX Purification & Assays

LOX from tomatoes [109], olive fruit [110], barley [111], avocado [112], canola seeds [113], peas [114], and banana leaf [115] has been reported but the most abundant and well-studied LOX is from soybeans [116]. It consists of four isozymes with different characteristics (**Table 1.3**). LOX-3, and LOX-4 have similarities and are therefore considered as one isozyme. All isozymes have the same molecular weight, but their optimum pH and pI are very different [117].

Table 1.3: Summary of the characteristics of the 4 isozymes of soybean LOX, adapted from Alturaihi et al. (2011) [117].

Enzyme characteristics	LOX-1	LOX-2	LOX-3
Molecular weight (Daltons)	98,000	98,000	98,000
pH optimum	9.00	6.5	4.5-9.0
pI	5.68	6.25	6.15
Co-oxidation activity	low	high	-

Plant enzymes can be extracted by green leaves, fruits, and other vegetable tissues [118]. The benefits of enzyme extraction are high degree of purity, high overall recovery of the enzyme activity and reproducibility. Extraction techniques enable the release of the wanted enzyme apart from the other enzymes, proteins, nucleic acids, and polysaccharides, which tends to increase the viscosity of the solution [119].

Several methods of purification and isolation of enzymes are employed [120], such as

(1) Molecular-weight based separation process – involves dialysis and ultrafiltration, gradient centrifugation of density, size exclusion chromatography.
(2) Separation process based on solubility differences – isoelectric precipitation, and solubilization or precipitation of proteins by salting in or out, respectively
(3) Separation processes based on electrical charge of the molecule
(4) Separation of proteins by selective adsorption
(5) Separation based on specificity of binders: affinity chromatography

To measure the activity of the LOX various methods have been employed by assessing; the adsorption of oxygen, the loss of the substrate, the formation of hydroperoxides and the formation of secondary oxidation products [121]. Techniques to determine the formation of hydroperoxides tend to be the mostly used because they are simple, easy fast and do not require expensive equipment.

The analysis of hydroperoxides can either be by chemical methods or physical methods. Chemical methods include the iodometric assays and the formation of iron complexes. Physical methods consist of the detection of conjugated dienes by UV or by the use of FTIR to identify an intense oxide peak produced by the reaction of hydroperoxides and triphenylphosphine [121].

Kuo *et al.* (2006) mentioned the purification of LOX from banana leaf by ammonium sulfate precipitation, hydroxyapatite column separation and gel filtration on Superdex 200 [115]. The activity of the enzyme was determined by the production of hydroperoxides based on their retention time and absorbances on the NP-HPLC. Chen and Whitaker (1986) reported on the use of DEAE-cellulose, Sepharose 6B-100 and polybuffer exchangers 94 chromatography for the purification of English peas [114]. The enzyme activity was determined spectrophotometrically by UV, for the identification of conjugated diene of the hydroperoxides.

Karout *et al.* (2007) recorded the use of ammonium sulfate precipitation for the purification of LOX from soybean seeds, succeeded by chromatography of CM C50 and DEAE Trisacryl [122]. The activity of the enzyme was also determined spectrophotometrically.

1.8 Immobilization

The use of enzymes in immobilized form has advantages such as enhanced stability, convenient handling, recovery, and reuse of the enzyme [123]. Most enzymes are very expensive and therefore, immobilization is also beneficial economically [124]. There are five main types of immobilization; adsorption, covalent binding, entrapment, encapsulation, and cross-linking.

Adsorption (non-covalent interactions) immobilization comprises the attachment of enzymes to a matrix through hydrogen bonding, van der Waals forces, or hydrophobic interactions [123]. Supports such as coconut fibres, microcrystalline

cellulose, Kaolin, and micro/mesoporous material have been used for various reactions. This type of immobilization is reversible due to nature of the forces which are affected by changing conditions (pH, temperature, ionic strength, and polarity of the solvent). It is one of the simplest methods of immobilization, mild, and preserves enzymes catalytic activity. However, it may fall short when the interactions are weak causing enzyme leakage from the matrix [123,125].

Covalent binding immobilization forms strong and stable bonds between the enzyme and matrix. This method is advantageous because the enzyme cannot be leached out of the matrix due to strong binding, is highly heat stable, and facilitates contact between the enzyme and substrate [123,125]. It has been employed where there are strict requirements for the absence of enzyme in the product. The main drawback is that both the matrix and the enzyme cannot be regenerated and that, due to limited mobility, there is decreased enzyme activity [124].

Entrapment immobilization is a method in which a polymeric network forms an occlusion of the enzyme, thereby allowing the substrate and products to pass through with the retention of the enzyme. One of the best reported materials, chitosan, is non-toxic, biocompatible, amenable to chemical modification, high affinity for proteins, and prevents friability [123,125]. The enzyme is often entrapped by using gels, micro-encapsulation, or fibre entrapping. The limitations of this method include mass transfer and enzyme leakage [126]. The encapsulation immobilization is actually the entrapment of the enzyme into the polymeric matrix. Therefore, the type of support used is similar to the entrapment technique. The benefits of this method are transportation of smaller sized compounds, continuous operation due to maintained cell density, and promotes cell separation and simplified downstream processes [124].

Cross-linking immobilization is a combination of covalent binding and entrapment techniques. Glutaraldehyde and bisisodiacetamide are used as cross-linking agents to form aggregates or crystals that are carrier-less macroparticles [124]. The advantages of this method are high enzyme load, strong biocatalyst binding and stability, prevents leakage and decreases desorption. The limitations include low diffusion, alterations to active site and loss of enzyme activity. [127]

LOX was immobilized by encapsulation in a form of silica gels with tetramethoxysilane together with methyltrimethoxysilane [122]. The activity of the encapsulated LOX was about 30% lower than the free LOX. However, the enzyme was recyclable but with each cycle the activity decreased further. Hsu *et al*. (1997) reported on LOX entrapped in alginate-silicate sol-gel matrix [128], and also the activity of the entrapped LOX was much lower than the free enzyme, even with efforts to restore the activity by addition of buffer with glycerol or organic solvent. The entrapped LOX showed activity after five cycles and was retained at ambient temperature for at least 25 days.

LOX was also immobilized by covalent binding using carbonyldi-imidazole activated support. The bound LOX was active even after seven cycles, but the stronger the binding the lower was the activity of LOX. Mitsuda and his team (1967) reported on the immobilization of LOX by natural polymer, cellulose, which was employed by entrapment technique [129]. This is a very inexpensive and versatile support.

1.9 Nootkatone Identification & Separation Methods

1.9.1 Chromatography

Chromatography is a method for separation, identification, and determination of compounds in complex mixtures. All types of chromatography have a stationary and a mobile phase, where the stationary phase is fixed, and the mobile phase moves through the stationary phase with the mixture to be separated. A stationary phase can be a solid phase packed column or a planar surface, and the mobile phase is usually a gas, liquid, or supercritical fluid. The separations are based on the differences in the migration rates of each compound in the mobile phase [130].

1.9.1.1 Column Chromatography

In column chromatography the sample mixture is dissolved in the mobile phase (liquid) and each compound moves according to their affinities for the stationary phase [130]. The elution occurs by pushing the sample through the column under gravity, by continuously adding fresh mobile phase **(Figure 4A)**.

1.9.1.2 Thin-layer Chromatography (TLC)

A TLC plate acts as a stationary phase while the solvent is the mobile phase. The detection of compounds of interest is by colour **(Figure 4B)** or by visualization of UV-active compounds under a UV light. Each compound is then identified by the comparison of the Rf (retention factor) values of the reference standards. Rf values are not reproduceable between runs or labs, due to various factors such as direction, volume, and composition of the mobile phase. The Rf value is calculated as the distance moved by the compound (A/ref) divided by distance moved by the solvent (Zf) [131].

$$Rf = \frac{distance\ travelled\ by\ compound}{distance\ travelled\ by\ mobile\ phase\ front}$$

This is the simplest method of detection that is also inexpensive, has good resolution and is fast. However, the disadvantages are detection inconsistency, low reproducibility, and lack of precision [132].

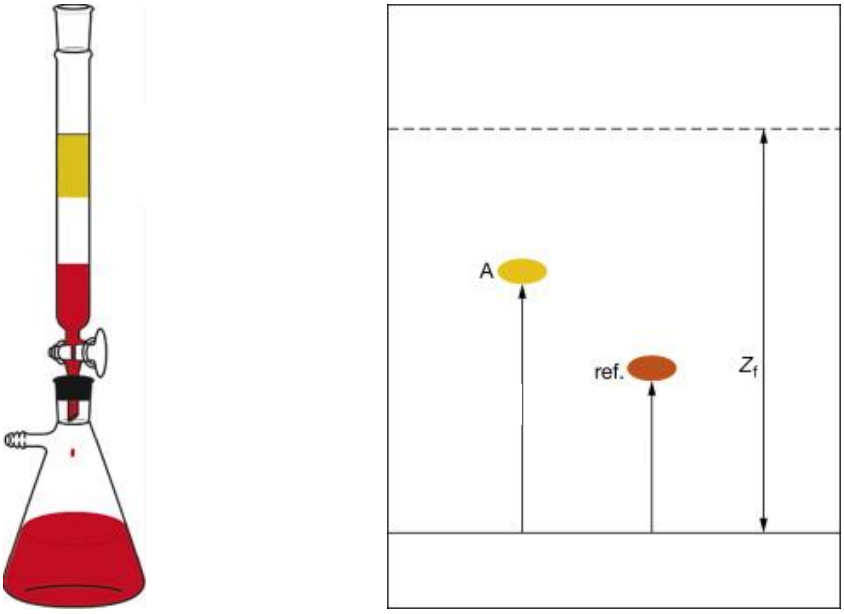


Figure 4: (A) Column chromatography and (B) Thin-Layer Chromatography, extracted from Sherma et al. (2003) [131]

1.9.1.3 Gas Chromatography

Gas chromatography is an efficient analytical technique for the separation and characterization of volatile mixtures [133]. It consists of high resolving power and detectors such as FID (Flame Ionization Detector) and MS (Mass spectrometry). The packed or capillary columns are the stationary phase, while the mobile phase is a carrier gas (helium, hydrogen, nitrogen). The identification of compounds is based on direct comparison of the retention times of the reference standards or from trusted libraries, e.g. the NIST mass spectral library.

GC is best known for high reproducibility, precision, accuracy, and sensitivity (ppm/ppb). GC also allows for fast analysis, and minimum thermal and catalytic decomposition of sensitive sample components [134]. The drawback of this technique is its limited application to volatile compounds, and it is not suitable for thermally liable samples, and it requires spectroscopy for peak identification [135].

1.9.2 Spectroscopy & Spectrometry

1.9.2.1 Ultraviolet-visible (UV-VIS) spectroscopy

It is defined by electromagnetic radiation that is represented by a wave with properties of frequency, wavelength, velocity, and aptitude [136]. This kind of spectroscopy is frequently used in enzymatic reactions to monitor product formation and to measure reaction rate. The absorption of light occurs in near-UV (180-390 nm) or visible (390-780 nm) radiation conditions by compounds in solution [137]. UV-VIS detection sometimes suffers from a lack of sensitivity and interferences during analysis [138].

1.9.2.2 Nuclear Magnetic Resonance (NMR) spectroscopy

NMR is a powerful tool utilized for the determination of organic structures, that is based on the interaction of magnetic moments of nuclei of various atoms with magnetic fields. The nuclei of ^{1}H, ^{13}C, ^{15}N, ^{19}F and ^{31}P contain an odd number of protons or neutrons and can therefore be observed by NMR [139]. The strength of the magnet that controls the precision of the magnetic fields is graded according to the frequency of the 1H NMR signals, such as 600, 500 or 400 MHz [140]. The disadvantage of this method is low sensitivity and resonance overlap caused by background compounds present in the sample [141].

1.9.2.3 Mass Spectrometry

MS is an analytical technique where the gaseous sample molecule is ionized, separated, and detected as ions according to the mass-to-charge ratio (*m/z*) [130]. The resultant mass spectral information of each separated compound is compared to reference standards. It is one of the best techniques in organic chemistry because of its high selectivity, analytical reproducibility, and simple sample preparation [142].

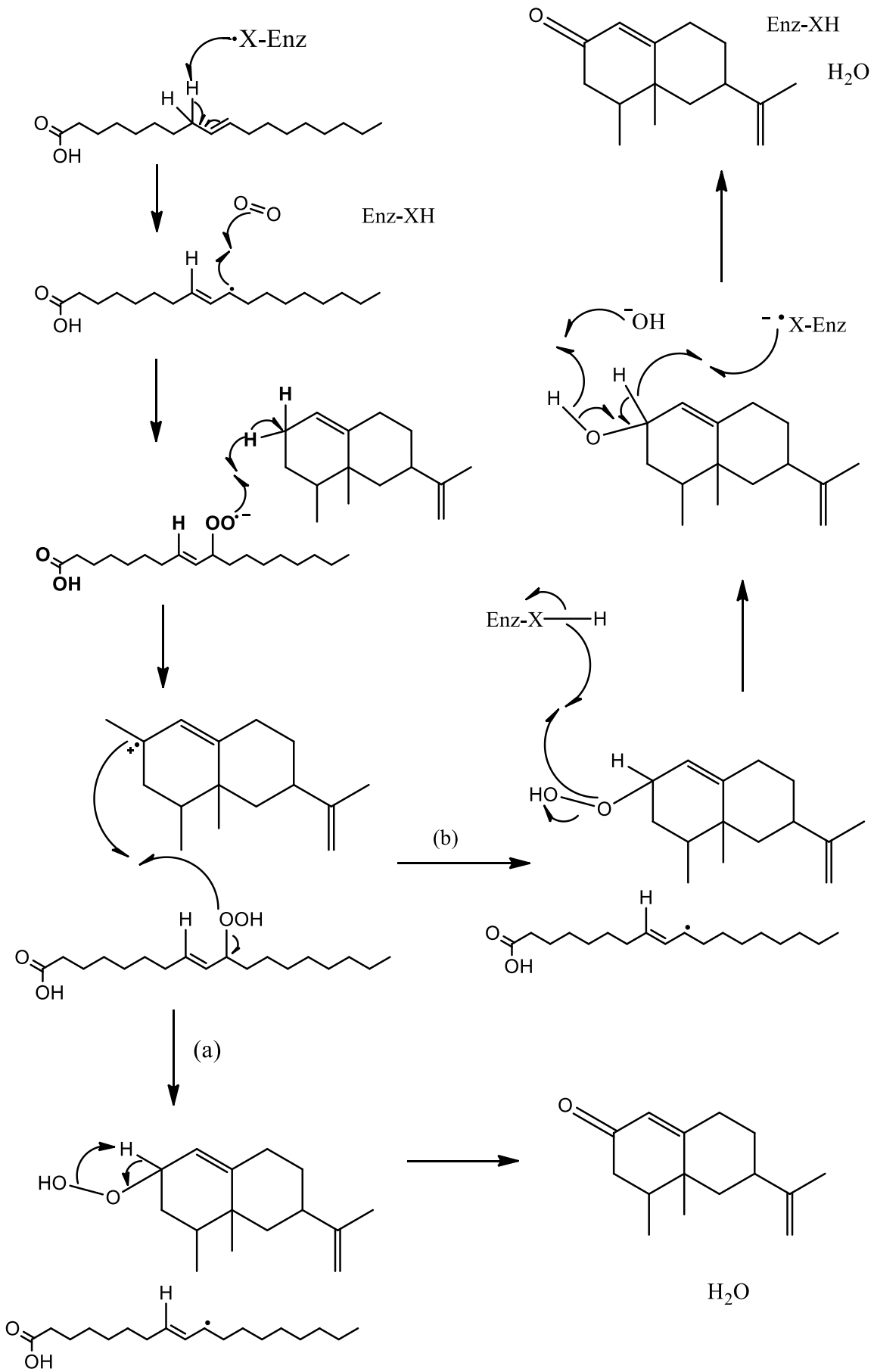
1.10 Aims and objectives

The aim of this project is to develop oxidative biocatalysts for the conversion of terpenes to value added products for commercial applications. Specific objectives include:

- Successfully extract lipoxygenase (LOX) enzyme from soybeans
- Determine optimum enzyme conditions for high enzyme activity
- Apply the enzyme in synthesis of commercially relevant compounds, however due to time constrains caused by the pandemic only one compound was synthesized.
- Identify and characterize the product using: GC-MS, UV-VIS, NMR, with careful evaluation of any impurities.
- Optimize the process steps to provide material of which the chemical and odour properties are fit for use in commercial products.
- Compare processes using crude LOX enzyme, partially purified LOX enzyme, and immobilised LOX enzyme.

1.11 Proposed mechanism

This project will focus on the biocatalytic conversion of (+)-valencene (VL) to (+)-nootkatone (NK), a highly sought-after grapefruit aroma. The proposed mechanism of this reaction **(Scheme 6)** involves the LOX enzyme that reacts with OA to form an unstable hydroperoxide intermediate. The hydroperoxide then oxidizes the VL at different positions, at C2 is the major product, NK **(Scheme 8a)** or forms NK via the formation of nootkatol **(Scheme 8b)**.



Scheme 8: Oleic acid is oxidized through the radical mechanism and (a) directly forms NK or (b) first forms nootkatol, which is then converted to NK.

CHAPTER 2

RESULTS AND DISCUSSION

In this chapter the aims and objectives of the project will be elaborated in detail through seven sections. The first section, **2.1**, focuses on the extraction of the crude lipoxygenase (LOX) enzyme from soya beans. In **2.2**, the enzyme activity of the Crude LOX was studied, while in **2.3** the Crude LOX was utilised in biocatalytic reactions was described. The intention of this project was to use the LOX enzyme to oxidize various terpenes that are commercially available, however, due to time constrains only focus on one compound (the conversion of VL to NK).

Then section **2.4** focuses on the reaction work-up optimization, followed by **2.5** the optimization of the biocatalytic reaction. In **2.6** a sample of NK was purified and characterized. Section **2.7** details the immobilization of the enzyme and describes the application of the immobilized enzyme in a biocatalytic reaction.

2.1 Crude LOX Enzyme Extraction

The preparation of the soya beans began with crushing because they are naturally hard and this is beneficial for the seeds as it provides a resistance against seed coat pathogens, protection from spoilage and helps delay seed germination [143]. A mortar and pestle were used to break the beans down into a coarse meal **(Figure 5A)**. However, this method was very labour intensive and time consuming.

Therefore, a coffee grinder was introduced as a tool to grind the beans with ease, fast and with no physical labour required. Unlike the coarse meal, the coffee grinder broke the seeds into a fine flour **(Figure 5B&C)**. Once the beans were crushed, they were suspended in a buffer and left overnight at 4°C.

Soybeans are the richest source of LOX enzyme [144] and unsaturated fatty acids [145]. The bean oil is the most widely harvested oil in the world for human consumption, animal feeding and biodiesel production. When beans are broken down completely, the cell walls are ruptured. This then improves the oil and enzyme extraction that can be washed from the cells and recovered as an emulsified cream or free oil [146]. Therefore, the more emulsified the supernatant the more enzyme was extracted.

As shown in **Figure 5:A** coarse meal in sodium borate buffer (pH 8.5, 100 mM) and **C** fine flour in Tris buffer (pH 7.1, 100 mM) was evaluated, the resulting supernatants were clear with the enzyme in free oil. However, supernatant **B** of fine flour in sodium borate buffer (pH 8.5, 100 mM) was viscous showing that the recovered enzyme was in an emulsified cream. A clear supernatant such as **A** or **C** was the most preferred because a viscous supernatant as **B** was very difficult to work with in reactions that present a major challenge in separating the product from the enzyme.

The separation of the supernatant from the soybean flour was difficult for **B** but easier for **C**. At first, a Buchner funnel with filter paper (Whatman filter paper – 110 mm circles, Merck), was tested but was unsuccessful. Then, the filter paper was replaced with a filter pad of cotton wool, but it was not satisfactory. This was because in both methods of filtration procedure was blocked by the emulsified cream.

A cheese cloth was finally introduced, which proved to be the best method. It was easy, did not require the Buchner funnel and there were no blockages as experienced in the previous methods. The filtered supernatant was stored at -20°C and used in subsequent biocatalysis reactions as the crude LOX enzyme.

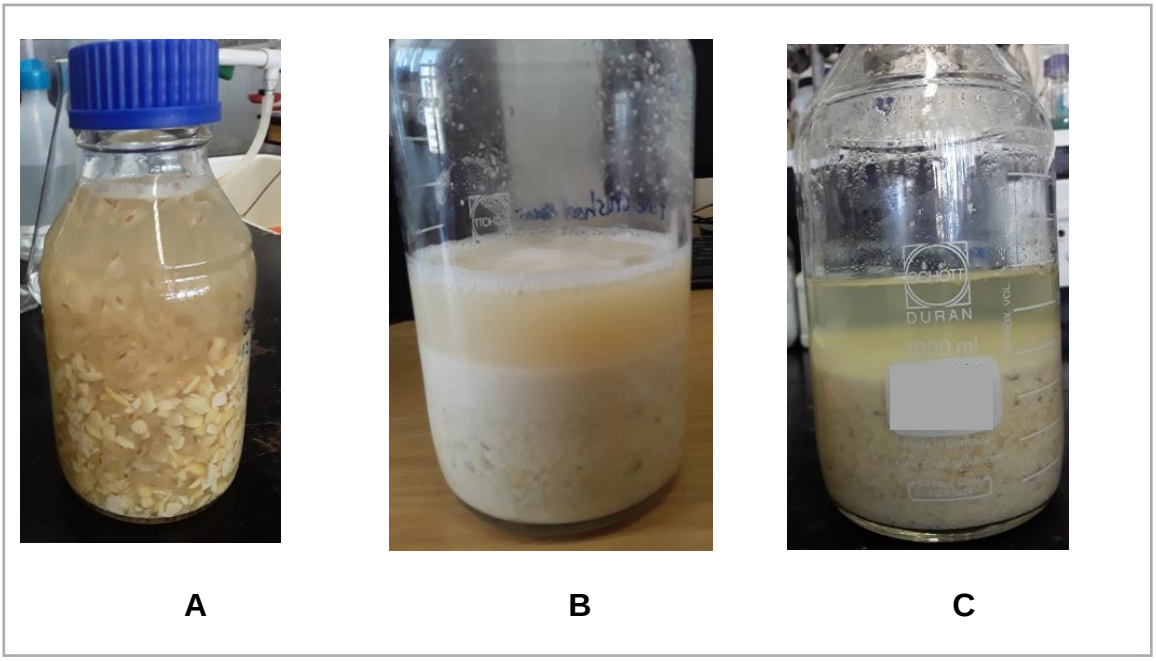


Figure 5: Photographs of soybeans crushed to a coarse meal using a mortar and pestle **A** and ground into flour by a coffee grinder that were extracted in sodium borate buffer **B** and Tris buffer **C** were left overnight at 4°C to form a LOX-active supernatant.

The use of cheese cloth to separate the extracted LOX from the soybeans was used throughout the project because of its easy, effective and industry adoptable traits. Once the most efficient method of extraction was established, we needed to study the activity of the enzyme in each extract. Therefore, in the next section assays that determine the formation of hydroperoxides were explored because of their simplicity and short result turn-around time. Subsequently, the best assay technique will be used to determine the optimum conditions of the enzyme by varying the buffer type, molarity, and pH of the buffer.

2.2 Analysis of hydroperoxides formation

An enzyme assay was important as a guide to achieve the best conditions for the highest activity of the LOX enzyme. Therefore, the buffer's molarity and pH were varied while the reaction temperature remained constant. Since the assay extractions were from 2 g soybean flour, in order to extract the enzyme by the

quickest way, it was placed on the shaker for 30 min at room temperature. Then the flour was allowed to settle at 4°C for 30 min.

According to literature, the 3 main soybean LOX isozymes are found at a pH range of 6.8-9.0 [100]. Therefore, for the activity assay the crude LOX was extracted with different buffers within the pH range to determine the best buffer for the extraction of LOX. The three buffers were sodium borate buffer (pH 6.7, 7.6, 8.1), Tris buffer (pH 7.0, 7.5, 8.1) and phosphate buffer (pH 6.7, 7.7, 8.3). The buffer molarity was 10, 50, 100, 150 and 200 mM at each pH.

The extraction of crude LOX from plant material has been done in various buffers at different molarity and pH. The phosphate buffer was used to extract LOX from poppy seeds (100 mM, pH 6.5) [147], avocado (20 mM, pH 7.2) [112], green peas (2.5 mM, pH 6.0) [114], soybeans (200 mM, 9.0) [148].

2.2.1 Substrate solution

Before each assay, a substrate solution was prepared to form hydroperoxides which gave an indication of the enzyme activity. In the assays used, the hydroperoxide presence changed the colour of the dye used. Thus, spectrophotometrically, the enzyme activity was determined based on the intensity of the colour change. The five samples of extracted crude LOX were used as biocatalysts in the presence of OA, iron sulphate, and manganese chloride at 37°C. After 30 min, the substrate solution (**Figure 6)** was allowed to cool and was used immediately due to the instability of the hydroperoxides [121].

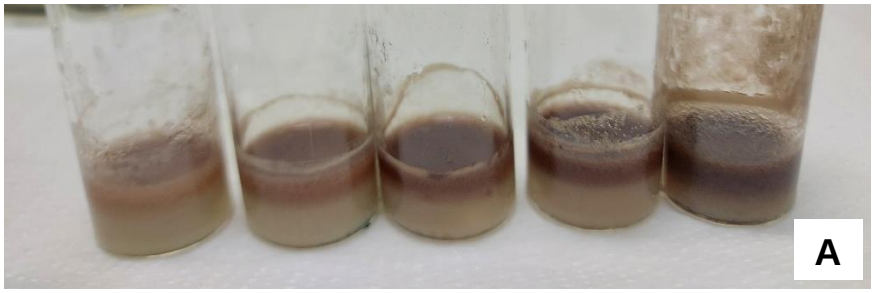




Figure 6: The substrate solution of sodium borate buffer at pH 8.1 **(A)** and phosphate buffer at pH 8.3 **(B)** at 10, 50, 100, 150, 200 mM.

Soyabeans are packed with isoflavones such as genistein, daidzein and glycitein [149]. Hence, these phenolic compounds may account for the 'darker' colour of the supernatants with the higher molarities. It is assumed that, as the buffer molarity increases, so do the phenolic compounds extracted into the sodium borate and Tris buffer. However, this was not the case for the phosphate buffer as there was no colour change at all molarities.

2.2.2. Ferrous Oxidation – Xylenol Orange (FOX) assay

The FOX assay was used to measure the presence of hydroperoxides formed in the substrate solution. When Ferrous (Fe^{2+}) from lipoxygenase is oxidized to Ferric (Fe^{3+}) ions by the hydroperoxides, the Fe^{3+} ion binds to the ferric-sensitive dye xylenol orange forming a Ferrous Oxidation-Xylenol Orange (FOX) complex [150]. The xylenol orange dye is brown and when the complex is formed it turns purple. The amount of hydroperoxides produced by each sample was directly proportional to the activity of the enzyme.

The formation of the FOX coloured complex required the substrate solution to be purified first. Therefore, brine (100 g NaCl in 200 mL water), 1M NaOH with EtOAc as an extraction solvent were used, and upon concentration a clear oil was isolated. The required amount of sample was diluted with MeOH to make 100 µL and the other reagents for the FOX assay were also prepared as described by Parra-Diaz (1993) [150]. The FOX reagent was reported to be very unstable, therefore a new solution had to be prepared daily. All the reagents were added and after 30 min, the

colour changed from orange (dark brown) to purple [150], **Figure 7**. This purple colour confirmed that the complex formed successfully [150].

The FOX-complex samples were analysed spectrophotometrically at 596 nm on the Agilent Cary 100 UV-VIS spectrophotometer, and samples were ran in triplicates. This was within the reported range of 540-600 nm, but the measured absorbance dropped drastically within 5 min making the results very inconsistent. The reasons may be continued oxidation of the sample even though an antioxidant, BHT, was added [149].

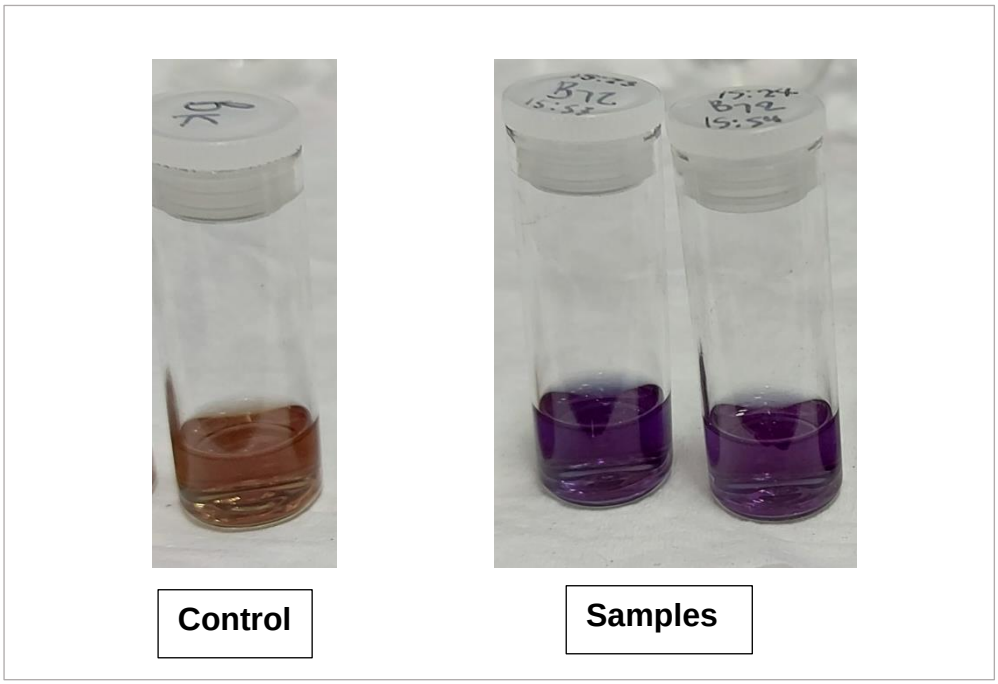


Figure 7: The FOX reagent blank that was brown (Left) and FOX sample (Right) after 30 min turned purple.

Being a green chemistry project with the intentions to use chemicals that are non-hazardous and are in line with the GCPs, the FOX assay was inappropriate as it used harmful chemicals such as sulfuric acid and BHT [151], it also required daily preparation which created waste if not used up. Therefore, an alternative assay was explored that was not harmful to humans or the environment but effective, and this turned out to be the methylene blue assay. Interestingly, methylene blue itself has several biological benefits [152,153].

2.2.3 Methylene blue (MB) assay

For the MB assay, the hydroperoxides formed by the OA and crude LOX enzyme reduced the MB by converting it to MB-H, and in this way bleaching it from blue to colourless. A calibration curve was prepared with 100 µM stock solution of MB and diluted to the 0.5-5 µM concentrations. The R^2 value was 0.99789 and utilised in studying the activity of the crude LOX enzyme in the three buffers at different pH values and molarities.

These MB samples were prepared by modifying the method described by Suda *et al*. (1995) [148]. Before the initiation of each MB sample, an aliquot of 100 µM MB was diluted down to a concentration of 5 µM. Then to initiate the redox reaction 10 µL of the substrate solution was added with no need for work-up, then allowed to bleach for 30 min before taking a reading at 664 nm on the Agilent Cary 100 UV-VIS spectrophotometer. The sample was not completely colourless **(Figure 8)** after 30 min. However, it turned into a lighter blue which with time became colourless and this took hours.

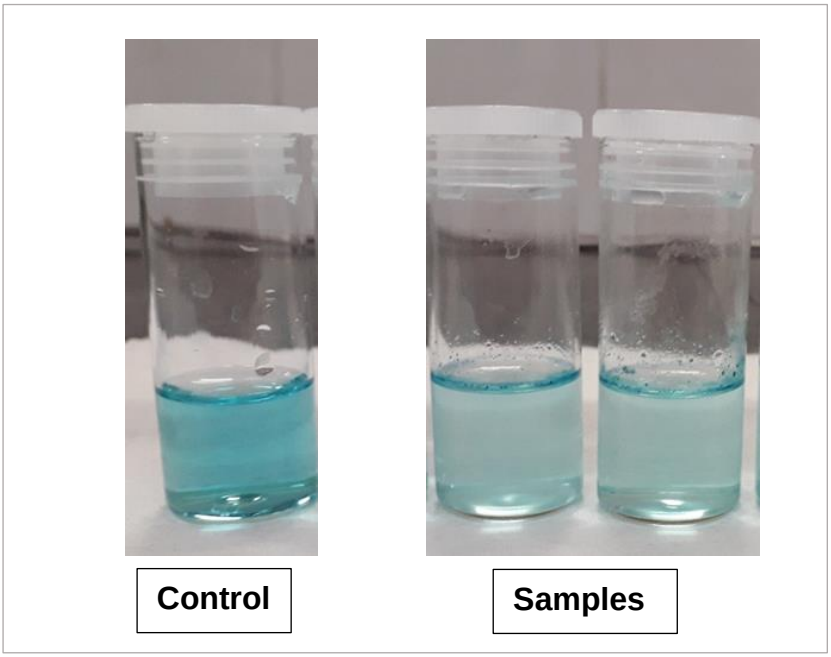


Figure 8: The MB assay bleaching, (Left) shows the control and (Right) the bleached sample.

The graph in **Figure 9** represents the concentrations of the bleached MB from all the MB samples of sodium borate, Tris, and phosphate buffer at pH range of 6.5-8.3 and molarity range of 10-200 mM. The highest concentration in each sample was 5 µM, which was expected to bleach to as low as 0.5 µM.

The Tris buffer showed the highest concentration of bleached MB at 2 µM between pH 7.0 (10, 50, 100 mM) and 7.5 (150 mM) which is 40% of the initial MB concentration. The maximum at pH 8.1 was 1.6 µM, however the lowest concentration for the Tris buffer was pH 7.5 (10 & 50 mM) with a very low 0.6 and 0.4 µM, respectively. The phosphate buffer showed the lowest enzyme activity amongst the three buffers. The maximum concentration was below 1.5 µM at pH 6.7 (10 & 50 mM), and at pH 7.7 (50 & 100 mM) there was no activity at all.

On the other hand, the borate buffer showed the best activity amongst the three buffers with the highest concentration of 3.3 µM at pH 8.1 (150 mM). This was followed by 2.9 µM at 10 mM at the same pH. However, at pH 6.5 the concentration decreased to less than 1.5 µM, thus not the most suitable for the borate buffer. These assays were a great success because most assays published on LOX assays use linoleic or linolenic acid [148] as their source of fatty acids. OA has the lowest rate of oxidation because the rate increases by the degree of unsaturation in the fatty acid molecule [154].

Therefore, the best buffer to extract crude soybean LOX was the borate buffer at pH 8.1 (10 & 150 mM). Followed by Tris buffer at pH 7.0 (50 & 100 mM) and lastly the phosphate buffer at pH 6.5 (10 & 50 mM). To extract LOX at pH lower 7.5 and still retain the activity of the enzyme, Tris buffer was the best option. However, at a higher pH of 8.1 the borate buffer showed very high activity. At the highest molarity of 200 mM, the borate buffer showed the highest activity of 2 and 2.3 µM at pH 7.6 and 8.1, respectively.

The substrate solution of both the Tris and borate buffer became dark in colour with increasing molarity **(Figure 6A)**. This was accounted for by phenolic compounds which may act as co-enzymes assisting in the catalytic action of the LOX enzyme found in soybeans. In all phosphate buffer substrate solutions **(Figure 6B)**, there

was no change in colour from white to brown. The lack of extracted phenolic compounds may have affected the activity of the enzyme in the phosphate buffer. These effects however depend on the physicochemical state of the substrate and the type of LOX used [149]. Therefore, the colour of the substrate solution changing from white to brown does determine the activity of the enzyme.

The MB assay was the best technique because it did not require hazardous chemicals as the FOX assay. The best conditions for each buffer were: sodium borate buffer, pH 8.1, 150 mM; phosphate buffer, pH 6.7, 50 mM; Tris buffer, pH 7.0, 50 & 100 mM. It was also a very fast, easy and effective method.

The development of this assay took several months to master, therefore the reactions in section **2.3** were used as a form of investigation. To best gather information about the most suitable conditions for optimum product synthesis. This was very difficult because the reactions were ran for more than 24 h, while a functional enzyme assay took less than 2 h to get results from. Therefore, the results from the MB assay were applied in the purification and immobilization of the enzyme in section **2.7**.

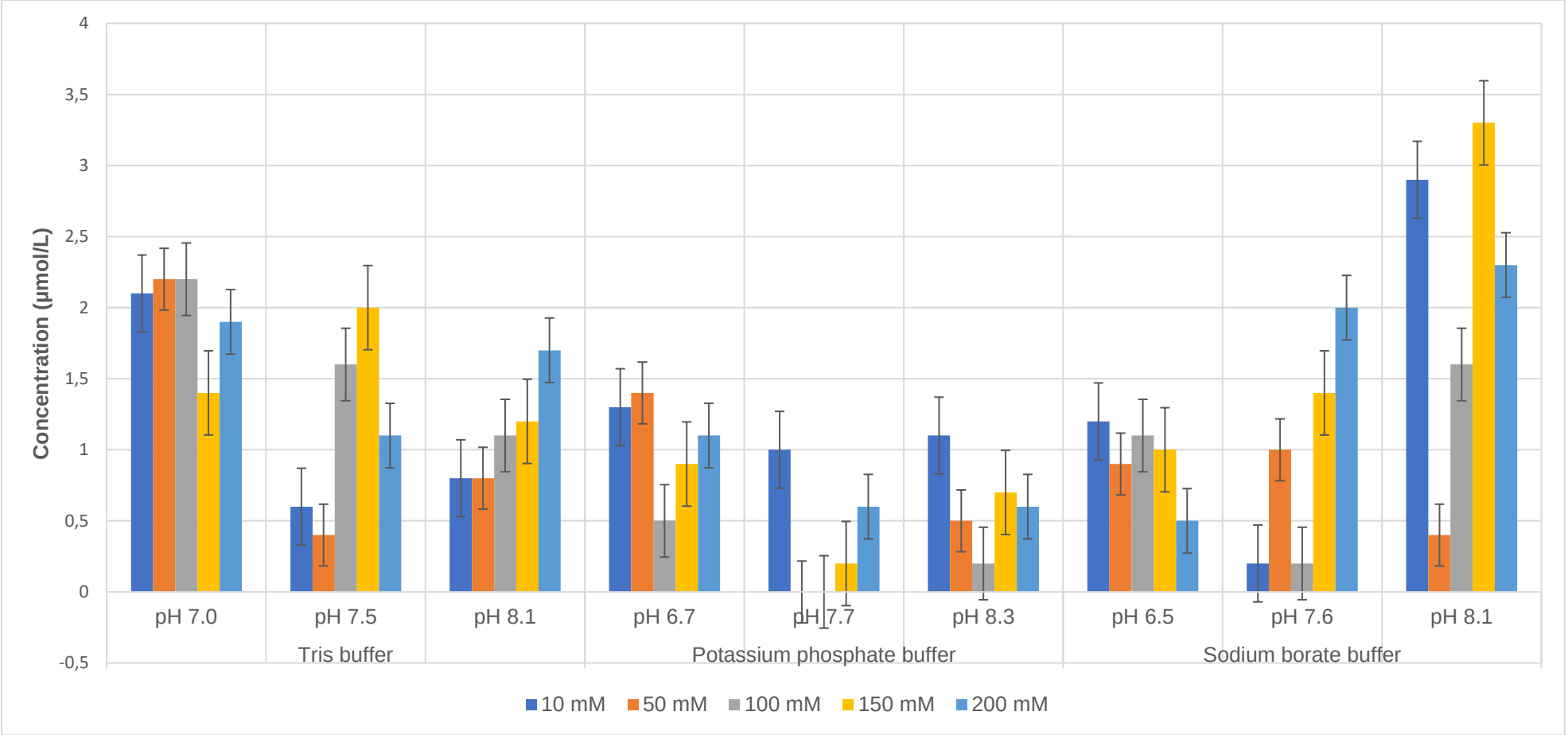


Figure 9: The concentration of bleached MB by crude LOX from the Tris (pH 7.0, 7.5, 7.0), phosphate buffer (pH 6.7, 7.7, 8.3) and borate buffer (pH 6.5, 7.6, 8.1) by extraction at the molarities of 10, 50, 100, 150, 200 mM. The error bars represent mean ± SD of three replicate samples (3n).

2.3 Crude LOX Biocatalytic Reactions

In this section, the biocatalytic application of the extracted crude LOX to from NK from VL through various reactions is described. The VL to NK reaction was done as another form of investigation because the MB assay in **2.2.3** was only completed towards the end of the research project. Therefore, these reactions were important to give us a better understanding of the activity of the enzyme and used the best conditions in the optimization of the VL to NK conversion in **2.5**.

The VL used throughout this project was from Evolva and its purity was >65%v/v. When the VL was analysed by GC-MS, the only impurity that was detected was aristolochene, although by column chromatography there were other impurities detected. For this research, the only focus was on the conversion of VL to NK.

2.3.1 The Biphasic reaction

The first reaction conducted was previously developed from our laboratory (unpublished) that was a biphasic reaction with hexane and the crude LOX enzyme in sodium borate buffer (100 mM, pH 8.5). VL (112.55 mmol), OA (39.61 mmol), $FeSO_4.7H_2O$ (0.899 mmol) and $MnCl_2.4H_2O$ (1.12 mmol) were also added to the reaction. It was maintained at constant temperature of 35°C, air flow and stopped after 7 days. The pH of the reaction was not monitored due to limited equipment.

On each day of the reaction, a sample was directly dissolved in HPLC grade methanol and dried with $MgSO_4$ before injection into the Agilent 7890 GC coupled with Time-of-Flight (TOF) MS for analysis. The concentration of each sample was not determined before analysis, therefore the results in **Table 2.1** seem as though VL amount decreases and then increases again. However, this was not the case because the GC peak area of each analyte reported was in relation to the area and intensity in every chromatogram. Therefore, before absolute quantification was done, our main interest was in relative quantification, thus checking how much of the

reacted VL formed NK, or any NK intermediates, by quoting relative % of total peak area during the GC run.

On the first day of the reaction, NK was only 4% **(Table 2.1)**, β-nootkatol 1.0%, α-nootkatol 4.6%. The biocatalytic conversion of VL to NK proceeds indirectly **(Scheme 3)** and *via* a regioselective hydroxylation of VL at C2, thus producing α- and β-nootkatol that are oxidized to NK [155,156]. The NK conversion was very low, however there was anticipation that an increase with time can afford more of the desired products, but there was rather a decrease. By day 2, NK had dropped to 1.8% and β-nootkatol 0.4%, α-nootkatol to 0.9%

Table 2.1: The GC-MS peak area (%) of the biphasic reaction from Day 1-7

Compound	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Valencene (%)	45	78	78	40	57	75	84
Aristolochene (%)	40	17	12	18	30	24	15
Nootkatone (%)	4,1	1,8	3,1	19	2,5	0	0
β-Nootkatol (%)	1,0	0,4	0,8	5,4	0,9	0	0
α-Nootkatol (%)	4.6	0.9	2.5	9.4	3.4	0	0

The activity of the enzyme continued to decline, by Day 3, the peak area of NK was lower than on Day 1. Therefore, more enzyme was added to the reaction to increase the enzyme activity. The highest peak area of NK was 19%, β-nootkatol 5.4%, α-nootkatol 9.4% achieved after 4 days. These results showed that on days 1 and 4, when the enzyme was freshly added NK and nootkatol were at their highest. However, as time increased the NK and nootkatol decreased drastically.

On day 5, there was a drastic drop in NK area to 2.5%, β-nootkatol 0.9%, α-nootkatol 3.4% and completely disappeared on day 6 and 7. **Figure 10**, shows how there was NK and nootkatol on day 4 and by day 7, all the NK and nootkatol had disappeared. Therefore, these results show that NK can be synthesized in less than a week, and when the reaction carried on long enough NK reacted further to form products that were not detectable by GC-MS, i.e. decomposition was suspected to occur over time.

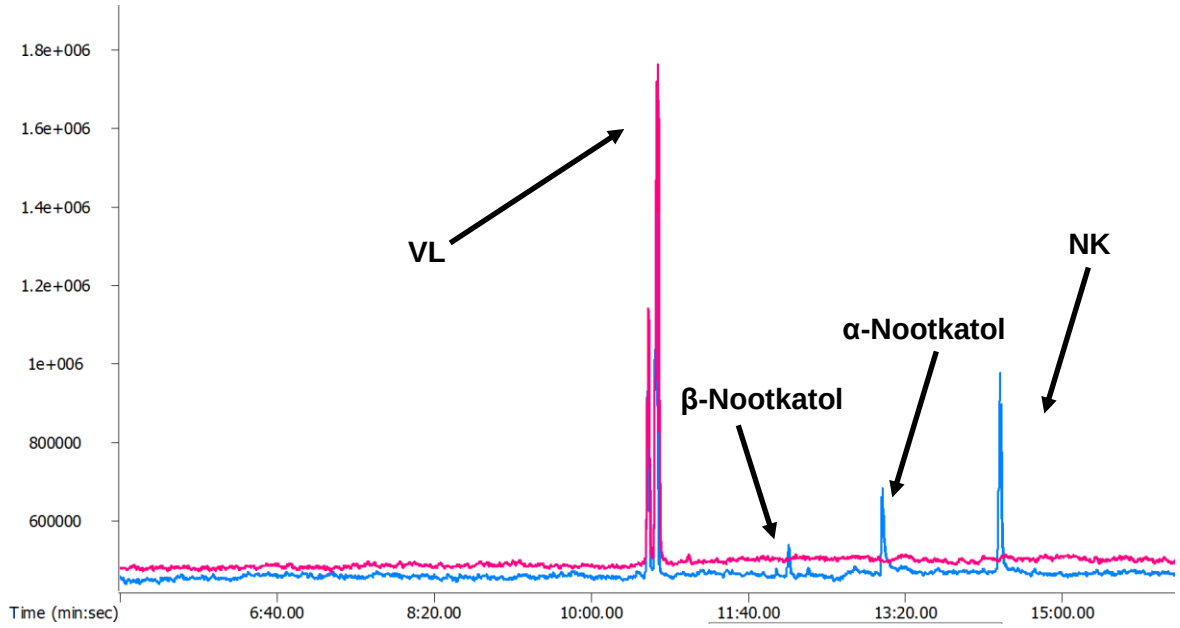


Figure 10: Comparison: GC chromatograms with peak area 39% VL and 19% NK on day 4 **(Blue)** with 84% VL and NK 0% on day 7 **(Magenta)**

Therefore, through this reaction these were the challenges encountered:

1. The reaction was carried out for a week and had less than 50% conversion.

2. The reaction became dry, as the buffer and hexane evaporated due to the air flow.

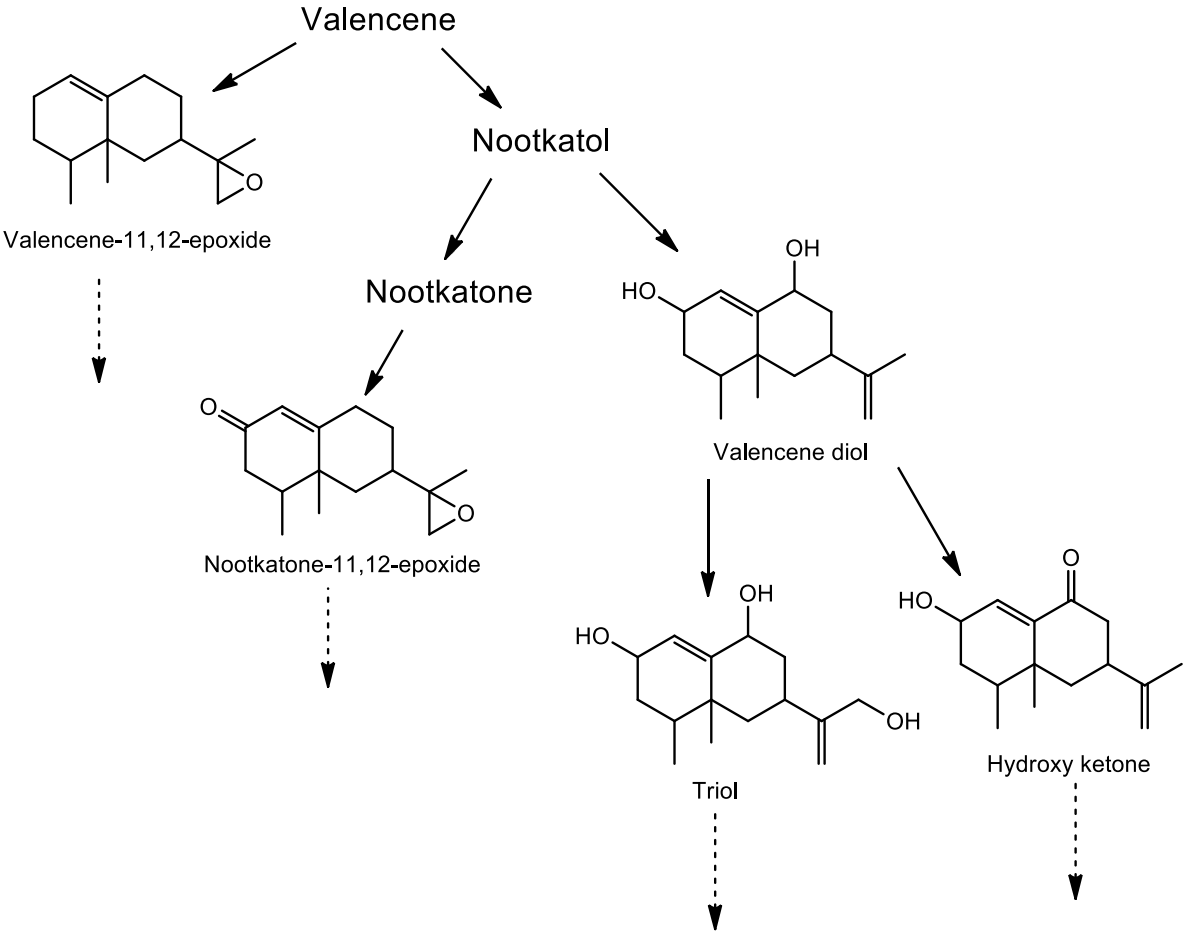
3. The enzyme became inactive after 3 days, and the existent NK over-oxidized to form other products that are not detected by the GC-MS.

4. The isolated product had an off-flavour or bad-odour which could be from soybean [157], OA, and other side products.

The reaction was modified by shortening the reaction time to 2 days with all the reaction conditions kept constant. The reaction work-up remained a nightmare and the recovered mass yield was 65% of a dark brown oil. Fortunately, the product formation improved because after 2 days the GC peak area of NK was 17%, β-nootkatol 5.8%, α-nootkatol 16.6% compared to 4% NK, β-nootkatol 1.0%, α-nootkatol 4.6% in the previous reaction. This was a significant improvement since the reaction time was reduced, however 17% was not good enough for reaction

optimization. The reaction work-up to isolate the product for all reactions was explained in detail in section **2.4**.

The biphasic reaction showed low conversion of NK. This was in line with the report by Cankar *et al*. (2004) that proved that the production of NK by biphasic system was not desirable [158], suggesting that VL and nootkatol may not be completely exposed to the enzyme for full oxidation. In contrast, Leonhardt and Berger (2014), reasoned that a better yield of NK was achieved in a biphasic system because the products dissolved in the organic phase and were protected from overoxidation [159] **(Scheme 9)**. These products are too polar to be used as flavour compounds and may have been produced by LOX in this study.



Scheme 9: Overoxidation of NK by CYP450 (2014) [159].

2.3.2 Singe-phase reaction

In light of the biphasic reaction not being suitable for NK production and also, seeking to create greener industrial processes, a single-phase reaction with no hexane was conducted. Besides, hexane has high selectivity for oils, limited energy cost and easy evaporation, and long-term exposure to hexane has negative implications on humans and the environment [160].

The reaction conditions were VL (56.28 mmol), OA (19.83 mmol), $FeSO_4.7H_2O$ (0.45 mmol) and $MnCl_2.4H_2O$ (0.56 mmol) with the duration of 2 days. The scale of the reaction was reduced by half to save reagents. Every sample taken was worked-up with 1M NaOH, brine and extracted with EtOAc, then upon drying an oil product was isolated. The oil was then weighed and diluted in HPLC-grade MeOH to a concentration of about 100 ppm before injecting into the GC-MS. This procedure was applied to all other reactions for more qualitative data.

The GC peak area of NK after 2 days was 15%, β-nootkatol 7.4% and α-nootkatol 20%. These results were similar to those mentioned in 2.3.1 however, no solvent was required. Therefore, this was an advantage because (i) this made the process greener by not including any solvents and (ii) it reduced the cost of the process. These results confirm what Gargouri *et al.* (1996) stated in a report that the regioselectivity of the lipoxygenation reaction is the same in both biphasic and monophasic media [161]. The recovered VL was 35%, which meant the other 65% was converted with low selectivity for NK. Other products were not identified by GC-MS.

2.3.3 Variation in buffer type:

Other buffers that have been used to extract LOX are phosphate and Tris [162] buffer. Therefore, the reactions were conducted with these buffers and the conditions were maintained at constant temperature of 37°C, air flow and stopped after 2 days. The reagents added to the reaction were the same as those in **2.3.2**, with just the change in buffer.

Tris buffer

The crude LOX enzyme in Tris-(hydroxymethyl) aminomethane (Tris) buffer (100 mM, pH 7.1), after the first and second day, showed a constant peak area of NK, at 3% only. This was much lower compared to the results in the previous reactions when sodium borate buffer was used. The mass yield was also very disappointing at 28%.

Potassium phosphate buffer

The crude LOX enzyme in potassium phosphate buffer (100 mM, pH 7.4) showed peak area of NK was 6% on the first day, however on the second day it decreased to 2% **(Table 2.2)**. This was very bad because the very little NK was already converted to other products which were not detectable by GC-MS. The NK area was double that of the Tris buffer reaction, however, it was lower than the 15% achieved in reaction **2.3.2** with the sodium borate buffer.

Table 2.2: The comparison of the GC peak area of VL and NK between the Tris and potassium phosphate buffer after 2 days.

Buffer	**Day 1**		**Day 2**	
	Tris (pH 8.5, 100 mM)	*Potassium phosphate (pH 7.4, 100 mM)*	*Tris (pH 8.5, 100 mM)*	*Potassium Phosphate (pH 7.4, 100 mM)*
VL (%)	80	44	73	54
NK (%)	3	6	3	2

Phosphate buffer is reported to have these limitations (i) poor buffering capacity below pH 7.5, (ii) tends to precipitate polyvalent cations and (iii) acts as an inhibitor

in many systems [163]. On the other hand, Tris buffer also has poor buffering capacity at the same pH and often acts as an inhibitor [163,164]. Therefore, these limitations may have contributed to the low reactivity of LOX in these buffers. Therefore, sodium borate buffer was the most suitable buffer for the extraction of LOX as it gave the highest NK peak area compared to both the Tris and phosphate buffer.

2.3.4 Substrate concentration variation

The effect of the substrate concentration on the activity of the enzyme was also explored. The reaction was carried out with the crude LOX enzyme in sodium borate buffer (100 mM, pH 8.5). OA (19.83 mmol), $FeSO_4.7H_2O$ (0.45 mmol), $MnCl_2.4H_2O$ (0.56 mmol) and only half VL 28.14 mmol instead of 56.28 mmol used in the previous methods. It was maintained at constant temperature of 37°C, air flow and the pH of the reaction was monitored every 24 h.

After about 48 h, the reaction was stopped, and approximately 25% volume of the reaction mixture remained as a homogeneous red suspension. This showed that most of the buffer water had been volatilised out of the reaction by the air flow through the reflex condenser. The product was isolated from the organic layer as a dark red oil with a good yield of 86%.

Amazingly, there was NK detected in acidic conditions of the reaction pH between 3-4. The initial pH of the reaction was the same as the buffer pH of 8.5, however as the reaction proceeded the reaction pH dropped drastically. Assuming that LOX converts OA to hydroperoxide such as 10-hydroperoxy-8-octadecenoic acid [165], which cause the reaction to be acidic. Therefore, this corresponds to the drastic drop of the reaction pH. The determination of these hydroperoxides by GC required them to be esterified with reagents such as diazomethane [165].

This reaction showed the best results since the beginning of the project, with NK peak at 70%, β-nootkatol 4.8%, and α-nootkatol 6.8% **(Figure 11)** after 2 days. The extracted product had a distinct smell, which was associated with NK. Muller *et al.* (1995) reported how the reaction pH below 8 for LOX produced higher levels of

nootkatol than NK, and thus maintained the pH at 8.5 by the addition of 5% NaOH solution throughout the reaction [105]. On the contrary, this reaction produced a very high NK peak when the reaction pH was more acidic, at pH 4, as opposed to alkaline at pH 8.

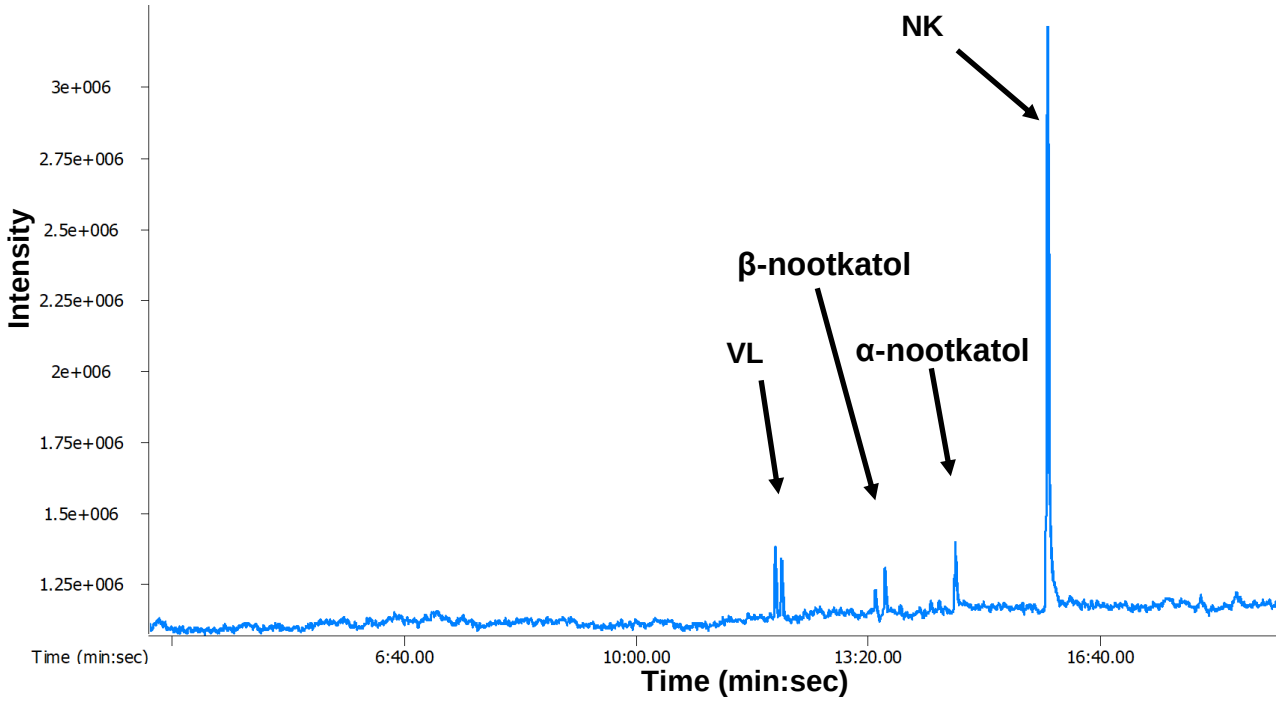


Figure 11: GC spectra of reaction 2.3.3, VL covered 4.8% peak area, β-nootkatol 4.8%, α-nootkatol 6.8% and NK 70%.

2.3.5 Variation in fatty acid

OA

Mahlala (2015) reported the biosynthesis of irones using soybean and he experienced a better conversion of irones when the unsaturated fatty acid (OA) used in the reaction was reduced by half [166]. Taking this into consideration, an investigation of whether OA was a possible inhibitor of LOX was carried out by reducing the OA by half, thus moving from 19.79 mmol to 9.91 mmol. The crude LOX enzyme in sodium borate buffer (100 mM, pH 8.5), $FeSO_4.7H_2O$ (0.45 mmol),

$MnCl_2.4H_2O$ (0.45 mmol) and VL (56.28 mmol) were added to the reaction. The reaction conditions were maintained at a constant temperature of 37°C and air flow.

The pH of the reaction also dropped to 4-5 within 48 h, and the isolated product was a yellow oil at 64% mass yield. On the first day, the NK peak was 71% and on the second day NK increased to 77% **(Figure 12)**. This proved that the OA added in the previous methods had been in excess and is a possible inhibitor of the enzyme [164].

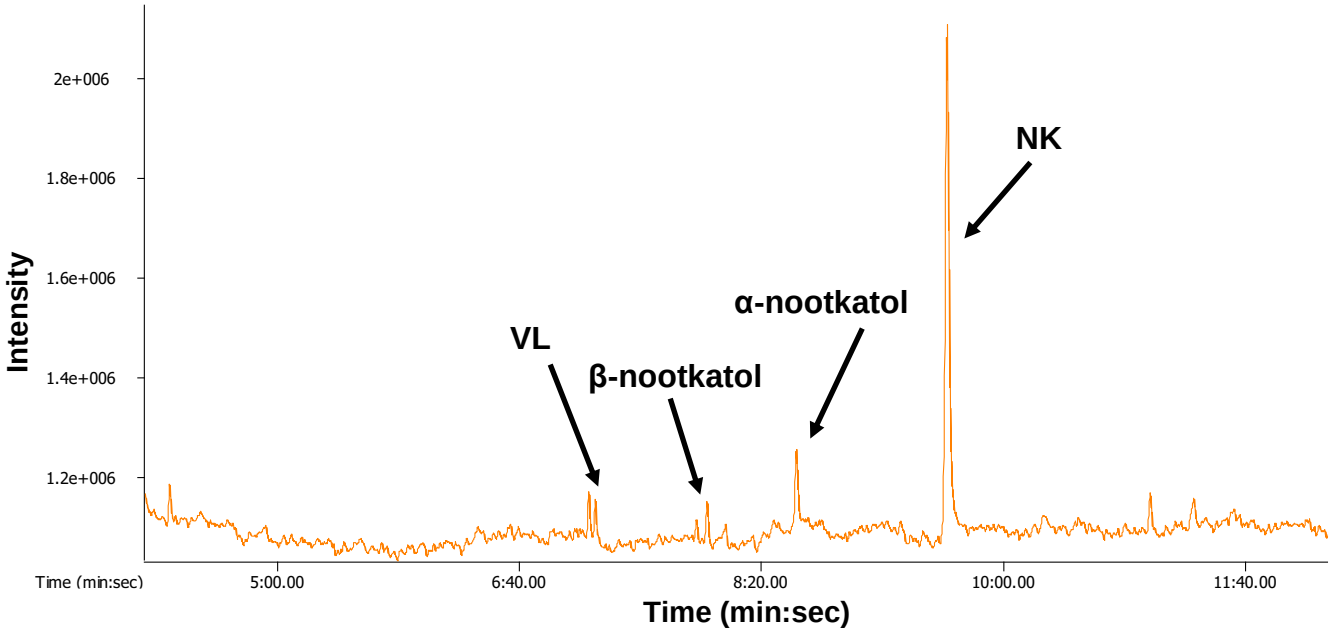


Figure 12: GC chromatogram of reduced OA after 2 days, the retention time differs to that of Figure 11 due to the changing or cutting of the column of the GC.

No OA

Another variation was done, where no unsaturated fatty acid was added at all. There was no activity, thus no NK detected. This proved that LOX was not the only enzyme in the conversion of VL to NK, because it requires unsaturated fatty acids to perform any oxidations on substrates.

Sodium oleate

The sodium salt form of OA, sodium oleate (NaOL), was used for this reaction. The VL, salts and sodium borate buffer (pH 8.5, 100 mM) were added, as previously. The temperature was 37°C and the pH of the reaction was 9-10 throughout. An antifoaming reagent was included to compensate for the vigorous foam caused by the sodium oleate powder.

As with all previous reactions, the volume of the reaction decreased with time due to air flow. However, in this reaction the sodium oleate was used as a powder thereby taking up the volume of the sodium borate buffer. This led to a complete dried-up reaction **(Figure 13)**, and very difficult product extraction because of the low solubility of the product in EtOAc.



Figure 13: Product formed as the dark-brown solid of NaOL reaction

The product solidification was possibly caused by the fact that NaOL is an anionic surfactant and upon addition of salts (NaCl, Na_2CO_3 or NaOH) wormlike micellar (WLM) structures are formed in aqueous media [167]. Tatini *et al.* (2021) reports on how the formation of these WLM species is dependent on pH, between pH 2 and 10

non-ionized (RCOOH) or anionic ($RCOO^-$) species are formed [168]. Suga *et al.* (2016) reported that at different pH values, metastable phases such as dispersed cubic phase (pH 7.5), vesicles (pH 8.5), cylindrical and spherical micelle (pH 9.7 & 10.6, respectively) [169].

After a total of 2 days only 28% mass yield was recovered as a brown sticky solid. Even with the difficulty in product separation, NK was converted but the peak area **(Figure 14)** was only 27%, β-nootkatol 4.0%, α-nootkatol 6.1% and VL 35%. This was impressive because NaOL proved to be a potential substrate of LOX in the production of NK.

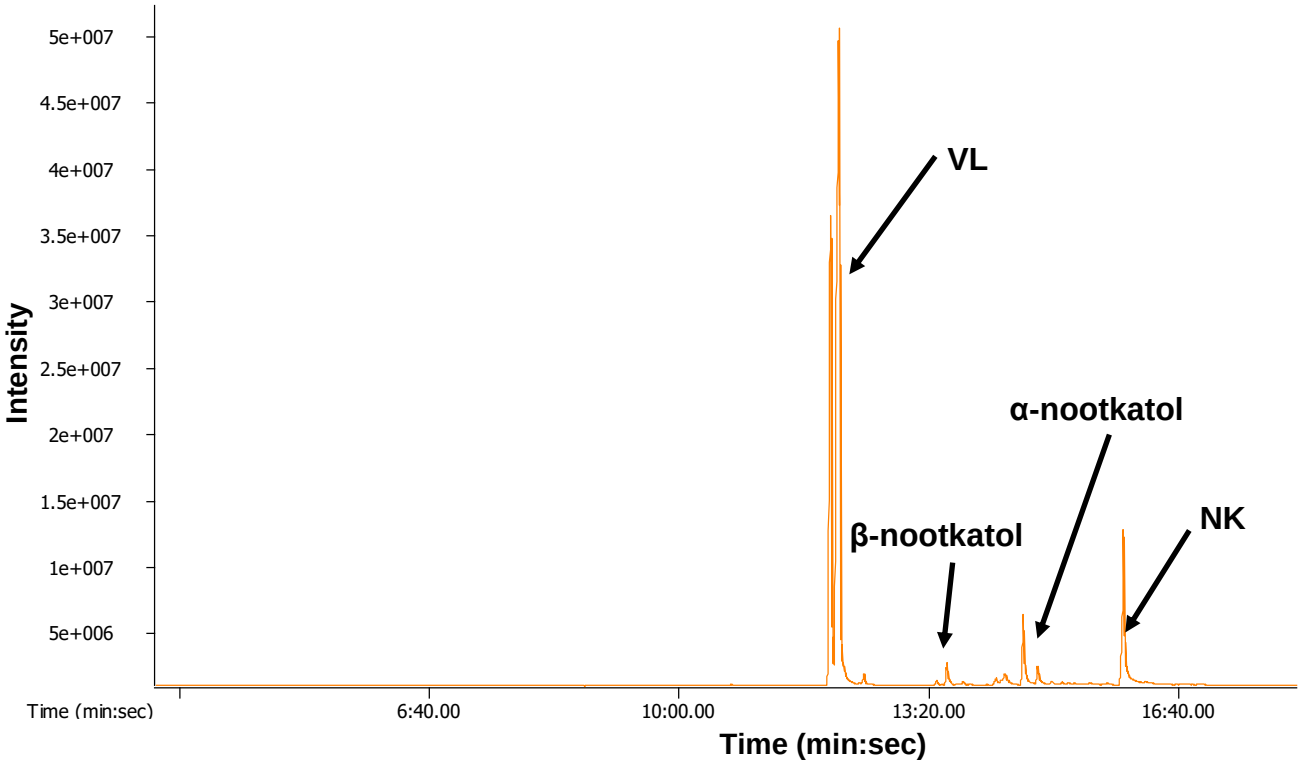


Figure 14: The GC chromatogram of sodium oleate reaction after 2 days, where NK covered 5.1% peak area, β-nootkatol 0.8%, α-nootkatol 2.0% and VL 69%.

2.3.6 Variation of salts concentration

Our next investigation was the effect of the salts such as $FeSO_4.7H_2O$ and $MnCl_2.4H_2O$ in the reaction. The $FeSO_4.7H_2O$ was doubled to 0.899 mmol and

$MnCl_2.4H_2O$ to 1.12 mmol. The crude LOX extraction procedure in borate buffer (pH 8.5, 100 mM) and the temperature remained constant (at 37°C). The pH remained at 3-4.

The reaction was stopped after 48 h and it was found that the NK peak area remained at a very low 0.6% throughout. This was extremely low and showed that the addition of the excess salts inhibited the activity of the enzyme. Although Fe^{2+} and Mn^{2+} act as activators for LOX [162], in excess they inhibit the activity of the enzyme. Koch *et al.* (1971) also agreed to this and emphasized the importance of Ca^{2+} as a more specific cation for the activation of the enzyme [170].

In summary, a biphasic reaction did not improve the product yield and extraction as expected. Therefore, we resorted to a single phase reaction which gave similar results as the biphasic reaction. The removal of hexane from the process was a bonus because it was now less hazardous and more economical. Tris and phosphate buffer both gave very low GC peak% of NK, this made sodium borate buffer the best buffer for the extraction of LOX. When the substrate was reduced, there was high GC peak area of NK of up to 70% but we failed to produce the same result. Then another fatty acid, NaOL was used but formed a solid that created difficulties in product extraction.

The addition of excess salts causes enzyme inhibition. We are also certain that the only enzyme operating in this biocatalytic reactions is LOX, because it is the only one that requires fatty acids. When no fatty acids are added to a reaction, there was no activity. When the amount of OA was reduced, there was light at the end of the tunnel because this reaction gave more than 70% peak area of NK. This was the best reaction conditions for LOX, as the substrate acted as an inhibitor in the previous reactions.

One of the biggest challenges encountered in most reactions was a creamy emulsion formation during work-up, which made it impossible to separate the product from the enzyme. Therefore, the optimization of the reaction work-up became extremely important, and this will be the focus in the following section. Several types of salts were used to break the emulsion and have a better separation of the product.

2.4 Reaction work-up optimization

2.4.1 General work-up

In an ideal biphasic reaction, one of the advantages would be the ease of separating the product from the enzyme. The general work-up procedure involved the addition of brine, 1 M NaOH, and ethyl acetate as an extraction solvent in a separating funnel by phase separation. However, in this case the addition of NaOH formed an emulsified cream which was impossible to separate **(Figure 15A).** Additional solvent was used to extract the product, **Figure 15B**, then the solution was dried with $MgSO_4$, and a dark brown oil was isolated after vacuum evaporation of the solvent by roto-evaporation. However, the colour was too dark, and the smell exhibited that of OA, not NK or VL.

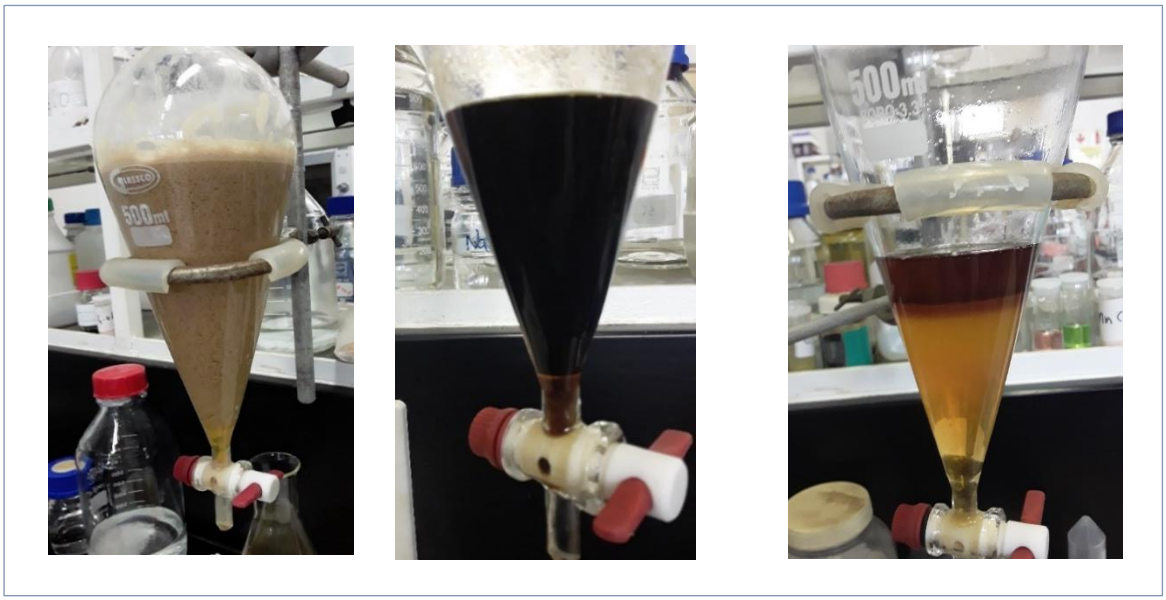


Figure 15: A cream-emulsion **A** formed during work-up, and after additional solvent added **B**. Single phase reaction work-up **C**.

When the single-phase reaction procedure was introduced for the first time there was a phase separation during work-up, **Figure 15C**. The same amount of ethyl

acetate, brine and 1M NaOH were added and, although an emulsified cream formed, it was a breakthrough to the separation challenges experienced in the past.

2.4.2 Variation in buffer

The product extraction from the phosphate and Tris buffer reactions was the easiest with brine, 1M NaOH, and EtOAc, and no emulsified cream formed. A new layer was observed between the aqueous (bottom) and organic layer (top), **Figure 16A**. This oily layer (middle) was isolated product as a yellow oil and contained traces of VL and NK (as determined by GC analysis). The sodium borate buffer extraction reaction showed the highest enzyme activity when compared to the Tris and phosphate buffers.

2.4.3 Variation in product extraction solvent

There are a few publications that report on the extraction of NK with diethyl ether (Et_2O) [105], and it is the well-known for the extraction of lipids. The difficulty or ease of their work-up procedure was not mentioned, probably because purified LOX was used. The work-up conditions were the same as **2.4.1**, however EtOAc was substituted with Et_2O. **Figure 16B**, shows a turbid top layer of Et_2O which proved that there was a high solubility of fat/lipids. This therefore made the work-up impossible, and thus selective extraction of the desired product was worse than when EtOAc was used. Therefore, other ways to improve the work-up procedure using EtOAc were explored.

2.4.4 Variation in aqueous salts

Sodium hydroxide (NaOH)

The addition of NaOH to fatty acids (OA) naturally formed salts **(Scheme 10a)** causing saponification. Ideally, the unreacted OA and unwanted side products would form a layer separate from the organic phase with no emulsion. Therefore, in an attempt to improve the work-up procedure NaOH addition was doubled from 1 M to 2 M NaOH. However, this caused a spontaneous exothermic reaction in the presence

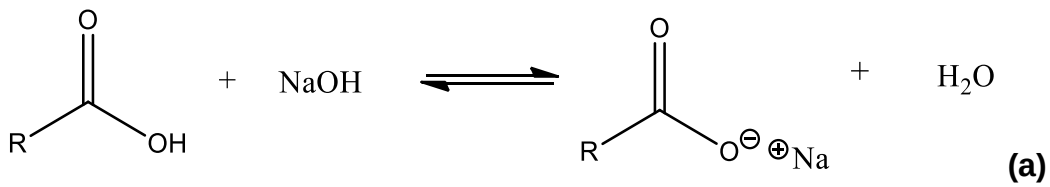
of the emulsified cream. When the emulsion finally broke, there were 4 layers instead of 3 layers as in the previous methods (i.e. the increase in the concentration of NaOH resulted in an additional layer). There was still an emulsified cream, and this proved that NaOH was not the most suitable salt to break the emulsions.

Saturated Potassium Carbonate (K_2CO_3)

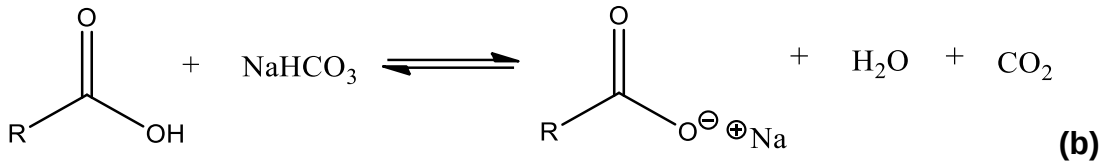
In light of NaOH forming a saponification reaction with fatty acids, it was replaced with saturated K_2CO_3. This is a milder base to handle that is easily available, eco-friendly, and non-toxic to nature [171]. For the work-up, EtOAc, brine and saturated K_2CO_3 were added. There was an improvement in how quickly the emulsion broke out, but the extract was turbid and did not become clear. Even after more brine was added, the extract remained turbid. This affected the appearance of the product after isolation.

K_2CO_3 and Sodium bicarbonate ($NaHCO_3$)

The work-up procedure was then improved with by using a mixture of brine and K_2CO_3, in a 4:1 V/V ratio. This created no turbidity, and the emulsion broke out easily. Afterwards, $NaHCO_3$ **(Figure 16C)** was used before drying with $MgSO_4$. The previous use of NaOH, caused an emulsion because of the strong ion-dipole bonds between the fatty acids and water molecules. [172]. The fatty acid tails, however, prevent complete solubilization of the fatty acid salts in the water phase. However, it is thought that the use of salts, such as $NaHCO_3$ and K_2CO_3, lower the general viscosity thereby causing the emulsion to break **(Scheme 10b)** [172]. The combination of the K_2CO_3 and $NaHCO_3$ together with NaCl gave the best work-up procedure for the VL to NK reactions. The advantages were no emulsified cream, turbidity or multiple layers formed.



(a)



(b)

Scheme 10: (a) The saponification of free fatty acids with NaOH **(b)** Proposed reaction of the formation of sodium salt, water, and carbon dioxide.

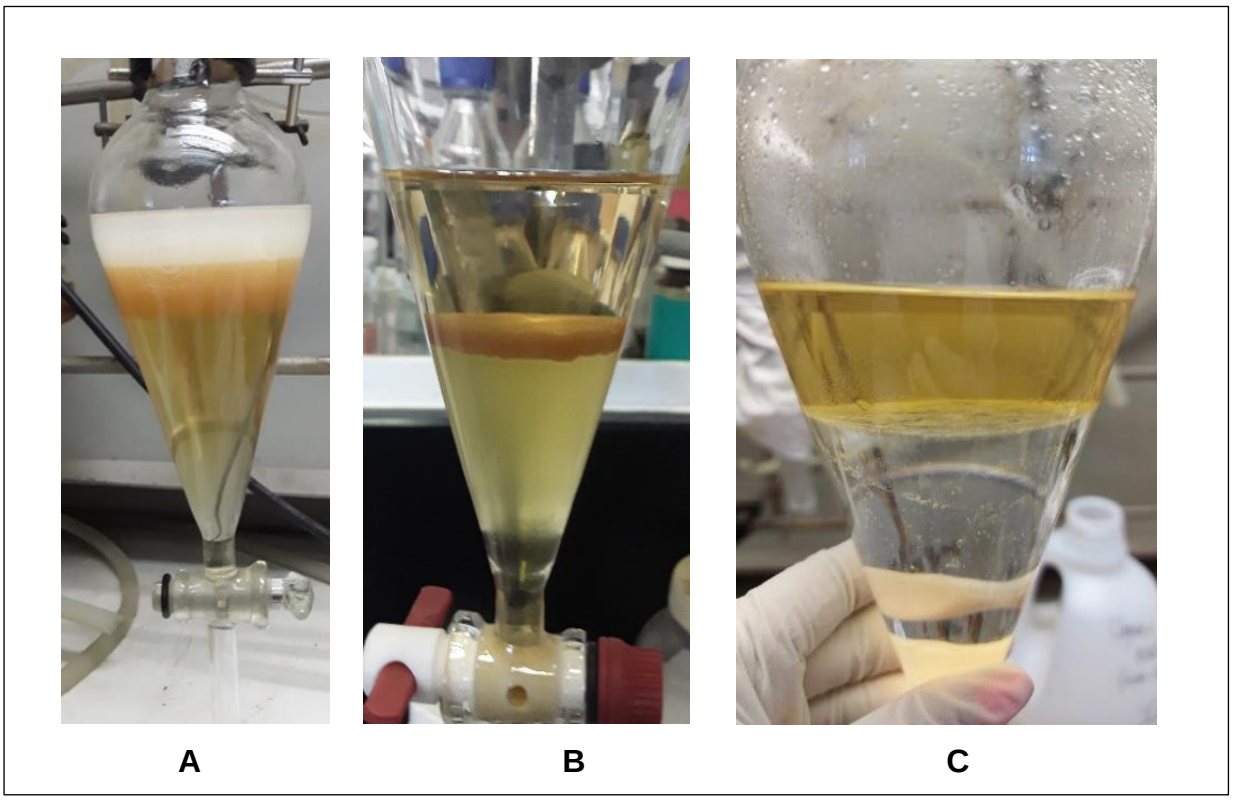


Figure 16: (A) Reaction work-up using Et_2O with turbid top layer. **(B)** A no emulsion work-up of the Tris and phosphate buffer. **(C)** The perfect product separation that contains no emulsion or layers after using brine, saturated K_2CO_3 and $NaHCO_3$ during work-up.

In summary, the combination of the K_2CO_3 and $NaHCO_3$ together with NaCl gave the best work-up procedure for the VL to NK reactions. The advantages were no emulsified cream, turbidity or multiple layers formed. This lead us to proceed to the optimization of the reaction to increase product yield in **2.5**. LOX was extracted with sodium borate buffer pH 8.1 and 7.5, and the reaction were carried out by varying

the buffer molarity and temperature. The resulting concentrations of NK were determined by GC-MS.

2.5 Optimization of Biocatalytic Reactions

In this section, reaction **2.3.4** was optimized because it showed the highest NK peak area within 2 days. The advantages of this reaction were, (i) the reduction of OA used in a reaction allowed the emulsion formed during work-up to break-up easily, (ii) the reaction required less than a week to form 70% NK peak area, and (iii) the colour of the products improved from dark to lighter oils and the scent became sweeter.

These optimization reactions were conducted using crude LOX enzyme in sodium borate buffer (pH 7.5 or 8.5), $FeSO_4.7H_2O$ (0.44 mmol), $MnCl_2.4H_2O$ (0.64 mmol), VL (56.28 mmol) and OA (4.95 mmol). The salts were added as stimulators of LOX, which improved the activity of the enzyme and the yield of NK. The reaction duration was between 72 and 96 h. A sample was taken every 24 h from each reaction and worked-up with EtOAc, K_2CO_3 and $NaHCO_3$ according to the procedure in **2.4.4**. Work-up was therefore the most time consuming and laborious part of the research, limiting the number of reactions that could be performed.

Once the product was isolated as an oil, it was kept at 4°C until the instrument was available for analysis. Before analysis, the oil was diluted in HPLC grade MeOH to a concentration of 100 ppm. Each sample (24-96 h) of each reaction was ran on the GC-MS in triplicates.

2.5.1 Crude LOX in sodium borate buffer (pH 8.1)

The reactions were conducted at various temperatures (21, 30 & 35°C), and two different buffer strengths (100 & 150 mM), and the reaction pH **(**Appendix, **Table A2)** of each reaction was recorded after every 24 hours.

At temperatures of 35°C, when the buffer molarity was 150 mM the concentration of NK was 66 mol% within 24 h and increased to 71 mol% in 72 h. Then in 96 h, there was a drastic drop to 17 mol% of NK product, because most of it was overoxidized to other products that were not detectable by GC-MS.

In comparison to the lower buffer molarity 100 mM at the same temperature of 35°C resulted in a maximum of 56 mol% after 24 h, then slightly decreased to 50 mol% in 72 h. The product was not allowed to overoxidize because the reaction was stopped in 72 h. The pH of the reaction medium decreased during the reactions, at 150 mM buffer concentration ranged between 4-6, while at 100 mM the pH was in the range 4-5 within 96 h.

When the reactions were conducted at a lower temperature of 30°C and at the buffer molarity of 150 mM, the conversion of NK reached a high of 69 mol% within 24 h and remained within this range for another 48 h. However, after 96 h the converted NK was beginning to overoxidise. The reaction pH was 6 for 72 h and dropped to 5 at 96 h. In a reaction at the same temperature with the buffer molarity decreased to 100 mM, NK was 73 mol% within 24 h. The reaction was stopped in 72 h and there was no overoxidation of NK. The pH of the reaction was 5 in 24 h and remained constant at 4 thereafter.

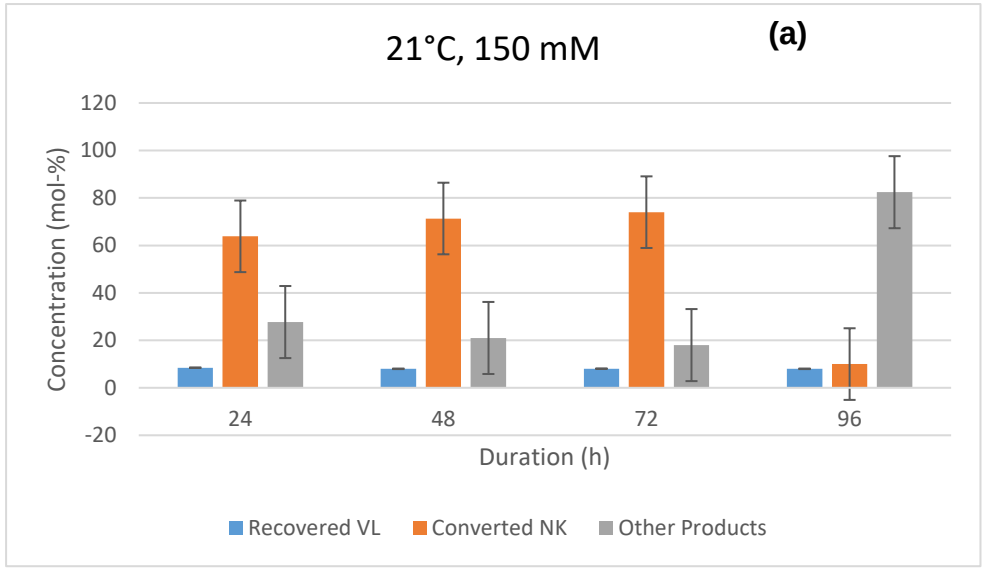
At a low temperature of 21°C, only a reaction at 150 mM buffer concentration was conducted. The concentration of NK reached 64 mol% and increased within 24 h. It continued to increase to 74 mol% in 72 h. This was a very good and steady production of NK. From this analysis, it was evident that the buffer molarity played an important role in the reaction pH. For all reactions at the buffer molarity of 150 mM, the initial pH was 6 then dropped down to 4 within 96 h. While for those at 100 mM, the initial pH was 5 and dropped to 4. Therefore, a slower drop in pH was vital for the conversion NK.

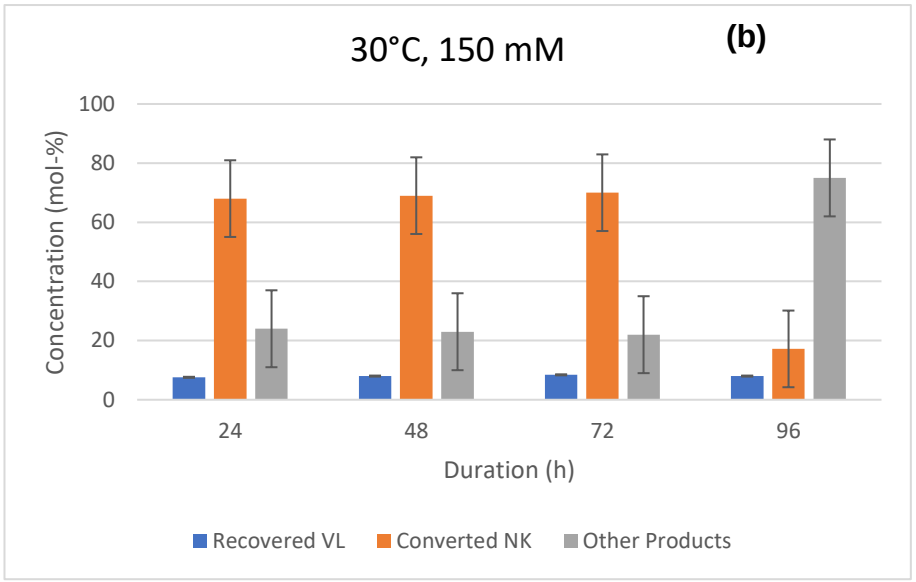
Therefore, the most important factors in these reactions were buffer molarity and reaction pH. The reactions were not dependent on the temperature **(Figure: 17)**. The best conversion of NK was at 150 mM buffer concentration and a pH range of 4-6,

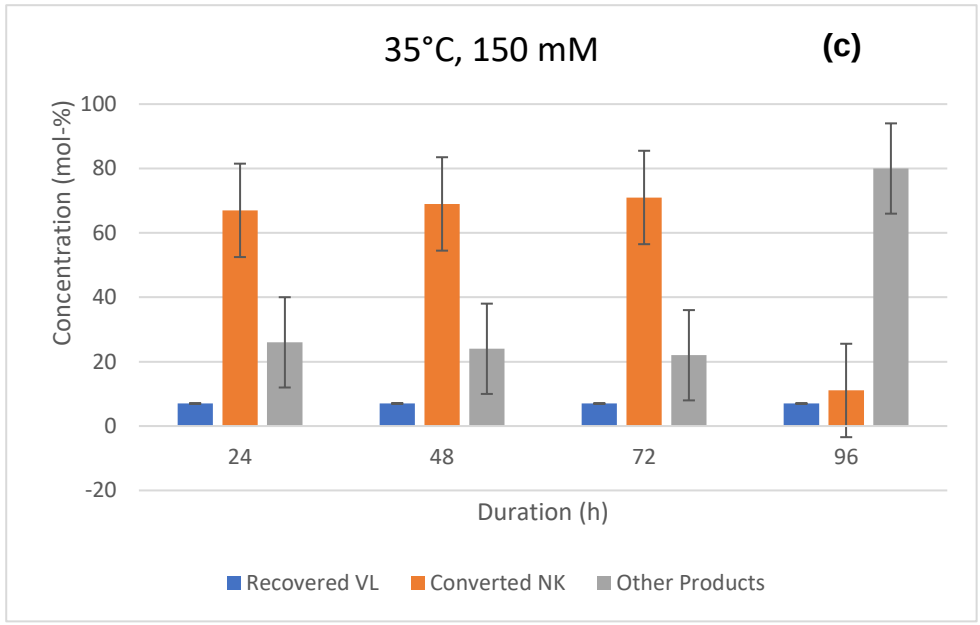
i.e. when the sodium borate buffer had a starting pH of 8.1. The duration of the reaction must be 24 h with a maximum of 72 h.

In all reactions, the converted VL concentration was higher than 90 mol% because less than 10 mol% was recovered. This was remarkable because it showed high enzyme activity, although other side products were produced from VL. A conversion of about 70 mol% to NK was very good and with continued research it can be optimized further. At this point the MB colorimetric assay had been developed, and the high activity of the enzyme seen in the biocatalysis reaction correlated with the results from the MB activity assay in **2.2.3**. The sodium borate buffer pH 8.1, 150 mM showed the highest concentration of bleached MB. Therefore, for both the assay and the reactions, high activity of the LOX enzyme was observed at pH 8.1, 150 mM sodium borate buffer.

In summary, extended reaction times are counter-productive as the NK product is overoxidised to by-products; the lower reaction temperature of 21°C provided slightly better conversion (as determined by yield) through reduced overoxidation to by-products compared to 30°C and 35°C; it was difficult to see any trend in the effect of buffer strength on conversion.


21°C, 150 mM
(a)
120
100
80
60
40
20
0
-20
Concentration (mol-%)
24
48
72
96
Duration (h)
Recovered VL
Converted NK
Other Products


30°C, 150 mM
(b)
100
80
60
40
20
0
Concentration (mol-%)
24
48
72
96
Duration (h)
Recovered VL
Converted NK
Other Products


35°C, 150 mM
(c)
100
80
60
40
20
0
-20
Concentration (mol-%)
24
48
72
96
Duration (h)
Recovered VL
Converted NK
Other Products

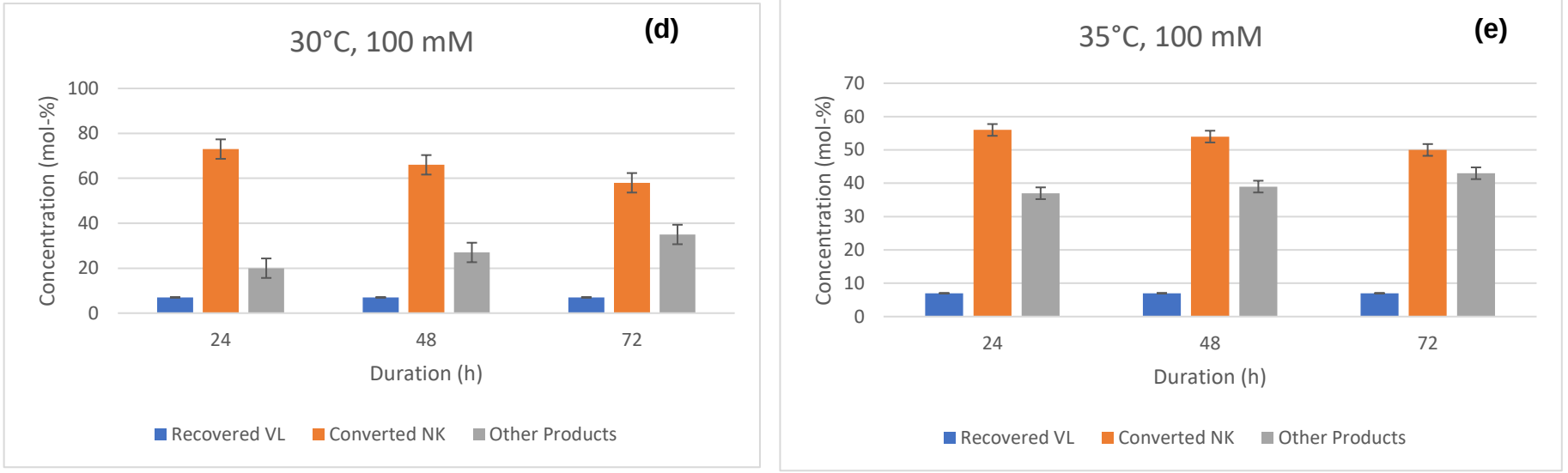


Figure 17: Concentration (mol%) vs Reaction duration (h) for unreacted VL, NK, and other products for reactions in sodium borate buffer at pH 8.5 at conditions (a) 21°C, 150 mM (b) 30°C, 150 mM, (c) 35°C, 150 mM, 30°C, (d) 100 mM and (e) 35°C, 100 mM. The error bars represent mean ± SD of three replicate samples (3n).

2.5.2 Crude LOX in sodium borate buffer (pH 7.5)

The buffer pH was adjusted to 7.5, to observe how the conversion of NK would be affected by a slightly lower pH. A pH of 7.5 is the lowest pH borate will effectively buffer at, and hence this experiment was to determine the impact of a lower starting pH and without changing the composition of the reaction medium. The reaction temperatures (21 & 30°C) and buffer molarity (10, 15, 100 & 150 mM) were used for these reactions (**Figure 18**, Appendix **Table A1**). When the reaction temperature was 30°C and 150 mM buffer molarity was used, NK was 46 mol% after 24 h which decreased steadily to 43 mol% after 72 h. The reaction pH was 5 for 72 h, then dropped to 4 thereafter. Then when the temperature was kept constant and the molarity lowered to 100 mM, there was a close to 20% increase in NK amount. After 24 h, NK was 59 mol% and 62 mol% in 72 h. The pH of the reaction was 5 within 24 h but dropped to 4 after this time.

At the buffer molarity 150 mM, when the temperature was decreased to 21°C, there was an increase in NK production. After 24 h, the pH was 6 then 5 after this time and NK amount was 66 mol%, but dropped slightly to 60 mol% in 72 h. At the same temperature, when the molarity was reduced to 100 mM, the concentration of NK was 63 mol% in 24 h and increased to 69 mol% in 72 h. The reaction pH was 5 in 24 h, then remained at 4 after this time.

In all these reactions, the overoxidation of NK occurred commonly after 96 h, which was also observed in reactions at a buffer pH of 8.1 in **2.5.1**. Therefore, the maximum time of a reaction must be no more than 72 h. Also, more than 90 mol% of VL was converted but did not selectively convert to NK. Similar to the reactions in 2.5.1, the buffer molarity and reaction pH play a vital role in the good conversion of VL to NK. For the reaction at 21°C and 30°C, at buffer molarity of 100 and 150 mM, the reaction pH range was 4-6.

Therefore, the optimum reaction pH for sodium borate buffer (pH 7.5) was 4-6 at the buffer molarity of 100 mM. An investigation was then carried out to find out whether the enzyme can convert VL to NK at a reaction pH lower than 4. The reaction was

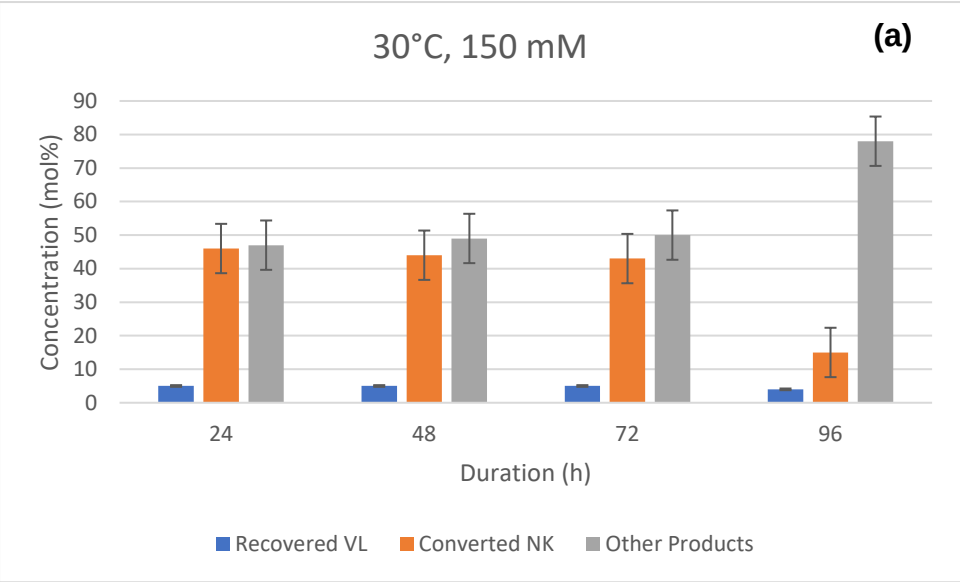
conducted at the temperature of 30°C with low molarity of 10 and 15 mM, using the sodium borate buffer at pH 7.5.

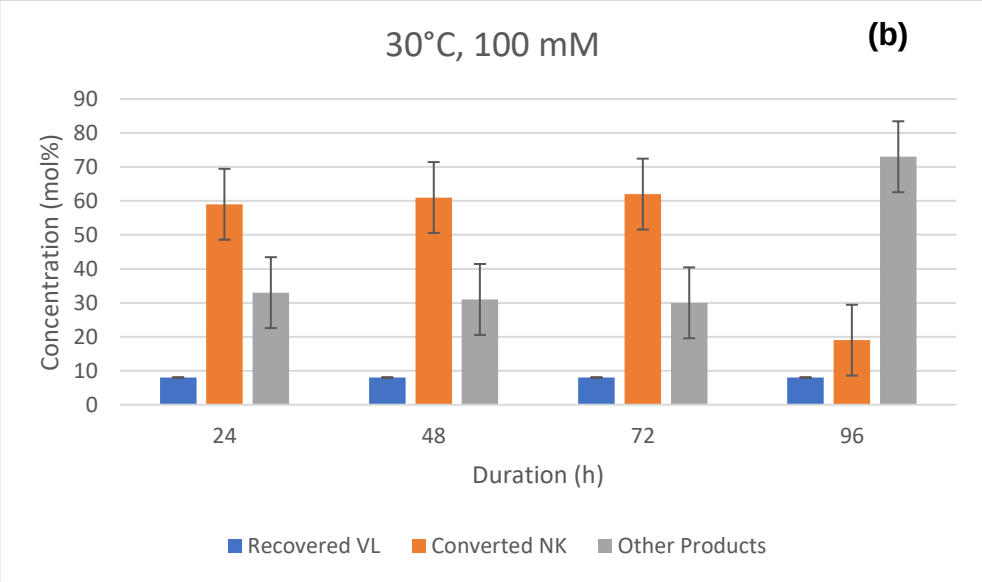
At the molarity of 10 mM, the reaction pH was down to 2 throughout the reaction period. These were extremely acidic conditions but surprisingly the enzyme was active and able to convert VL to NK. After 24 h, NK was 49 mol% which increased to 51 mol% after 72 h. When the molarity was 15 mM, the reaction pH remained constant for 96 h at 3 and within 24 h the concentration of NK was 61 mol%.

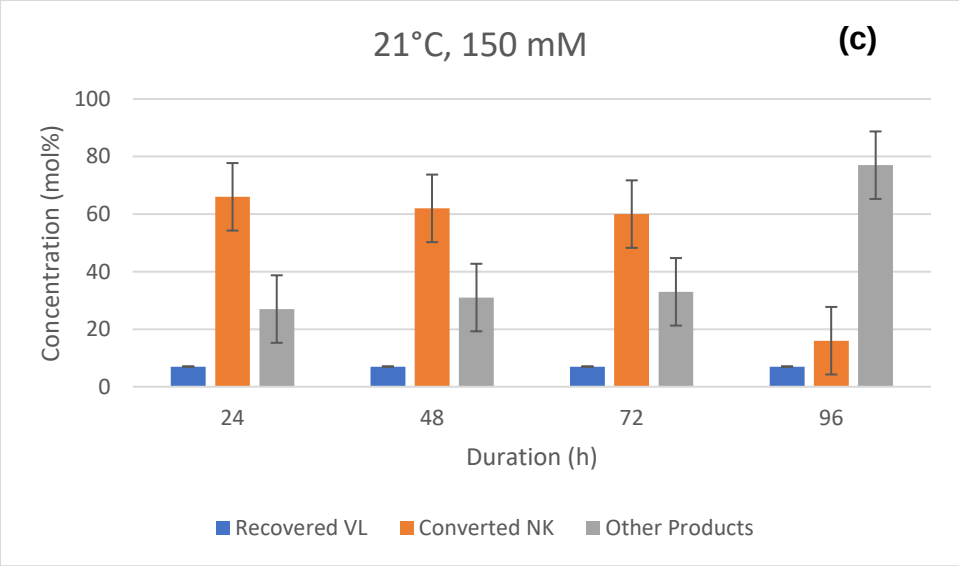
These results were surprising because according to the MB assay in **2.2.3**, at the molarity of 10 mM, buffer pH 7.5, the enzyme showed the lowest activity. The possible explanation for this is that there was a slow production of hydroperoxides and therefore, over a period of time there are enough to convert into NK.

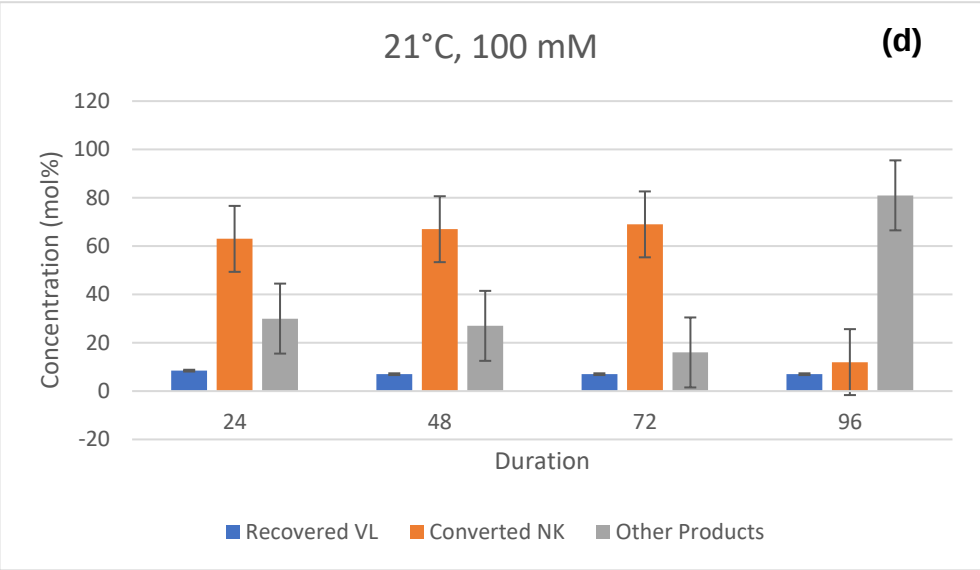
The concentration of the converted VL was close to 90 mol% in both reactions (10 & 15 mM), and the converted NK was overoxidized after a period of 72 h. The reaction pH of 2 gave an average of 50 mol% NK and at pH 3, 60 mol%, thus the pH range was extended to 3-6 for the optimum enzyme activity. The good conversion of NK at very low pH range was amazing because, to date, LOX has been reported to be active only between the pH ranges of 8.0-9.0 [105].

In summary, extended reaction times are counter-productive as the NK product is overoxidised to by-products; the lower reaction temperature of 21°C provided better conversion through reduced overoxidation to by-products; lower conversions are seen at both low and higher buffer strengths. The lower starting pH had little influence on the maximum conversion compared to the previous set of experiments. In the next section, a product sample of NK was purified by chromatography and characterized by NMR and GC-MS.


30°C, 150 mM
(a)
Concentration (mol%)
90
80
70
60
50
40
30
20
10
0
24
48
72
96
Duration (h)
Recovered VL
Converted NK
Other Products


30°C, 100 mM
(b)
Concentration (mol%)
90
80
70
60
50
40
30
20
10
0
24
48
72
96
Duration (h)
Recovered VL
Converted NK
Other Products


21°C, 150 mM
(c)
Concentration (mol%)
100
80
60
40
20
0
24
48
72
96
Duration (h)
Recovered VL
Converted NK
Other Products


21°C, 100 mM
(d)
Concentration (mol%)
120
100
80
60
40
20
0
-20
24
48
72
96
Duration
Recovered VL
Converted NK
Other Products

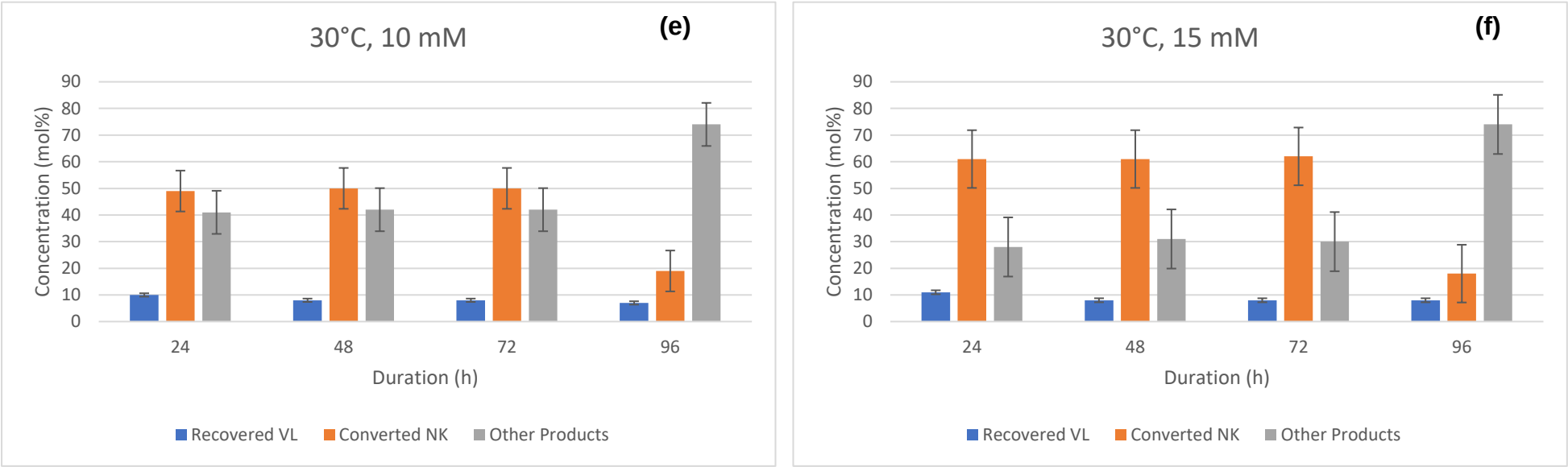


Figure 18: Concentration (mol%) vs Reaction duration (h) for unreacted VL, NK, and other products for reactions in sodium borate buffer at **pH 7.5** at conditions **(a)** 30°C, 150 mM **(b)** 30°C, 100 mM, **(c)** 21°C, 150 mM, **(d)** 21°C, 150 mM, **(e)** 30°C, 10 mM and **(f)** 30°C, 15 mM. The error bars represent mean ± SD of three replicate samples (3n).

2.6 Sample purification and characterization

The oil product of the converted NK was purified by column chromatography for further characterization by NMR and GC-MS. The stationary phase was flash silica gel (Sigma) and the mobile phase consisted of 5% EtOAc/Hexane. Each fraction was monitored by TLC and the solvent system used for TLC monitoring was 20% EtOAc/Hexane. The TLC spots were visualized by potassium permanganate stain and heated until the spots were yellow/brown. The starting material (VL), isolated product and a standard of NK (Sigma) were spotted in **Figure 19**. The TLC confirmed that NK was successfully synthesized, as the synthesized NK had the same Rf as the standard. The Rf of VL was 0.94 and for NK was 0.26, because of the relative higher polarity of NK it travelled slower than VL.

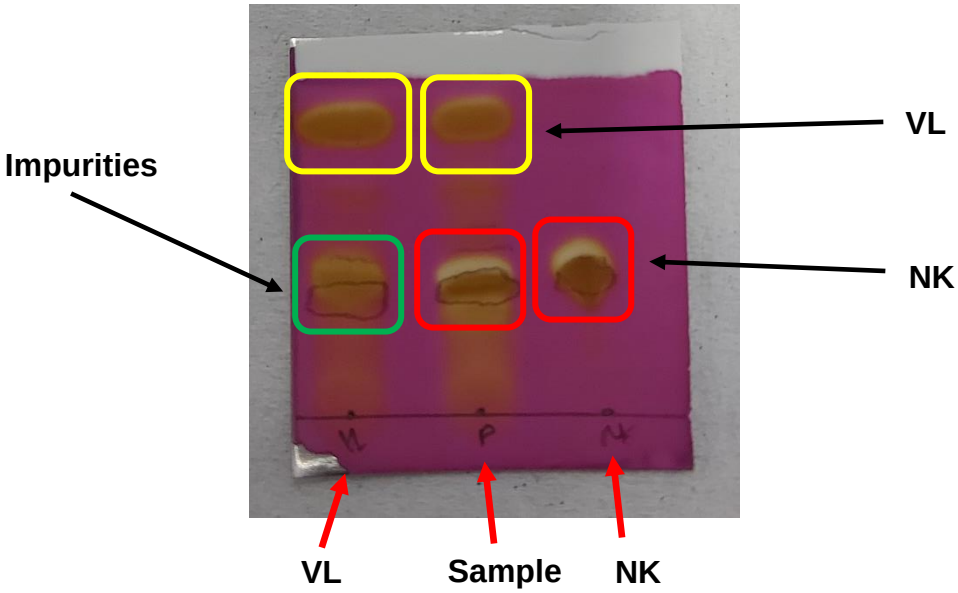


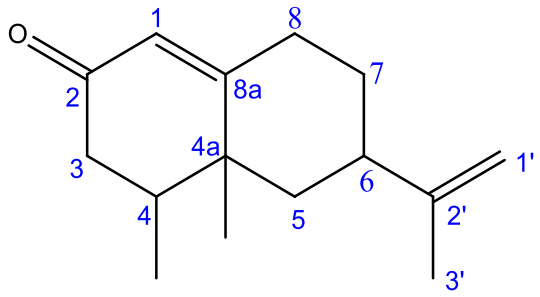
Figure 19: TLC plate showing VL and NK spots, where VL represented valencene, P, sample product and N, nootkatone standard.

All fractions collected were combined (according to purity as determined by the TLC analysis), dried by vaccum and analyzed. Only two distinct signals were observed in the ^{1}H NMR spectra for NK **(**Appendix, **Figure: B3)**. The singlet downfield at 5.76 ppm intergrated for 1 proton that was due to the cyclic vinyl proton, H**1**. The proton was confirmed to have travelled downfield because on the VL 1H NMR **(**Appendix,

Figure B1) the proton was observed at 5.33 ppm. Additionally, there was a disappearance of the proton at 4.70 ppm of the VL ^{1}H NMR that integrated for 2 protons.

Although, there were impurities of two protons that integrated as a doublet at 1.34 ppm and at 1.67 ppm as a singlet that appears as one proton. Also, instead as 3 protons at 1.13 ppm, there were 4 protons. All the 22 signals expected were observed and summaried in **Table 2.3**, thus confirmed the formation of NK.

Table 2.3: A summary of the key signals of ^{1}H NMR for NK



Cyclic region (ppm)	**Aliphatic region (ppm)**
5.76 (1H, s, H**2**), 2.51 (1H, ddd, H**6**), 2.25-2.34 (4H, m, H**1**, H**8**), 1.92-2.02 (4H, m, H**5**, H**7**)	4.73 (2H, d, H**1'**), 1.73 (3H, s, H**3'**), 1.13 (4H, m, CH_3), 0.96 (3H, d, CH_3)

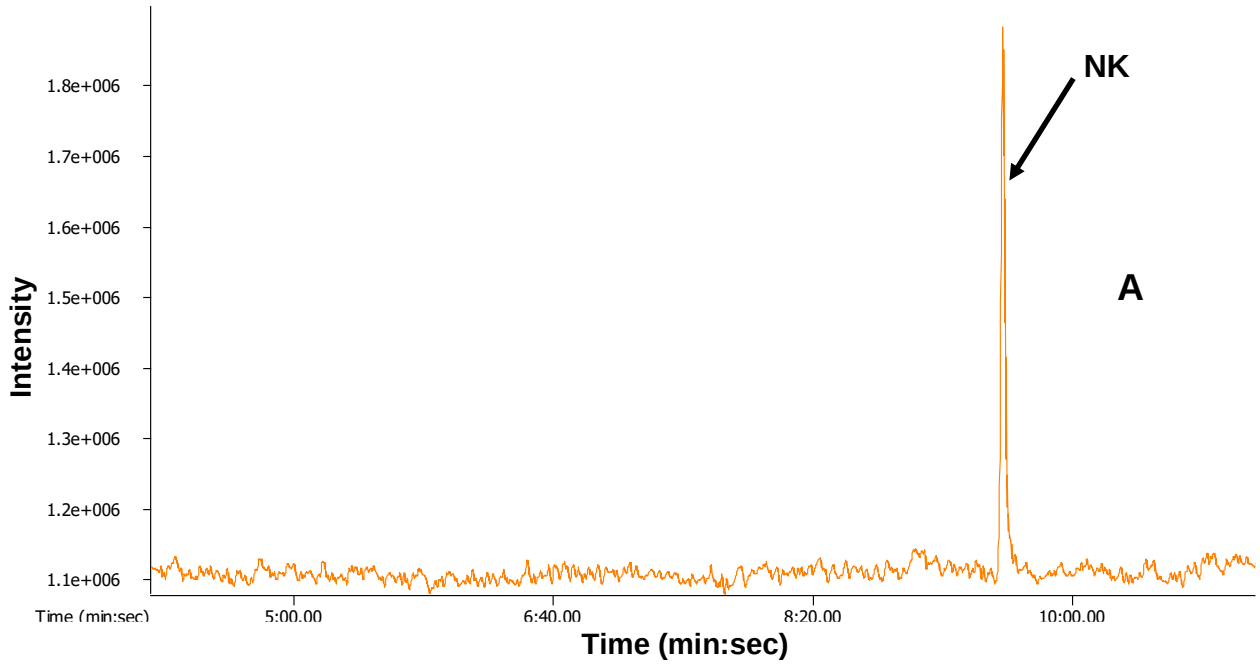
The ^{13}C NMR spectrum further confirmed the formation of NK **(**Appendix, **Figure B4)**. The signal downfield at 199.70 ppm was due to the carbonyl carbon, C**2**. The two quaternary carbons, enone, C**8a**, gave rise to the signal at 170.57 ppm and olefinic, C**2'** brought about a signal at 149.10 ppm. The methine carbon closest to the carbonyl group, C**1** caused a signal at 124.69 ppm and the upfield signal was from the methylene carbon of the olefinic group, C**1'** at 109.25 ppm. Other cyclic and

methyl signals are summaried in **Table 2.4**. Both the ^{1}H and ^{13}C NMR signals observed of NK corresponded with data provided by Melo *et al.* (2021) [173].

Table 2.4: A summary of the key signals of ^{13}C NMR for NK

C=O (ppm)	C (ppm)	CH (ppm)	CH_2 (ppm)	CH_3 (ppm)
199.70, C**2**	170.75, C**8a**	124.69, C**1**	42.08, C**3**	14.92, CH_3
	149.10, C**2'**	40.46, C**6**	43.92, C**5**	16.86, CH_3
	39.33, C**4a**	40.32, C**4**	31.62, C**7**	20.84, C**3'**
			33.04, C**8**	
			109.25, C**1'**	

The presence of Nootkatol was only confirmed by GC-MS **(**Appendix, **Figure C3** and **D3)**, as the isolated product showed high levels of impurities by NMR analysis of the semi-purified product **(**Appendix, **Figure B7-9)**. Therefore, further purification steps were required for accurate characterization by NMR. NK was further identified by 2D NMR, HSQC **(**Appendix, **Figure B6)** and DEPT135 **(**Appendix, **Figure B5)**.



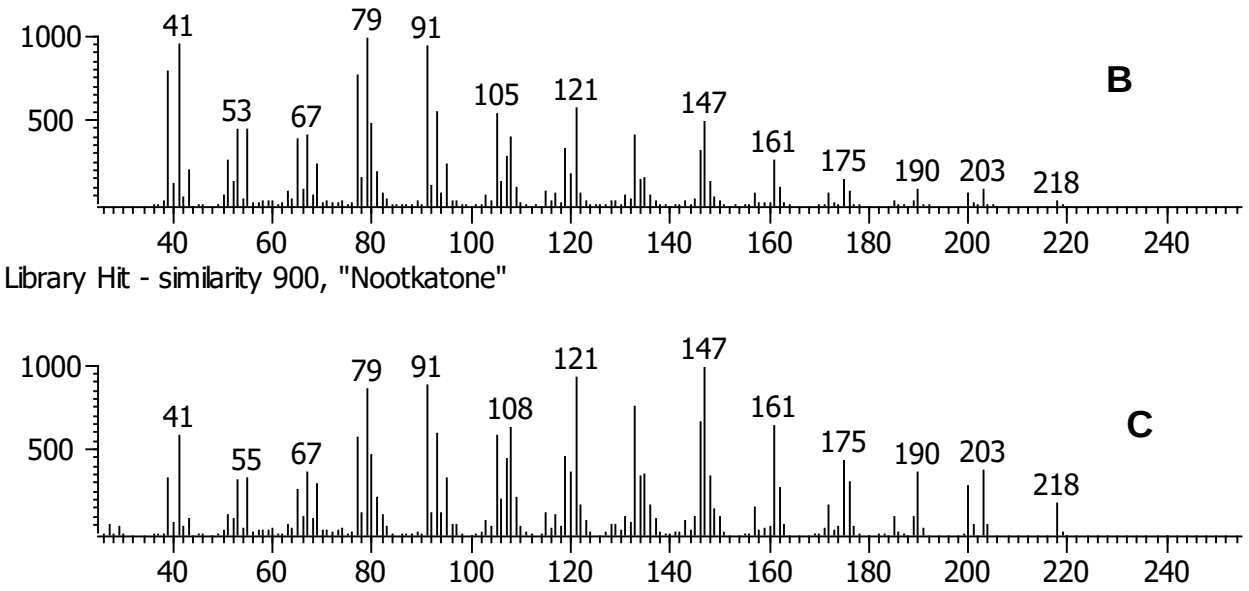


Figure 20: (A) GC chromatogram of purified NK, **(B)** the MS spectra of the synthesized NK and **(C)** the NIST library matched NK.

In summary the NMR and Mass Spectrometry data confirm the product to be Nootkatone. The immobilization of crude LOX by cross-linking to form CLEA will precede this section and the LOX CLEA will also be used in the biocatalytic reaction under similar as for the crude LOX, **2.5.1**.

2.7 The biocatalytic conversion of VL to NK with immobilised LOX CLEA

Glutaraldehyde is a simple but powerful cross-linking agent, that has been successfully applied to enzymes such as nitrile hydratases [174], lipases and nitrilases [123]. In our knowledge, there is no published work on the cross-linking of LOX with glutaraldehyde. Therefore, in this section partially purified LOX was immobilized using glutaraldehyde, **Figure 21** illustrates the formation.

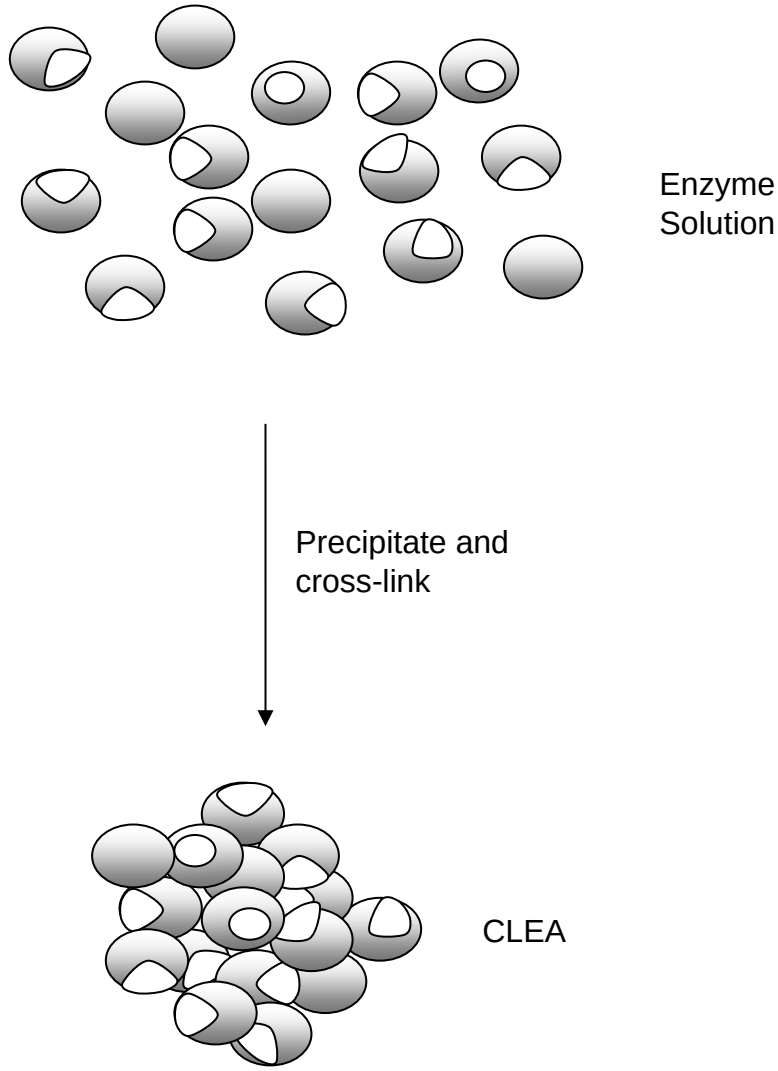


Figure 21: Illustration of the formation of LOX CLEA.

2.7.1 The partial purification & CLEA immobilization of LOX

Since the crude LOX enzyme in sodium borate buffer (pH 8.1, 150 mM) showed high activity, both in the activity assay **(Figure 9)** and in the reactions in Appendix: **Table A3**, it was used to form the LOX CLEA aggregates. The precipitation LOX CLEA began with ammonium sulfate precipitation as an aggregation agent, then cross-linked with glutaraldehyde.

The initial precipitation was 40 wt% saturation [175], followed by 70 wt% saturation of ammonium sulfate and, after centrifugation, the resultant pellet and the supernatant were used to form LOX CLEAs **(S. Richardson and I.M Makhubela, unpublished work)**. The supernatant is usually discarded because it no longer contains the enzyme and is thus considered inactive. However, the supernatant was not discarded initially, to check if all the LOX had precipitated.

According to several reports, the investigation of the optimum concentration of the glutaraldehyde for the enzyme was important because it affected the activity, stability and enantioselectivity observed in CLEA catalysed reactions [176-178]. Therefore, the concentration of the glutaraldehyde was investigated for its effect on the LOX enzyme activity recovery.

The LOX CLEAs were prepared from 6 different concentrations (1.25-20% v/v) of glutaraldehyde. This method was adapted and modified accordingly from a paper by van Pelt *et al.* (2008), where nitrile hydratase was immobilized as LOX CLEAs [174]. Once the LOX CLEAs were formed, there was a total of 6 samples of the precipitated pellet (Undialyzed LOX CLEA) and another 6 samples of supernatant (LOX CLEA supernatant). A portion of the precipitated pellet was dialyzed (Dialyzed LOX CLEA), i.e. 6 samples in total, to remove all salts that may interfere with the enzyme activity.

The enzyme activity of the LOX CLEAs was investigated by a MB assay mentioned in 2.3. Therefore, a substrate solution was formed for each LOX CLEA at the different concentrations. This process was very laborious and took a long time to complete. Upon bleaching the enzyme activity was measured spectrophometrically at 664 nm, and the initial concentration of MB used was 5 µM. Therefore, each LOX CLEA was required to bleach a maximum of 5 µM to show the maximum enzyme activity.

The LOX CLEA supernatant and the dialyzed LOX CLEA showed the highest activity with concentration of 1.4 µM of bleached MB (**Figure 22**) at the concentration of

1.25% glutaraldehyde. This was low compared to the crude LOX concentration of 3 µM, however it meant that approximately 50% of the activity was recovered. The loss of activity of the enzyme after purification and immobilization is common [174], however with continued investigation on the best conditions suited for the LOX enzyme the optimum activity is achievable.

Although there was high activity at 1.25% glutaraldehyde, at 2.5% there was a drastic drop for both the dialyzed LOX CLEA and LOX CLEA supernatant. As the concentration of the glutaraldehyde increased, there was a rise and fall in the concentration of bleached MB for the LOX CLEAs. Barbosa *et al.* (2014) emphasized the moderate use of glutaraldehyde to avoid excessive modification of the enzyme [179]. Wilson *et al.* (2008) however believed that the ratio of the enzyme to the glutaraldehyde does not affect the activity of the CLEAs but does affect the product yield [176].

The bleaching of MB was evident across all three types of LOX CLEAs at the glutaraldehyde concentration of 1.25%. This confirmed that the less cross-linking agent used, the better [176]. The dialyzed LOX CLEAs were more active than the undialyzed LOX. The purpose of dialysis was purification by removal of salts or to reduce the concentration of salts from enzyme solution [180]. Therefore, the undialyzed salts of ammonium sulfate affected the activity of the enzyme greatly. Therefore, the dialysis of the precipitated pellet plays a very important role in the recovery of the enzyme activity.

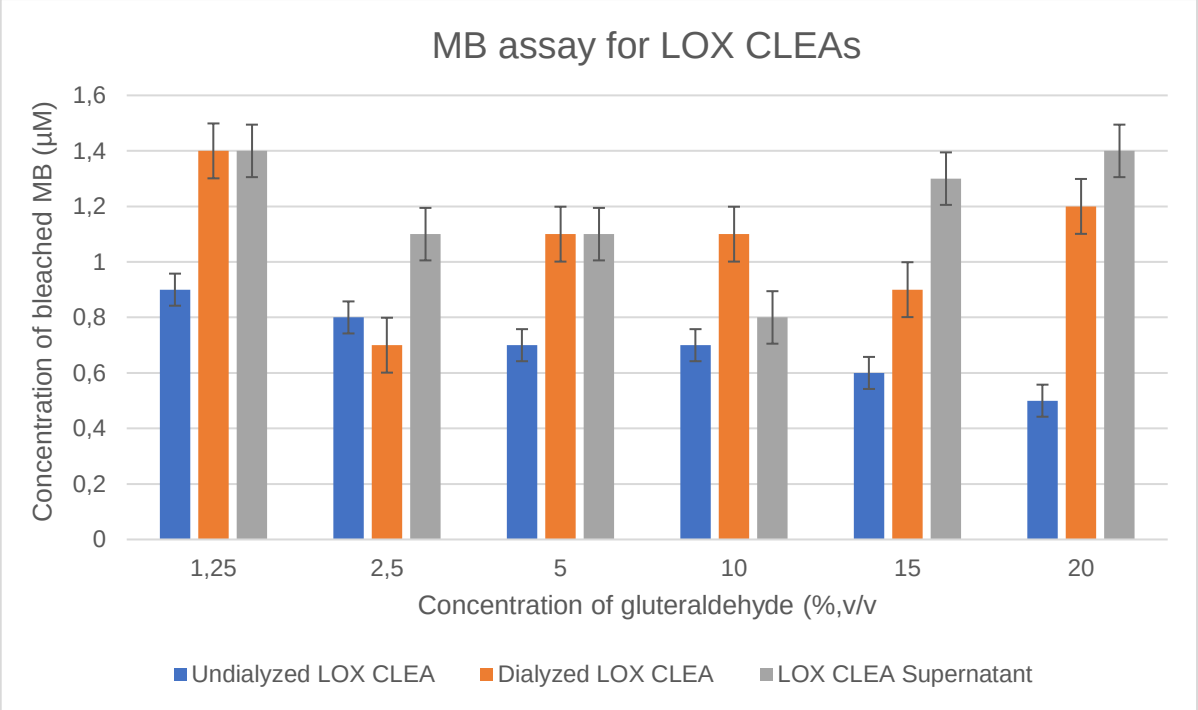


Figure 22: The MB bleaching assay of the immobilized LOX CLEA at different concentrations of glutaraldehyde. The error bars represent mean ± SD of three replicate samples (3n).

The assay also showed that LOX did not precipitate completely as was evident from the activity observed in the LOX CLEA supernatant. In light of this, the precipitation of LOX was adjusted to 50 wt% and 80 wt% saturation of ammonium sulfate. The resulting supernatant was tested and showed no activity. Therefore, this was the best precipitation method and overall, this was a good starting point for the LOX CLEAs and with further research can be optimized.

2.7.2 Reactivity of partially purified LOX CLEAs

As prepared in **2.7.1**, the reactivity of the LOX CLEAs to convert VL to NK was investigated by using two types of LOX CLEAs, undialyzed and dialyzed, in separate reaction flasks. The reaction was initiated by the addition of LOX CLEA, VL, OA (9.9 mmol), $MnCl_2.4H_2O$ (0.56 mmol) and $FeSO_4.7H_2O$ (0.45 mmol), while keeping the reaction temperature at 37°C. An air pump was used for air flow as an oxygen

source and the reaction continued for 48 h. The reaction created so much foam that it overflowed out of the reaction flask into the reflux condenser, but this did subside once VL was added. A sample was taken every 24 h, which was worked-up and isolated as an oil, before GC-MS analysis.

The reaction suspension of the undialyzed LOX CLEA became an emulsion **(Figure 23A)** during work-up, which was very difficult to break-up. The work-up procedure used was **2.4.4**, with K_2CO_3 and $NaHCO_3$. According to GC results in **Table 2.4**, by the end of a 48 h period, only 25% NK by peak area was produced. This was much lower than the crude LOX that gave more than 70% NK peak area. The partial purification and immobilization of the enzyme had a great impact on its reactivity [178].

Table 2.5: The GC-MS peak area (%) of VL, NK and nootkatol the LOX CLEAs within 48 h period

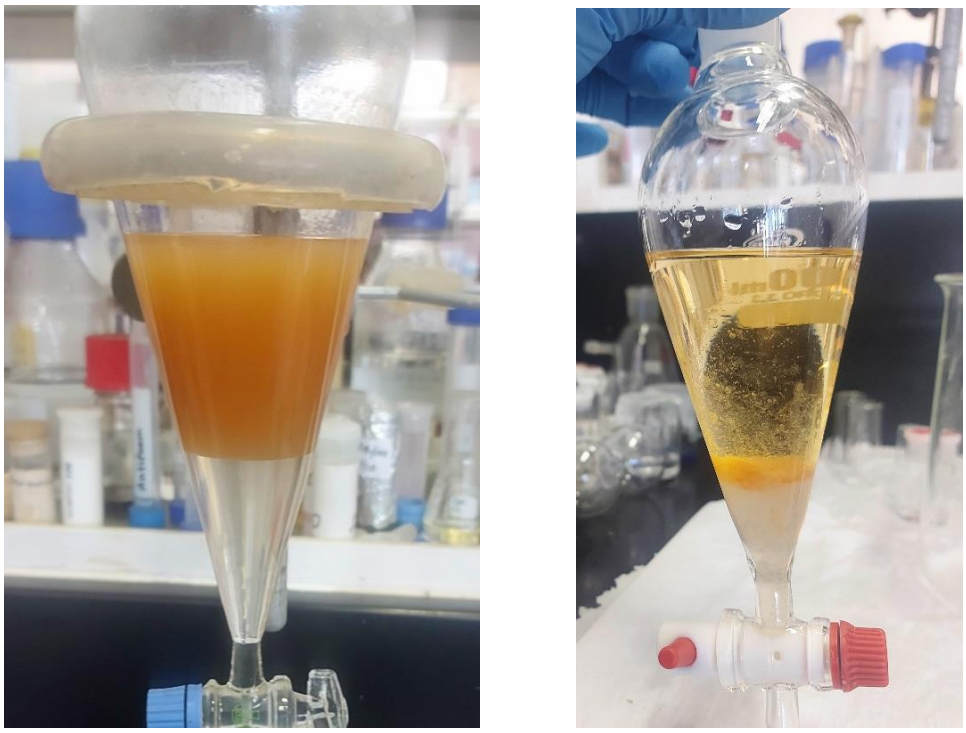
LOX CLEAs	**Duration (h)**	**Reaction pH**	**VL (%)**	**α-Nootkatol (%)**	**β-Nootkatol (%)**	**NK (%)**
Undialyzed LOX CLEA	24	4	72	0.8	1.3	2.7
	48	4	45	3.4	5.1	25
Dialyzed LOX CLEA	24	2	72	1.0	1.4	1.8
	48	2	58	4.8	4.0	8.4

Pelt *et al.* (2007) also experienced similar problems with the nitrile hydratase CLEA, were there was loss of activity during preparation of the CLEA. To mitigate this problem the cross-linking agent was changed to dextranpolyaldehyde, which did increase NK yield from 21% to 40-45%. However, the challenge was low dispersion of the CLEA in the reaction and the difficulty of separating the CLEA from the reaction mixture resulting in low recyclability [174].

Although, the overall NK peak area was very low for the undialyzed LOX CLEA, the drastic increase of approximately 20% NK between the 24 h and 48 h period was

very impressive. Not only did NK peak area increase but that of α- and β-nootkatol as well. The pH of the reaction was constant at 4 throughout the 48 h, which was the determined optimum pH for the enzyme.

The reaction conditions of the dialyzed LOX CLEA were the same as for the undialyzed LOX CLEA. There was less foam formed, also the work-up was the best **(Figure 23B)** with no emulsions and very easy separation of the product from the enzyme. The GC peak area for NK was after 24 h at 1.8% which was comparable to the undialyzed LOX CLEA at 2.7%. However, after 48 h it showed a very small increase of 8.4%. This was disappointing because, according to the MB activity assay, the dialyzed LOX CLEA showed better activity than the undialyzed LOX CLEA.



A **B**

Figure 23: Reaction work-up of the **(A)** undialyzed LOX CLEA and **(B)** Dialyzed LOX CLEA.

The undialyzed LOX CLEA was within the optimum reaction pH of 4, which resulted in a steady increase in the production of NK. However, for the dialyzed LOX CLEA the reaction pH was 2 throughout the 48 h period and resulted in slower production

of NK. This confirms that the purification or removal of salts by dialysis affected the activity and conditions of the enzyme.

Although the dialyzed LOX CLEA has lower activity compared to the undialyzed LOX CLEA, it showed the best recovery after work-up and was reusable. Therefore, the ideal LOX CLEA would be one with easy recovery, recyclability, and high activity. At this point it would be more attractive to optimize the dialyzed LOX CLEA to improve its conversion of VL to NK.

2.7.3 Reactivity of purified LOX CLEA

Sasha Richardson (unpublished work) purified the crude LOX by precipitation and ion exchange chromatography, then cross-linked the purified LOX with glutaraldehyde to form CLEAs. The purified LOX CLEA was then dialyzed before we used it in a reaction to convert VL to NK. The recyclability of the purified LOX CLEA was tested in reactions according to conditions in **2.7.2**. Therefore, after the initial reaction the purified LOX CLEA was centrifuged and resuspended in buffer before reuse (Cycle 1).

Table 2.6: GC peak areas of the purified LOX CLEA

Reactions	**Reaction pH**	**VL (%)**	**β-Nootkatol (%)**	**α-Nootkatol (%)**	**NK (%)**
Initial run (24 h)	2	67	2.5	0.6	2.1
Cycle 1 (24 h)	2	64	0.4	0.6	2.3

The reactivity of the purified LOX CLEA was comparable to the dialyzed LOX CLEA mentioned in 2.7.2. After 24 h, the GC peak area of NK was 2.1% for purified LOX CLEA and 1.8% for dialyzed LOX CLEA. Even after the first cycle it was 2.3%. This showed that the enzyme retained activity after recycling. The reaction pH remained

at 2 for both reactions. The purified LOX CLEA work-up gave a very good separation of the enzyme and reaction mixture. Since the reactivity of both the purified and partially purified LOX CLEAs exhibit corresponding results, the optimization of the partially purified LOX CLEA may be more attractive. This would reduce the cost and time involved in the purification of the enzyme.

In summary LOX can be self-immobilised using the CLEA process and use to synthesise nootkatone. Considerable optimisation of the method would be required to provide a better catalyst than the extracted enzyme. Enzyme purification did not provide a more active immobilised biocatalyst, but the LOX-CLEA particles generated were demonstrated to be recycled in a subsequent reaction with no loss of activity.

CHAPTER 3

CONCLUSION AND RECOMMENDATIONS

3.1 Conclusion

The extraction of LOX from soybean fine flour resulted in higher enzyme and protein content that soybean coarse meal. Therefore the milling of the seeds to produce a flour played a vital role in the extraction process. This, however, worked against us during biocatalytic reactions where the highly viscous, emulsified cream of extracted LOX from soybean flour made product separation impossible. The best method of separation of the crude LOX in buffer was by using cheese cloth, since it avoided filtration blockages resulting from emulsified cream. This extraction method was very easy, cost effective and non-laborious.

We also investigated which buffer was the best for extraction amongst the Tris, potassium phosphate and sodium borate buffers. A methylene blue assay was used to determine the best extraction buffer, measuring the highest formation of hydroperoxides. Sodium borate buffer was evidently the best extraction buffer.

There are seven biocatalytic reactions for the conversion of VL to NK, for which we deduced the best reaction conditions before optimization. Variations of fatty acids, salts and substrate concentration formed part of these reactions. The best GC peak area for NK was 70% and was achieved when the substrate concentration was reduced. We failed in an attempt to optimize this reaction. Therefore, we reduced the fatty acids added and achieved a 10% improvement in NK to 77% GC peak area. This method was then adapted for optimization and showed excellent results. Interestingly, the final reaction pH in both reactions was 4, yet most literature emphasises that the suitable pH for LOX is 8-9 [105].

We carried out the optimization reactions according to the conditions described in **2.3.4** and varied the temperature, buffer molarity and pH. Special attention was placed on the buffer molarity and the pH in relation to NK conversion (mol%). When the buffer pH was 8.1 the influence of molarity was similar at both 100 and 150 mM at 30°C. At 21°C and 150 mM, 74 mol% NK was obtained after 72 h and the final reaction pH was 4-6. At pH 7.5, the optimum molarity was 100 mM, 69 mol% NK was obtained within 72 h and final reaction pH was 3-6. The duration of these reactions was best at 72 h, as longer than this created the overoxidation of NK and formation of other side products.

The separation of the product from the enzyme was very difficult due to the viscous emulsions that were impossible to break. To our knowledge, most publications do not outline the reaction work-up because isolated/purified LOX isozymes are commonly used [105] in small scale reactions, and we assume that the purified LOX does not form any of the emulsions that the crude LOX extract does. Therefore, we developed a work-up procedure that solved the challenges faced when using the crude LOX extract. The procedure was based on the utilization of non-toxic and cheap salts such as NaCl, $NaHCO_3$ and K_2CO_3.

In an attempt to improve the reactivity and recyclability of the enzyme, we partially purified the crude LOX enzyme by ammonium sulfate precipitation. We further immobilized the partially purified LOX by cross-linking with glutaraldehyde to form CLEAs. In literature, LOX has been immobilized in several ways [122,128, 129] besides cross-linking with glutaraldehyde. Not only did the LOX CLEAs suffer loss of activity but they were very hard to handle and not recyclable. However, this changed when the LOX CLEAs were dialyzed. As a result, they became easy to handle, stable and recyclable.

In conclusion, the reaction conditions at pH 3-6 with NK concentrations as high as 74 mol% has expanded the knowledge of the soybean LOX to biocatalytically convert VL to NK. Together with the use of inorganic salts to break the emulsion that made product separation impossible, which was developed for the first time through this project. The demand of natural NK has increased drastically due to the approval of the EPA (Environmental Protection Agency) as of August 2020 [64] as an excellent

repellent and insecticide because of its effectiveness, green and sustainable nature. Therefore, the scaled-up and commercialized would be attractive to benefit various industries that require natural NK.

3.2 Recommendations

- Since only one compound was synthesized therefore, we suggest soybean LOX biocatalytic oxidation of other terpenes and organic compounds that have greater commercial significance. Example the conversion of limonene to carvone.

- The main fatty acid used for this project was oleic acid, which is monounsaturated fatty acid, therefore we recommend the use of other types of fatty acids that have higher degree of unsaturation such as linoleic acid.

- The immobilization of LOX in glutaraldehyde CLEAs showed very low activity compared to the crude LOX. However, it showed good recyclability and handling, which would make the enzyme even more attractive to the commercial market. Therefore, would recommend the optimization of the dialyzed LOX CLEAs reaction conditions to produce higher concentrations of NK.

- Finally, we recommend the use of another type of chromatography, such as HPLC (High Pressure Liquid Chromatography) to identify and characterize the intermediate fatty acid hydroperoxides. This can assist in the optimization of the reactions, to produce more of the hydroperoxides that are essential for product formation.

CHAPTER 4

MATERIALS AND METHODS

4.1 General procedures

All chemicals used were purchased from Sigma-Aldrich or Merck (South Africa), unless otherwise stated.

4.2 Chemicals

Solvents used were HPLC grade methanol, ethyl acetate, hexane, and diethyl ether, together with deuterated chloroform (for NMR analysis). Hexane and ethyl acetate were distilled before use, and others were used without any further purification. Oleic acid (98% purity), sodium oleate (>89% OA), methylene blue, NK (>98% purity). Salts used were sodium chloride, potassium carbonate, sodium bicarbonate, magnesium sulphate, sodium hydroxide. VL, purity >65% v/v from Evolva (USA) and soya beans were obtained from Seeds for Africa (South Africa).

4.3 Instrumentation

4.3.1 Chromatography

Column chromatography was used to separate compounds, the stationary phase was packed with Macherey-Nagel silica gel (particle size, 0.063-0.200 mm) and the mobile phase was distilled EtOAc and hexane.

TLC was also employed to identify the compounds from product formed. The aluminium-backed Merck silica gel (60 F_{254}) plates were visualized under UV light or stained using potassium permanganate dip with subsequent heating using a hairdryer.

Gas chromatography (GC) analysis was performed on an Agilent 7890 GC with an auto injector, coupled with Leco Pegasus time-of-flight (TOF) mass spectrometer. The separation of the compounds was achieved by using a Restek Rxi-5Sil MS column made of 5% diphenyl/dimethyl polysiloxane with dimensions (30 m x 0.25 mm i.d x 0.25 µm) film thickness. The detection and analyte separation methods were optimized by adjusting the flow rate, temperature programs and spectra acquisition rate. Therefore, based on the modifications, the following method was adopted.

A 1µL sample was injected in split mode with a carrier gas (high-purity helium) at a constant flow rate of 1.4 mL/min. The GC oven temperature was initially set to 50°C (hold for 20 sec), then increased to 200°C (hold for 2 min) at a rate of 20°C/min and further increase to 300°C at a rate of 20°C/min. The temperatures of the transfer line and front inlet were 300°C and 200°C, respectively. The total run time was 14 min 7 sec. The mass spectrometer was operated in electron impact mode using an ionization voltage of 70eV, and the ion source temperature was 200°C. The mass spectral scan rate was 10 spectra per second at a set mass range of 35-450 m/z. The Leco ChromaTOF software and NIST library was used for data processing and peak identification.

4.3.2 Spectroscopic and Physical Data

The proton (^{1}H) and carbon (^{13}C) Nuclear Magnetic Resonance (NMR) data was recorded on 300, 400 and 500 MHz Bruker spectrometers.

The Ultraviolet-visible (UV-VIS) spectrophotometer used was Cary 100 UV-VIS from Agilent Technologies and the processing software was Cary WINUV. The optical system was a double beam, and the light source was tungsten halogen.

4.4 Sample preparation procedures

4.4.1 NMR

For NMR analysis, each product isolated in a form of oil was dissolved in deuterated Chloroform prior to analysis.

4.4.2 GC-MS

Every oil sample was dissolved in HPLC grade MeOH to make concentrations of 100-500 ppm, then diluted as needed. The standard curves of VL and NK were prepared in the range of 10 - 100 ppm. The calibration curves are shown in the Appendix, **Figure E2-3**. The LOD (Limit of detection) for VL was 5.80 ppm, LOQ (Limit of quantification) was 17.40 ppm, while for NK LOD was 11.66 ppm and LOQ was 33.78 ppm.

4.5 Enzyme extraction methods

Method A
The soybeans were prepared by crushing into a coarse meal using a mortar and pestle, or into flour by a coffee grinder. This coarse meal or flour were then suspended in a buffer and left in a refrigerator (4°C) with no form of agitation, overnight.

Method B
For smaller scale extraction, a 2 g soya flour was soaked in buffer at the various pH values and molarity. The soaked samples were placed on a shaker for 30 min at room temperature and allowed to cool for 45 min at 4°C.

4.6 Enzyme activity assay of LOX

4.6.1 Preparation of LOX enzyme substrate solution

The substrate reaction contained OA (0.895 g, 3.19 mmol), $FeSO_4.7H_2O$ (0.025 g, 0.09 mmol), $MnCl_2.4H_2O$ (0.025 g, 0.13 mmol) and the crude LOX extract in sodium borate buffer. The reaction was conducted in a small vial and was left open for 30 minutes at 35°C. The substrate solution was then used immediately for the enzyme assays, the solution was not stored due to the instability of the hydroperoxides.

4.6.2 Preparation of FOX activity assay

A stock solution of 100 mL Xylenol Orange (XO) reagent that consisted of 90% methanol (v/v), 10% H_2SO_4 (25 mM), 4 mM BHT, 250 µM ammonium ferrous sulphate, and 100 µM xylenol orange was prepared. Then in an Eppendorf tube, 2 mL of the XO reagent added and increased to 2.1 mL with ethanol. The isolated substrate solution oil (10 µL) was added to the tube and the sample was centrifuged for 5 min at 10000 g. The sample was allowed to form the FOX complexes for 30 min before analysis on the Cary 100 UV-VIS spectrophotometer at 596 nm.

4.6.3 Preparation of MB activity assay

A stock solution of 100 µM methylene blue (MB) reagent was prepared by dissolving 3.2 mg of MB in deionized water at room temperature. In an Eppendorf tube, the reagent was diluted to 5 µM and 10 µL of the substrate solution was added. The sample was centrifuged for 5 min at 10000 g and allowed to bleach for 30 min. Then the sample was analysed on the Cary 100 UV-VIS spectrophotometer at 664 nm. The calibration curve (Appendix) was prepared by diluting the stock solution to concentration of 0.5-5 µM.

4.7 Biocatalytic reactions procedures

4.7.1 The biphasic reaction

Method A

The reaction was initiated by the addition of VL (23 g, 112.55 mmol) and hexane in a 3-neck reaction flask. A reflux condenser was connected to cool the reaction and collect any volatile products/reagents. The temperature of the reaction was set to 35°C and an air pump (fitted with tubing bearing a glass capillary to blow air into the reaction liquid mixture) was also connected to the flask as a source of oxygen. Then OA (11.19 g, 39.61 mmol), $FeSO_4.7H_2O$ (0.25 g, 0.899 mmol), $MnCl_2.4H_2O$ (0.25 g, 1.12 mmol) and crude LOX (pH 8.5, 100 mM, sodium borate buffer) were added.

The reaction was monitored for a period of 7 days and a sample (1-2 mL) was taken from the reaction flask every 24 h. The sample was dissolved in 5 mL HPLC grade MeOH, dried with $MgSO_4$ and injected into the GC after filtration of the $MgSO_4$ salts. On the last day of the reaction, the suspension was extracted with EtOAc (x3) and worked up with 1 M NaOH and brine according to the procedure in **4.8.1**. The organic layer was dried with $MgSO_4$ and further concentrated by vacuum. The isolated product was dark brown and was subsequently analysed by GC-MS. The mass yield was 75%.

Method B

The same procedure as Method A was followed, however the reaction time was reduced to 2 days. The mass yield of the isolated brown oil was 65%.

4.7.2 Single-phase procedure

In a 3-neck reaction flask, VL (11.5 g, 56.28 mmol) and OA (5.6 g, 19.83 mmol) were heated to a constant temperature of 35°C. The air pump and reflux condenser were also connected. The scale of the reaction was reduced to save reagents. The crude LOX (pH 8.5, 100 mM, sodium borate buffer) together with $FeSO_4.7H_2O$ (0.125 g,

0.45 mmol) and $MnCl_2.4H_2O$ (0.125 g, 0.56 mmol) were added to the reaction flask. A sample was taken every 24 h, which was worked-up according to **4.8.1** and the isolated brown oil was analysed by GC-MS. The mass yield of the product was 68%.

4.7.3 Variation in buffer type

The same method as **4.7.2** was used but the buffer was replaced by the Tris and phosphate buffers. In one reaction flask it was crude LOX in Tris (pH 7.1, 100 mM) and in the other, phosphate buffer (7.4, 100 mM). The reactions were also worked-up according to the procedure in **4.8.1**. The Tris isolated product was a yellow oil with the mass yield of 28%, and the phosphate buffer oil was clear with a mass yield of 17%.

4.7.4 Variation of substrate

The method was the same as **4.7.2** and the work-up procedure as for **4.8.1**, but instead of adding 11.5 g (56.28 mmol) of VL 5.75 g (28.14 mmol) was added, which is half the amount used in the previous reactions. The pH of the reaction was monitored, and after 24 hours was found to measure 3-4. The pH strips were used and only gave approximate readings. The isolated product was red, and the mass yield was 86%.

4.7.5 Variation of fatty acid

Method A

This reaction procedure was the same as for **4.7.2** and the work-up procedure as for **4.8.1**, but the OA was reduced, and 2.80 g (9.91 mmol) instead of 5.60 g (19.83 mmol) was added. The isolated oil was yellow, and the mass yield was 64%.

Method B

The reaction was the same as for **4.7.2** and the work-up procedure as for **4.8.1**, but no OA or fatty acid was added. The yellow oil was isolated, and the mass yield was 56%.

Method C
The method was the same as for **4.7.2** and the work-up procedure as for **4.8.1**, but OA was replaced by sodium oleate (1.52 g, 5.0 mmol). An antifoam reagent was used to reduce the foam, but it did not decrease. It was only reduced by letting the air blown on the surface of the reaction and not through it. The mass yield of the obtained brown sticky product was 25%.

4.7.6 Variation of salts

The method was the same as for **4.7.2** and the work-up procedure as for **4.8.1**, but the amount of salts added was doubled to $FeSO_4.7H_2O$ (0.25 g, 0.899 mmol) and $MnCl_2.4H_2O$ (0.25 g, 0.64 mmol). The isolated clear oil had a mass yield of 40%.

4.8 Reaction work-up optimization procedures

4.8.1 General work-up procedure

In a separatory funnel, the reaction suspension was added together with 100 mL of EtOAc as the extraction solvent, 100 mL 1 M NaOH and 100 mL saturated NaCl (brine). This was repeated 3 times, to wash the organic solvent of unwanted products and reagents. Then $MgSO_4$ was added to the organic layer before drying by vacuum roto-evaporation. Once concentrated into an oil, the product was kept at 4°C until it was analysed.

4.8.2 Variation in extraction solvent

The work-up procedure was the same as for **4.8.1**, but the extraction solvent was Et_2O instead of EtOAc.

4.8.3 Variation in salts

Method A

The procedure was the same as for **4.8.1**, but the concentration of the 1 M NaOH was increased to 2 M.

Method B

The procedure was the same as for **4.8.1**, but NaOH was replaced with saturated K_2CO_3.

Method C

The procedure was the same as for **4.8.1**, but NaOH was replaced with a combination of 20 mL saturated K_2CO_3 (100 g in 200 mL water) and 80 mL brine, followed by saturated $NaHCO_3$ (100 g in 200 mL water).

4.9 Optimization reaction of VL to NK

These reactions were each conducted according to the following conditions. In a 3-neck reaction flask, VL (11.5 g, 56.28 mmol) and OA (2.80 g, 9.91 mmol) were added. The air pump and reflux condenser were also connected. The crude LOX (pH 8.5 or 7.5, sodium borate buffer) together with $FeSO_4.7H_2O$ (0.125 g, 0.45 mmol) and $MnCl_2.4H_2O$ (0.125 g, 0.56 mmol) were added to the reaction flask. The temperature of the reactions and the buffer molarity were varied. A sample (24-96 h) was taken, then worked-up according to the procedure described in **4.8.3**. The isolated oil of one sample was analysed by TLC, NMR and GC-MS. NK Rf (20% EtOAc/Hexane) 0.26. **^{1}H NMR (400 MHz, $CDCl_3$):** δ 5.76 (1H, s, H**2**), 4.73 (2H, d, H**1'**), 2.51 (1H, ddd, H**6**), 2.25-2.34 (4H, m, H**1**, H**8**), 1.92-2.02 (4H, m, H**5**, H**7**), 1.73 (3H, s, H**3'**), 1.13 (4H, m, CH_3), 0.96 (3H, d, CH_3). **^{13}C NMR (100 MHz, $CDCl_3$):** δ

199.70 **(C2)**, 170.75 **(C8a)**, 149.10 **(C2')**, 124.69 **(C1)**, 109.25 **(C1')** 43.92 **(C5)**, 42.08 **(C3)**, 40.46 **(C6)**, 40.32 **(C4)**, 39.33 **(C4a)**, 33.04 **(C8)**, 31.62 **(C7)**, 20.84 **(C3')**, 16.86 **(CH_3)**, 14.92 **(CH_3)**.

4.10 Oxidation of VL to NK with immobilized LOX

4.10.1 Partial purification

Method A

In a 250 mL beaker, solid ammonium sulfate sufficient to give 40% saturation was added to crude LOX (pH 8.5, sodium borate buffer) with continuous stirring for 30 min. After centrifugation at 20000 g for 30 min, the supernatant was brought to 70% saturation of ammonium sulfate with stirring for 30 min. Then, after centrifugation as above, the supernatant was kept, and the pellet was resuspended in the same buffer. All purification steps were carried out and stored at 4°C until required.

Method B

Same as above but the solid ammonium sulfate was adjusted to 50% for the initial precipitation and 80% for the precipitation of the supernatant.

4.10.2 CLEA Immobilization

The dissolved LOX pellet and supernatant were both divided into 6 aliquots, resulting in a total of 12 aliquots. In each aliquot concentration of 25% glutaraldehyde was varied between 1.25, 2.5, 5, 10, 15, 20% and these solutions were left to shake at 300 rpm for 3 h. After centrifugation for 30 min at 20000 g, the resultant supernatant was kept at 4°C for MB assay analysis. The pellet was resuspended in buffer (pH 8.5, sodium borate) and then divided into 12 equal aliquots. Only 6 aliquots were dialyzed against the same buffer, until the conductivity of the dialyzing buffer was the same as the original buffer. After dialysis, the aliquots were stored at 4°C until required. The activity of the CLEAs were analysed by first preparing the substrate

solution according to the procedure described in **4.6.1** and then by the MB enzyme assay, as described in **4.6.3**.

4.10.3 Reaction procedure

The reaction was conducted according to the procedure described in **4.9**, at a constant temperature of 35°C, and a duration of 48 h. And the work-up according to **4.8.3**.

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APPENDIX A: TABLES

Table A1: Reactions at different conditions with crude LOX in sodium borate buffer at pH 7.5 and the mole percentage (mol%) of the recovered VL, converted NK and other by-products.

Entry	Sodium borate buffer pH	Buffer Molarity (mM)	Reaction Temp. (°C)	Reaction duration (h)	Reaction pH	Recovered VL (mol%)	Converted NK (mol%)	Other Product (mol%)
1	7.5	150	30	24	5	7	46	47
				48	5	7	44	49
				72	5	7	43	50
				96	4	7	15	78
2	7.5	100	30	24	5	8	59	33
				48	4	8	61	31
				72	4	8	62	30
				96	4	8	19	73
3	7.5	150	21	24	6	7	66	27
				48	6	7	62	31
				72	5	7	60	33
				96	5	7	16	77
4	7.5	100	21	24	5	8	63	29
				48	4	7	67	26
				72	4	7	69	24
				96	4	7	12	81
5	7.5	15	30	24	3	11	61	28
				48	3	8	61	31
				72	3	8	62	30
				96	3	8	18	74
6	7.5	10	30	24	2	10	49	41
				48	2	8	50	42
				72	2	8	50	42
				96	2	7	19	74

Table A2: The statistical analysis as (Average, Standard deviation, and Standard error) of data in **Table A1**.

pH 7.5					
Entry	**Analysis**	**Duration (h)**			
		24	**48**	**72**	**96**
1	VL (ppm) Average	5.13	4.96	4.78	4.31
	VL Standard Deviation	0.19	0.68	0.43	0.34
	VL Standard Error	0.11	0.39	0.25	0.20
	NK (ppm) Average	31.28	39.68	30.27	5.03
	NK Standard Deviation	2.43	3.51	2.79	0.07
	NK Standard Error	1.40	2.03	1.61	0.04
2	VL (ppm) Average	5.77	7.32	5.41	5.65
	VL Standard Deviation	0.21	0.94	0.35	0.47
	VL Standard Error	0.12	0.54	0.20	0.27
	NK (ppm) Average	40.63	58.23	42.56	5.58
	NK Standard Deviation	2.15	4.20	1.02	0.18
	NK Standard Error	1.24	2.43	0.59	0.10
3	VL (ppm) Average	4.72	4.97	5.21	4.48
	VL Standard Deviation	0.47	0.63	0.45	0.35
	VL Standard Error	0.27	0.37	0.26	0.20
	NK (ppm) Average	45.89	40.26	41.82	4.69
	NK Standard Deviation	5.52	4.25	2.39	1.25
	NK Standard Error	3.19	2.46	1.38	0.72
4	VL (ppm) Average	5.60	4.63	4.87	4.78
	VL Standard Deviation	0.21	0.57	0.04	0.24
	VL Standard Error	0.12	0.33	0.03	0.14
	NK (ppm) Average	43.50	46.69	34.48	6.83
	NK Standard Deviation	2.50	1.89	2.58	0.26
	NK Standard Error	1.45	1.09	1.49	0.15
5	VL (ppm) Average	7.79	12.38	5.84	6.47
	VL Standard Deviation	0.60	1.10	0.39	0.75
	VL Standard Error	0.35	0.63	0.22	0.44
	NK (ppm) Average	40.58	77.40	42.57	5.15
	NK Standard Deviation	3.04	4.93	3.64	0.47
	NK Standard Error	1.75	2.85	2.10	0.27
6	VL (ppm) Average	7.08	5.43	5.30	5.56
	VL Standard Deviation	0.87	0.29	0.68	0.38

	VL Standard Error	0.50	0.17	0.39	0.22
	NK (ppm) Average	33.26	36.56	34.98	6.22
	NK Standard Deviation	8.05	4.69	6.20	1.06
	NK Standard Error	4.65	2.71	3.58	0.61

Table A3: Reactions at different conditions with crude LOX in sodium borate buffer at pH 8.1 and the mole percentage (mol%) of the recovered VL, converted NK and other by-products.

Entry	**Sodium borate buffer pH**	**Buffer Molarity (mM)**	**Reaction Temp. (°C)**	**Reaction duration (h)**	**Reaction pH**	**Recovered VL (mol%)**	**Converted NK (mol%)**	**Other products (mol%)**
1	8.1	150	30	24	6	8	68	24
				48	6	8	69	23
				72	6	8	70	24
				96	5	8	17	75
2	8.1	150	35	24	6	7	67	26
				48	5	7	69	24
				72	4	7	71	22
				96	4	7	11	80
3	8.1	150	21	24	6	8	64	28
				48	5	8	71	21
				72	5	8	74	18
				96	4	8	10	82
4	8.1	100	35	24	5	7	56	37
				48	4	7	54	39
				72	4	7	50	43
5	8.1	100	30	24	5	7	73	20
				48	4	7	66	27
				72	4	7	58	35

Table A4: The statistical analysis as (Average, Standard deviation, and Standard error) of data in **Table A3**.

pH 8.1					
Entry	**Analysis**	**Duration (h)**			
		24	**48**	**72**	**96**
1	VL (ppm) Average	5.05	4.37	5.90	6.24
	VL Standard Deviation	0.49	0.22	0.73	0.45
	VL Standard Error	0.28	0.13	0.42	0.26
	NK (ppm) Average	43.69	50.42	46.35	7.87
	NK Standard Deviation	8.00	7.39	5.40	1.24
	NK Standard Error	4.62	4.27	3.12	0.72
2	VL (ppm) Average	6.37	5.44	5.03	5.21
	VL Standard Deviation	0.45	0.64	0.47	0.22
	VL Standard Error	0.26	0.37	0.27	0.13
	NK (ppm) Average	48.96	49.83	39.37	8.01
	NK Standard deviation	9.90	3.39	4.74	0.47
	NK Standard Error	5.71	1.96	2.74	0.27
3	VL (ppm) Average	5.93	4.53	5.64	5.24
	VL Standard Deviation	0.54	0.12	0.17	0.32
	VL Standard Error	0.31	0.07	0.10	0.19
	NK (ppm) Average	43.70	46.78	54.73	12.19
	NK Standard Deviation	5.00	5.70	4.28	0.36
	NK Standard Error	2.89	3.29	2.47	0.21
4	VL (ppm) Average	5.00	5.09	5.16	-
	VL Standard Deviation	0.50	0.22	0.50	-
	VL Standard Error	0.29	0.13	0.29	-
	NK (ppm) Average	35.79	32.14	26.48	-
	NK Standard Deviation	7.61	7.60	7.17	-
	NK Standard Error	4.39	4.39	4.14	-
5	VL (ppm) Average	5.74	4.76	5.15	-
	VL Standard Deviation	0.60	0.57	1.06	-
	VL Standard Error	0.35	0.33	0.61	-
	NK (ppm) Average	50.47	31.46	39.86	-
	NK Standard Deviation	3.17	2.59	3.37	-
	NK Standard Error	1.83	1.49	1.95	-

APPENDIX B: NMR SPECTRA

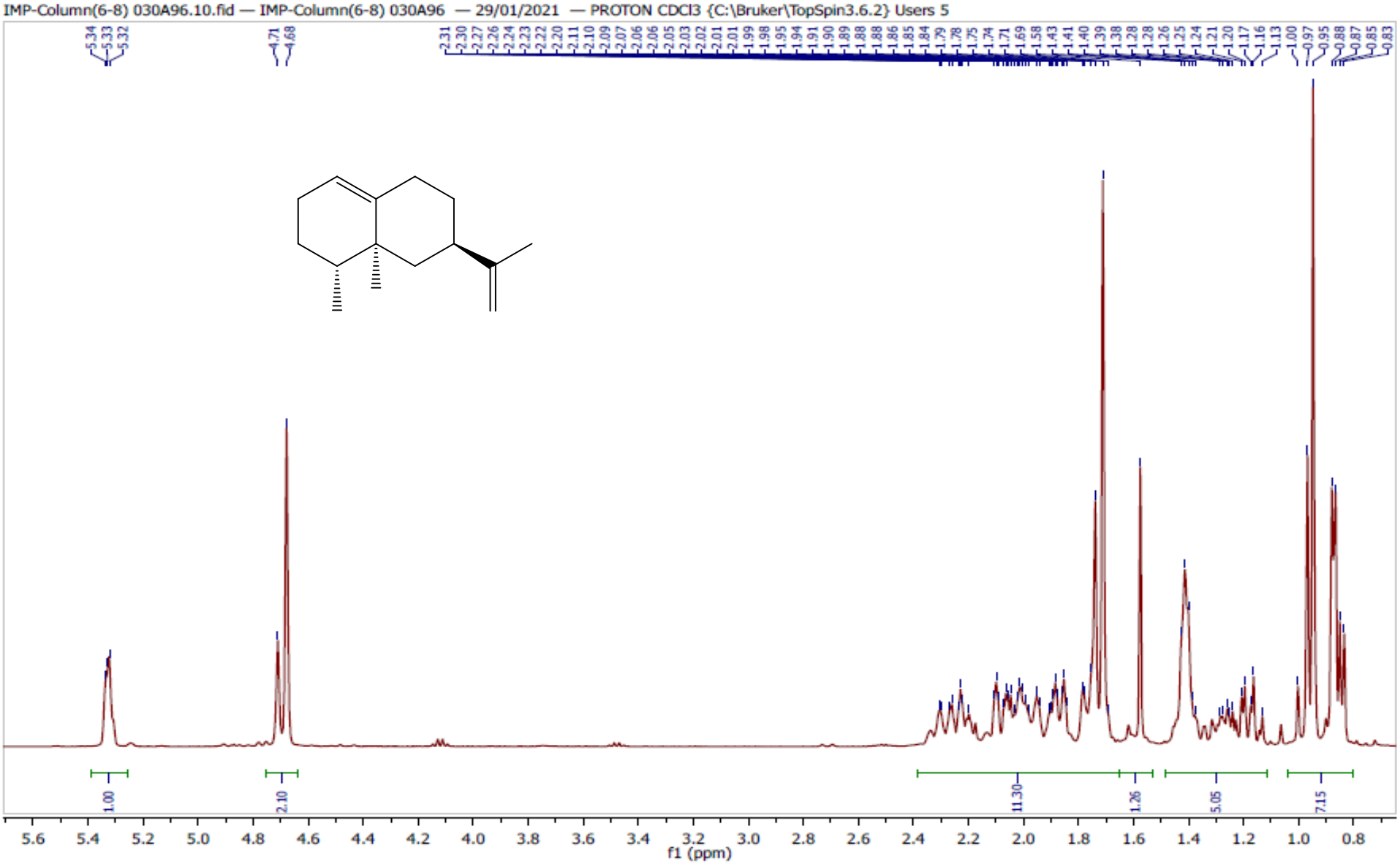


Figure B1: ^{1}H NMR spectra of Valencene

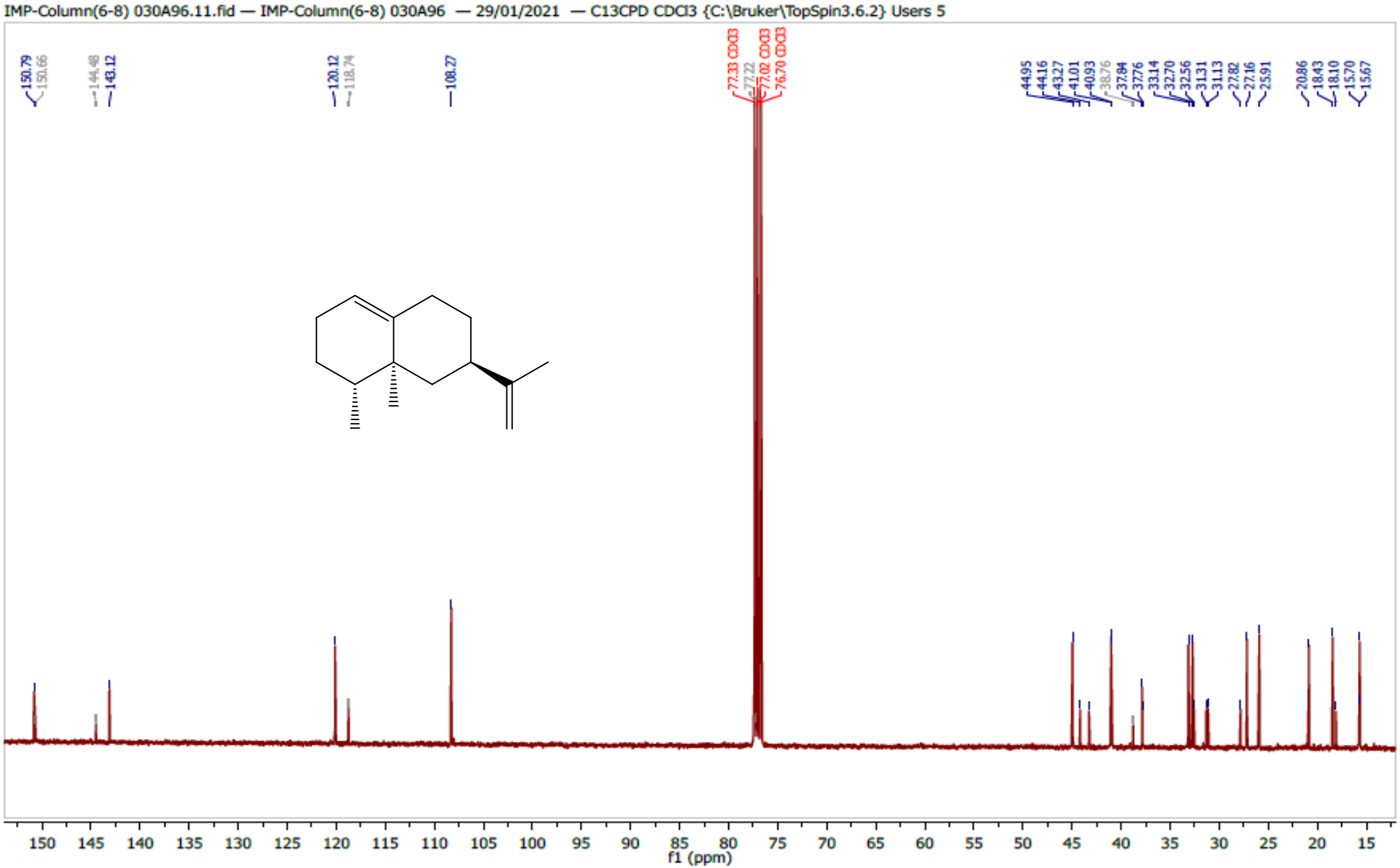


Figure B2: ^{13}C NMR spectra of Valencene

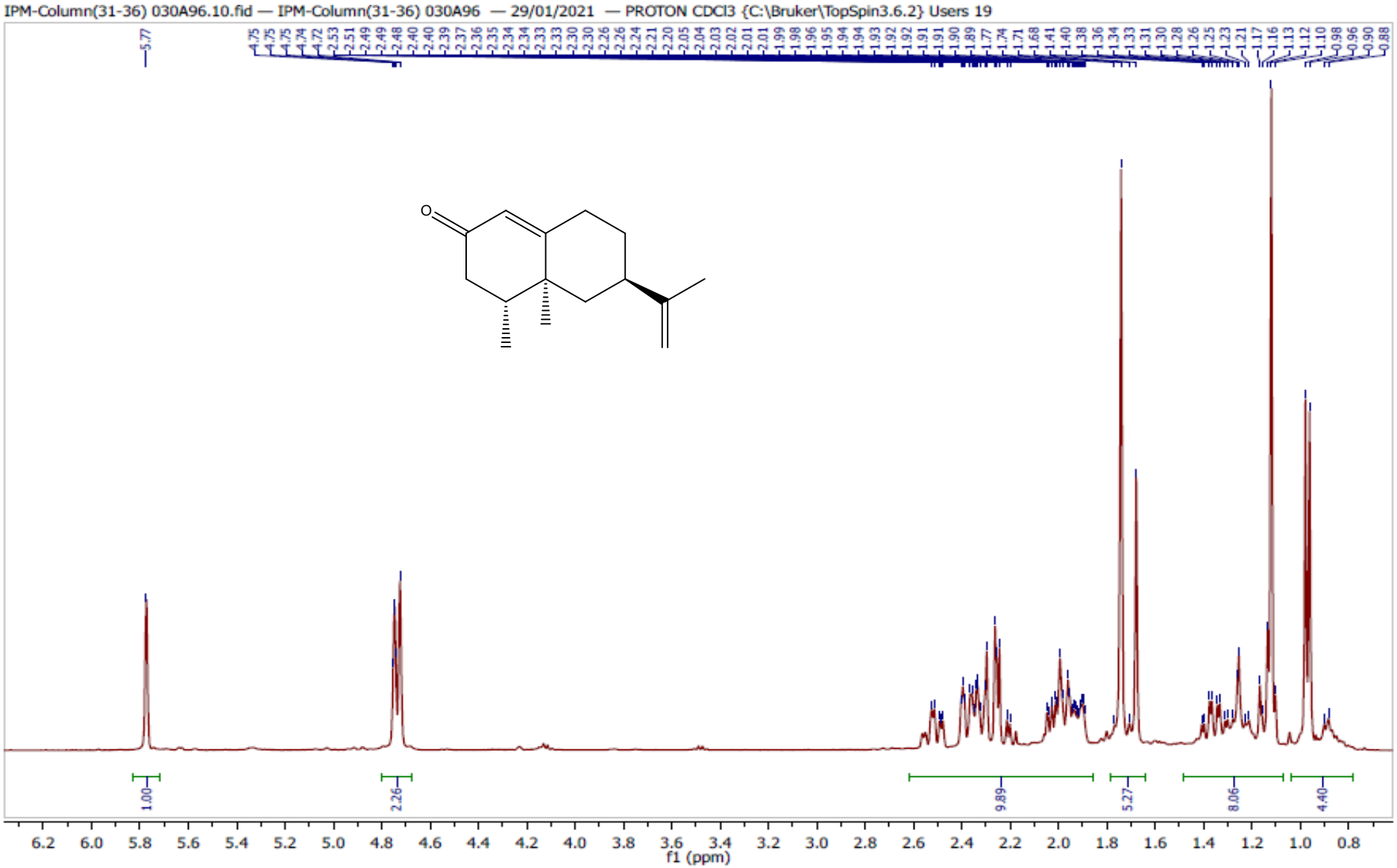


Figure B3: ^{1}H NMR spectra of Nootkatone

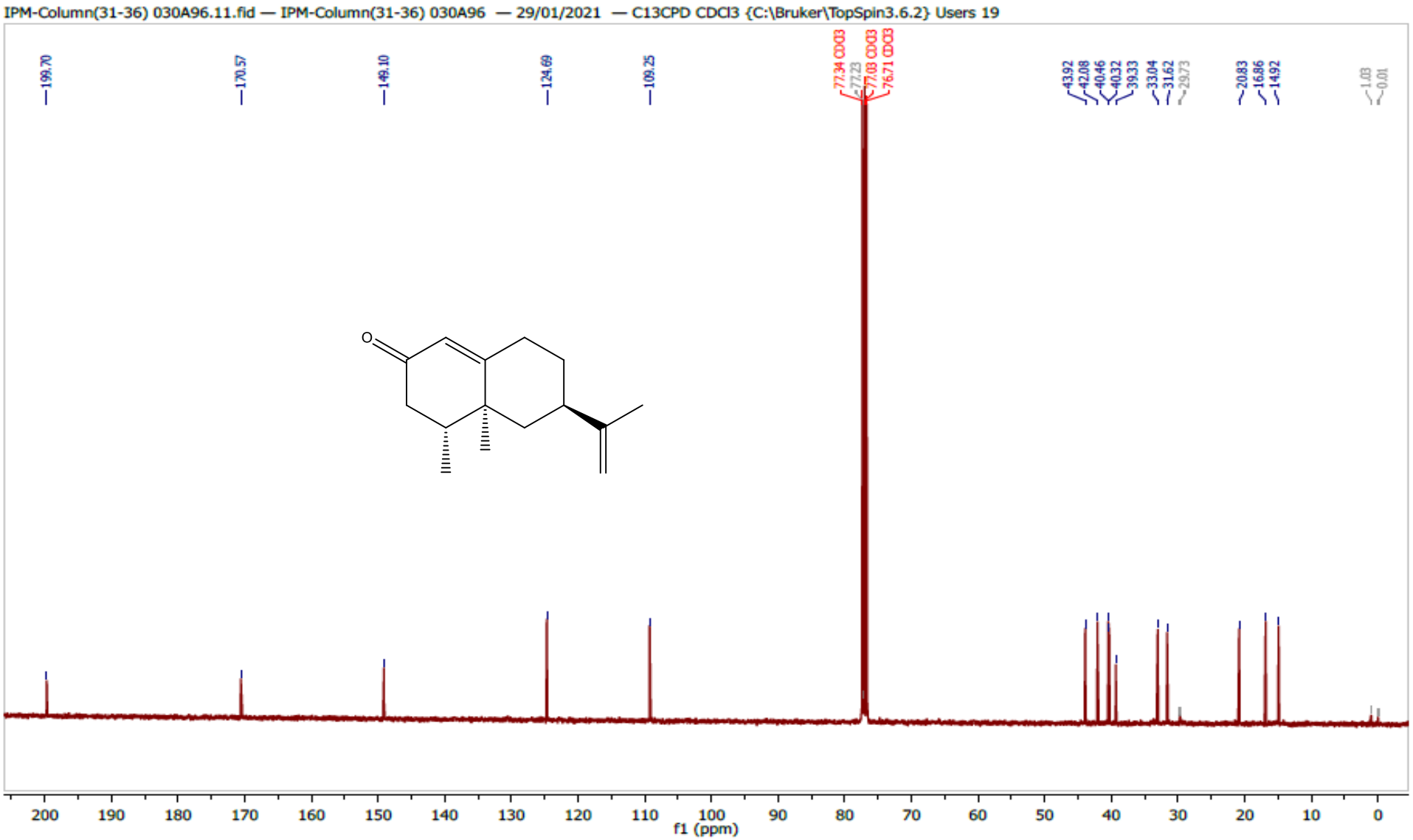


Figure B4: ^{13}C NMR spectra of Nootkatone



Figure B5: ^{13}C DEPT 135 NMR spectra of Nootkatone

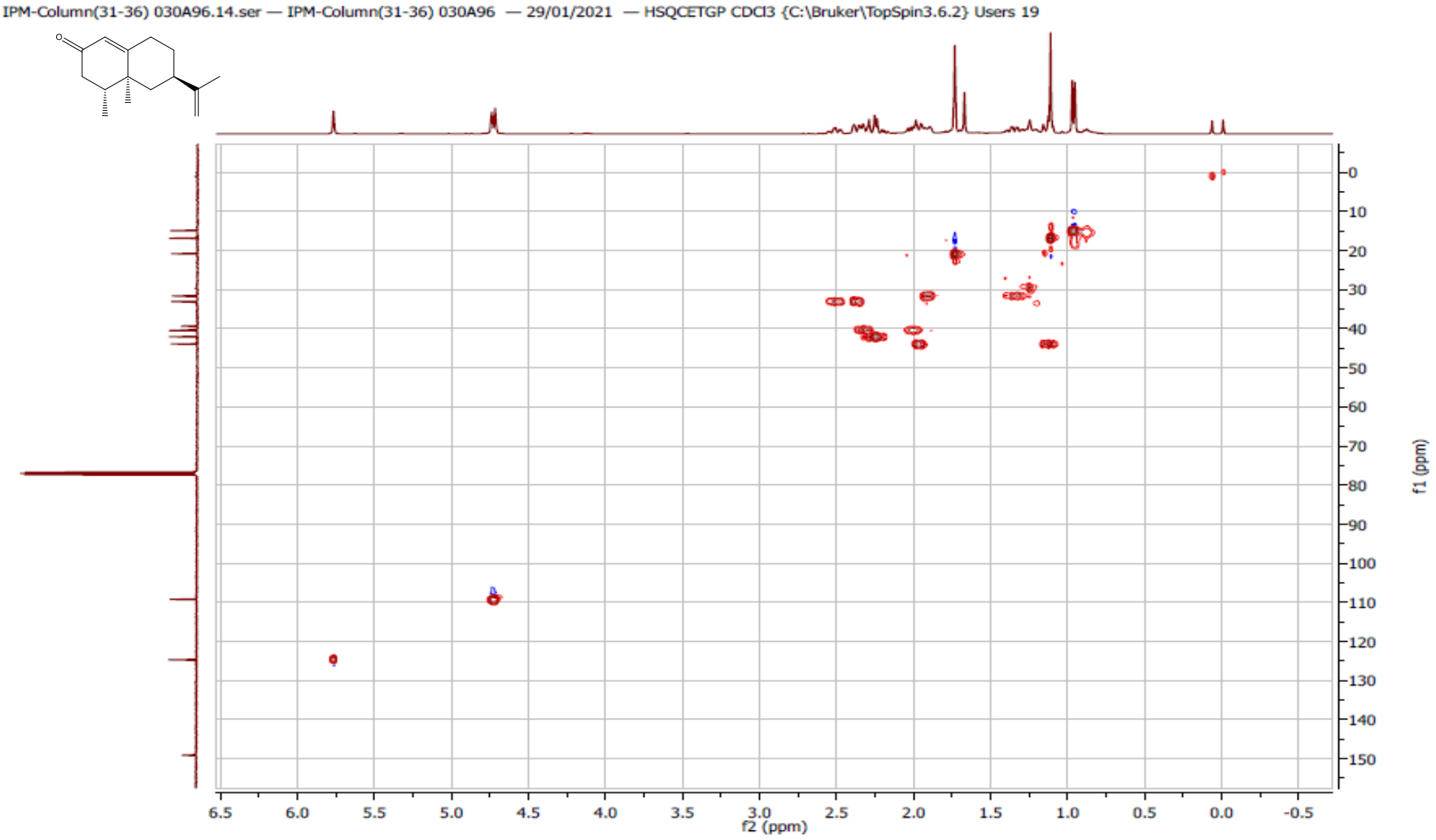


Figure B6: HSQC NMR spectra of Nootkatone

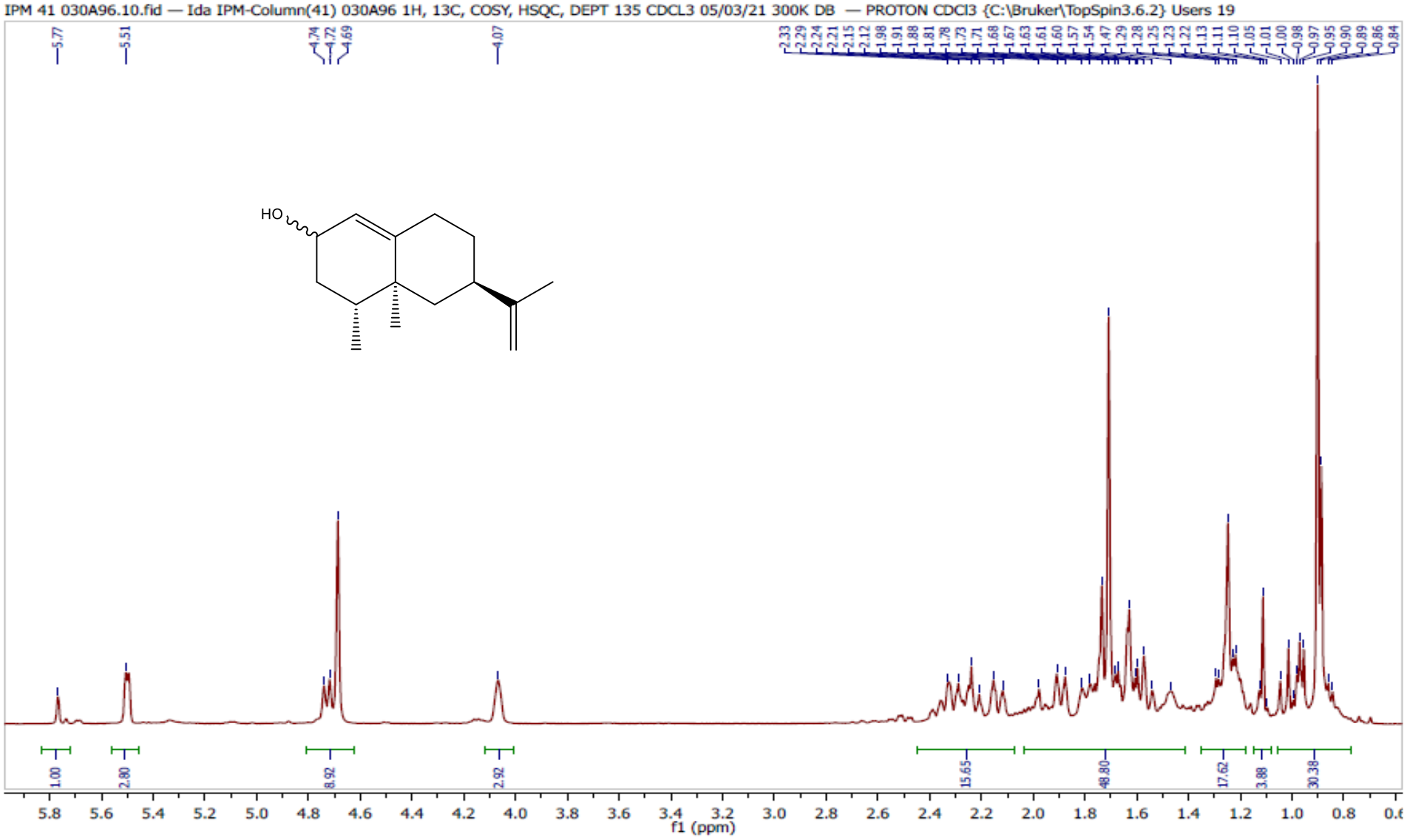


Figure B7: ^{1}H NMR spectra of Nootkatol

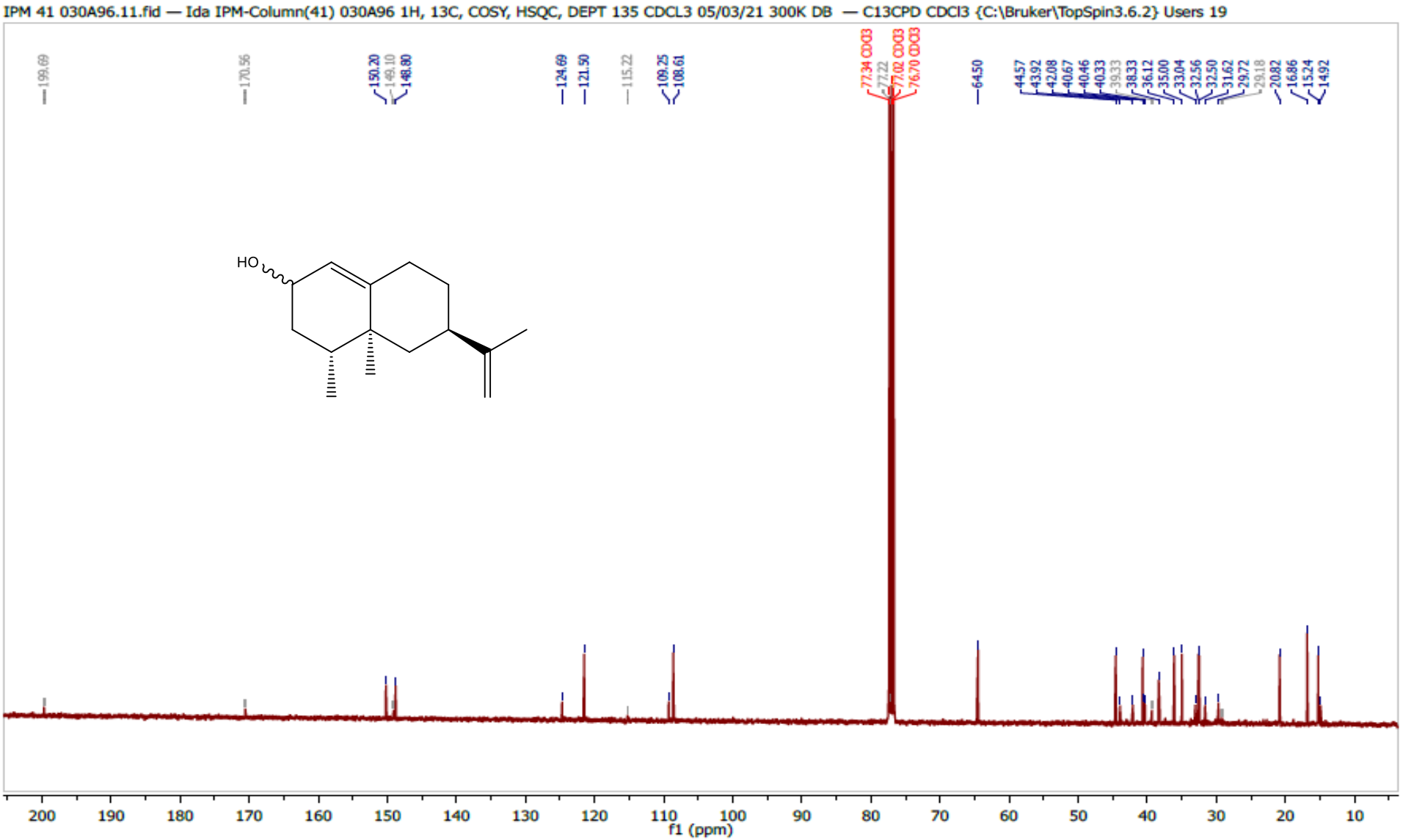


Figure B8: ^{13}C NMR spectra of Nootkatol

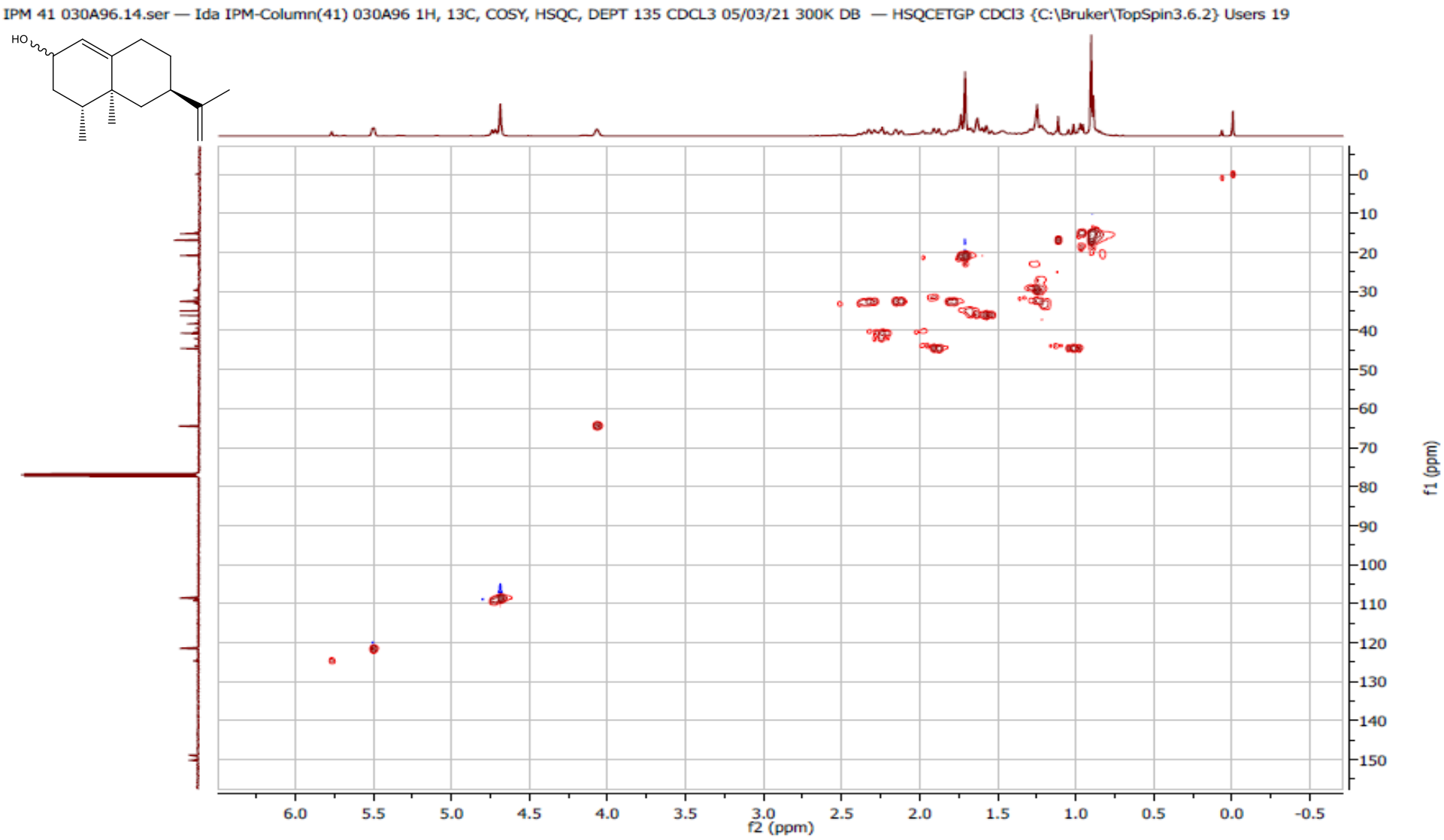


Figure B9: HSQC NMR spectra of Nootkatol

APPENDIX C: GC CHROMATOGRAMS

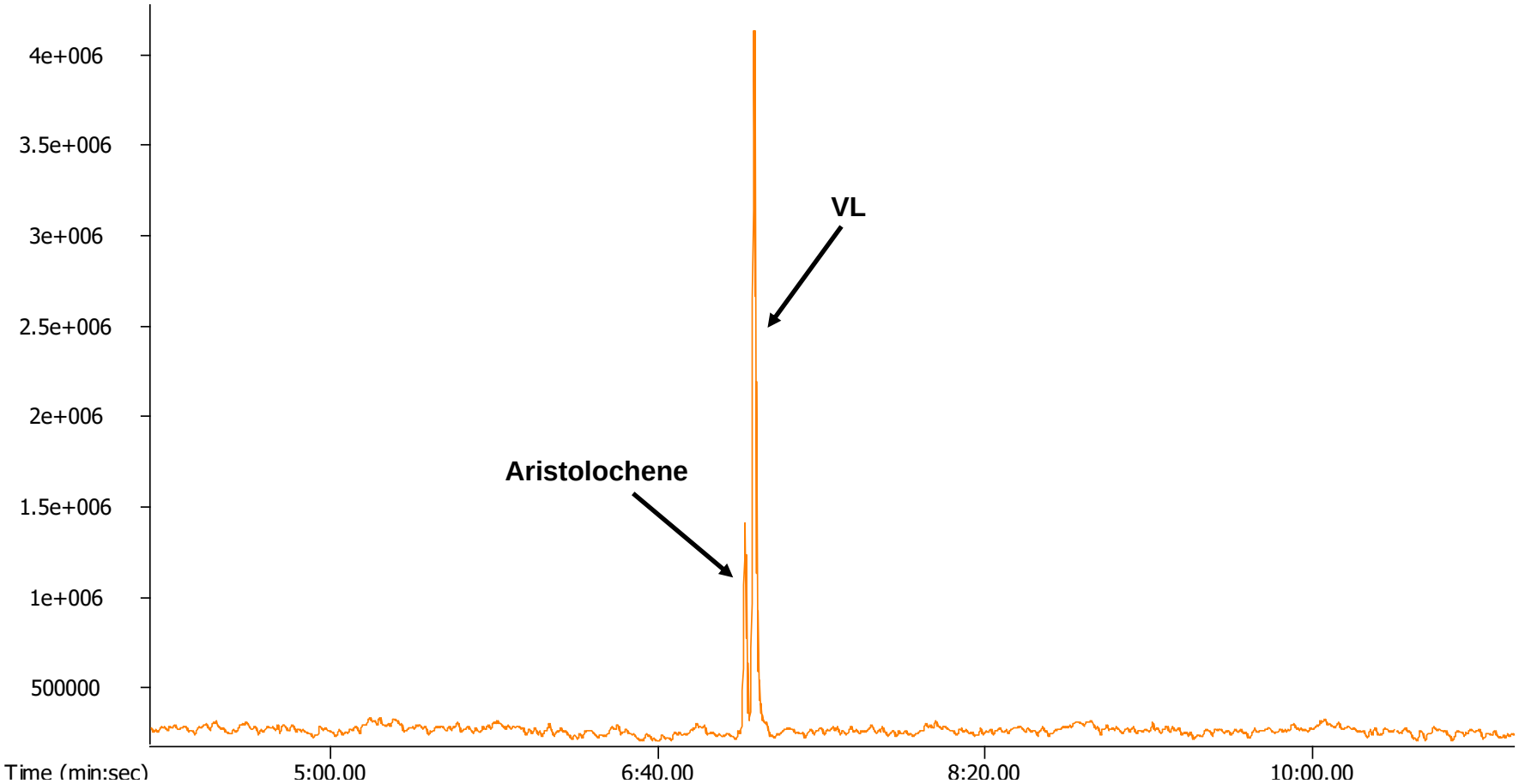


Figure C1: GC chromatogram of unreacted Valencene

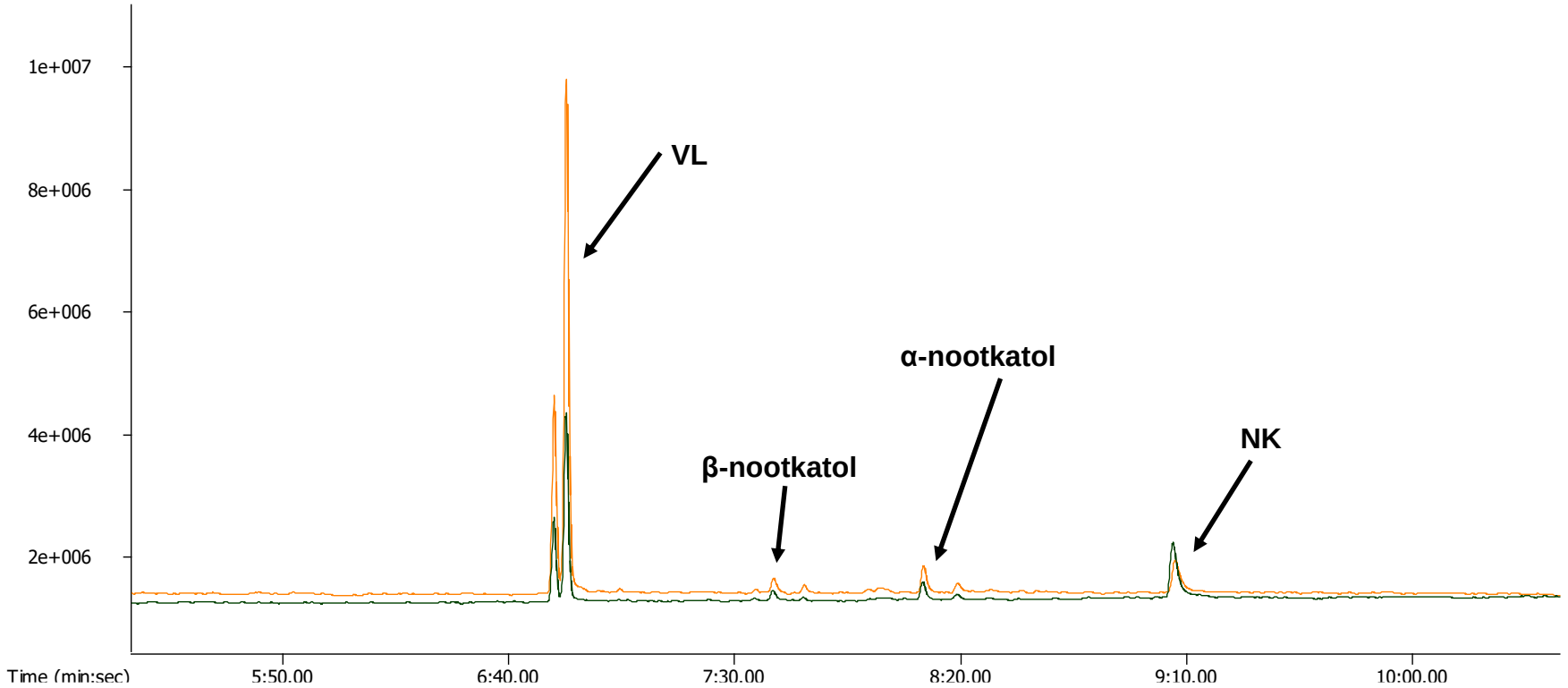


Figure C2: GC chromatogram of biocatalysed Nootkatone with the LOX CLEA pellet **(Orange)** and dialyzed LOX CLEA pellet **(Black)**

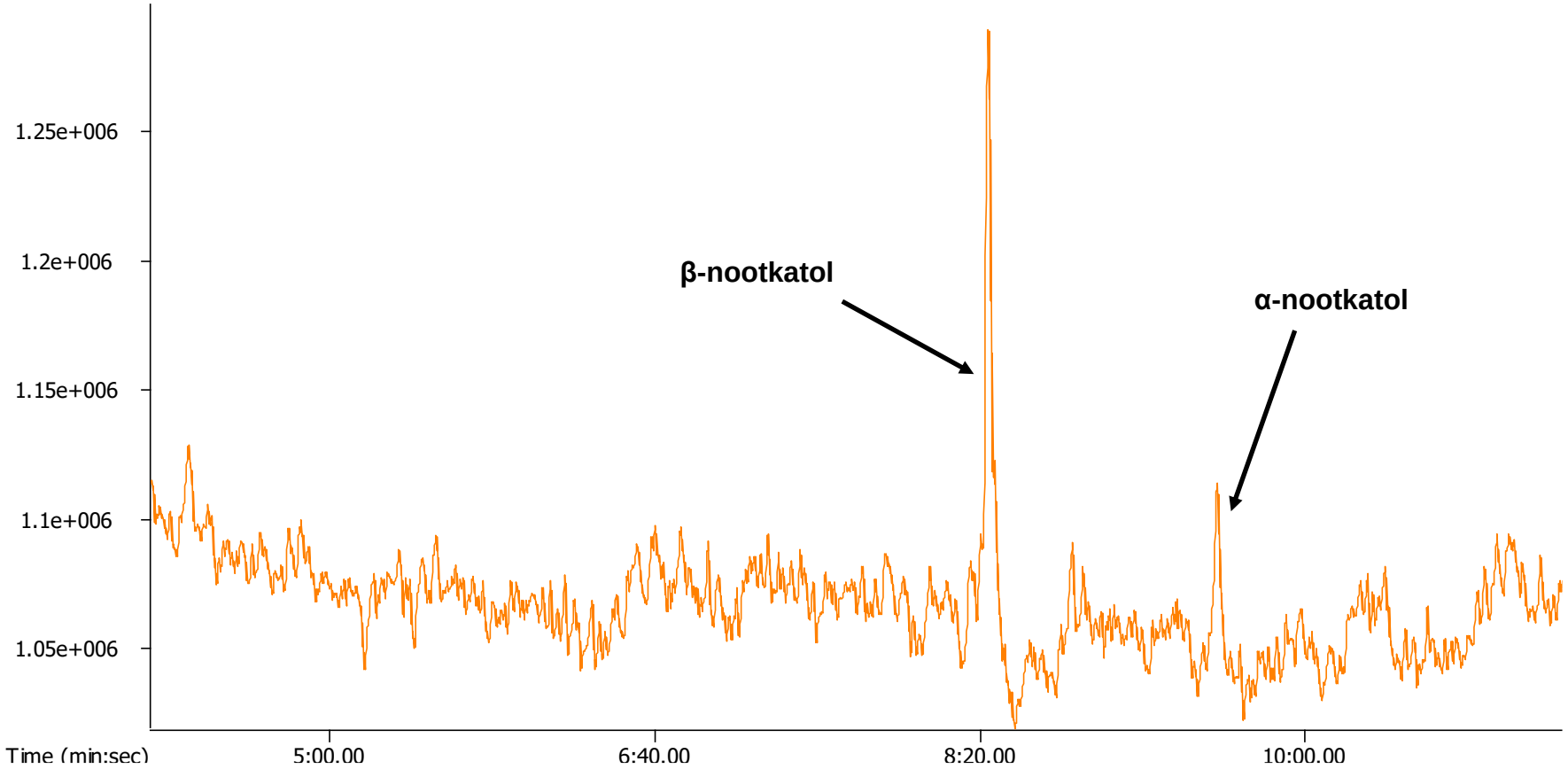


Figure C3: GC chromatogram of biocatalysed Nootkatol

APPENDIX D: MS SPECTRA

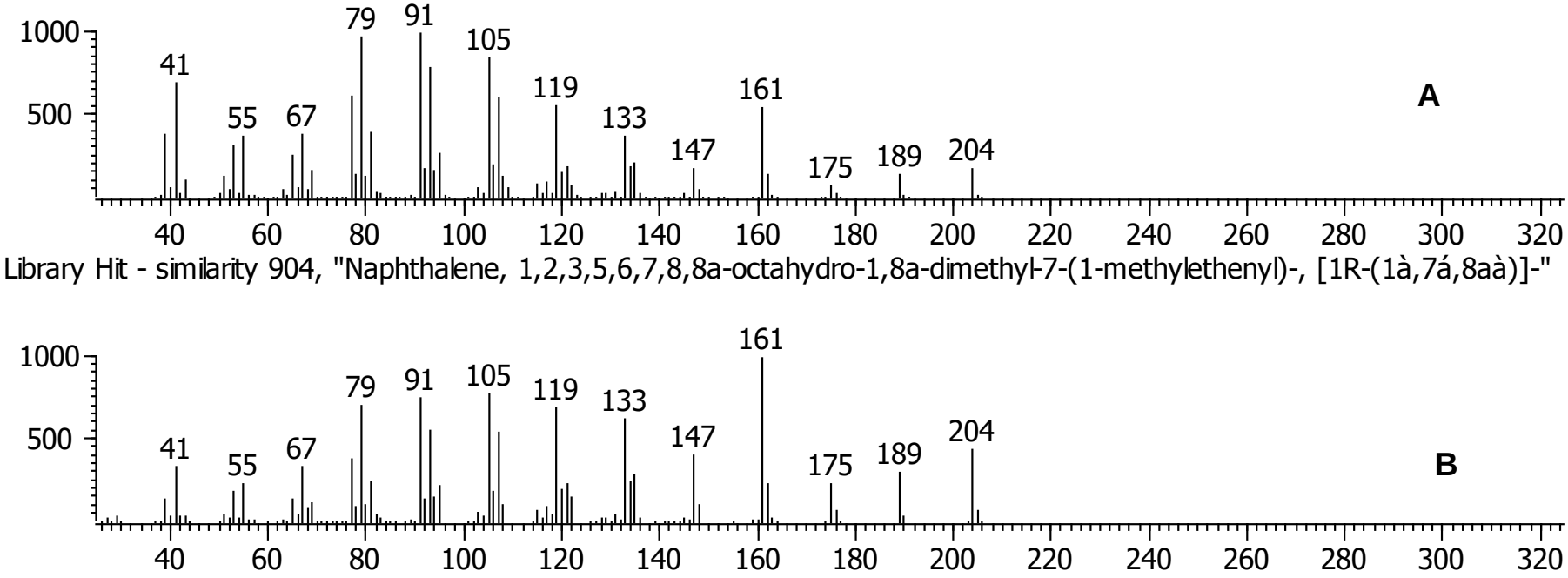


Figure D1: MS spectra of valencene **(A)** sample of VL and **(B)** the NIST library match of VL

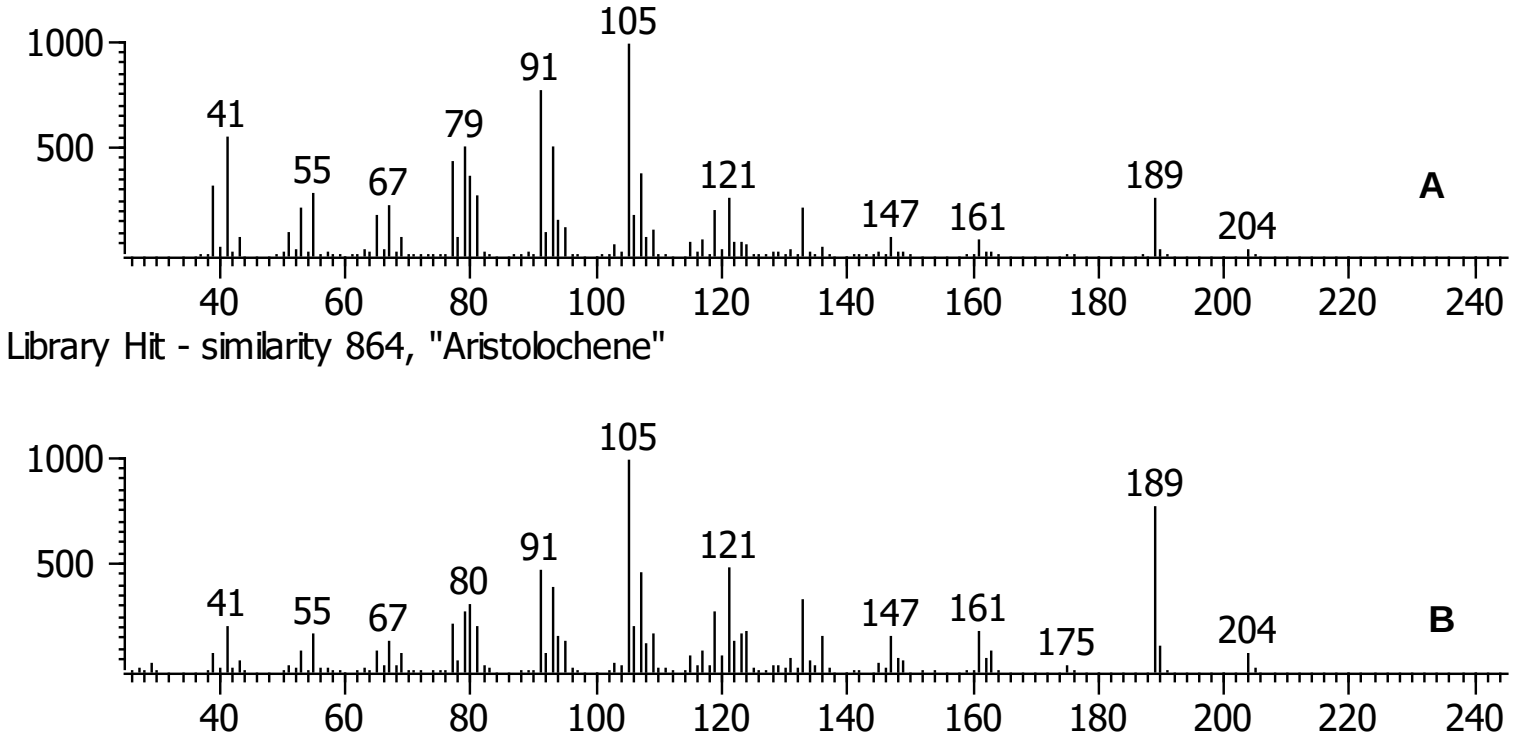


Figure D2: MS spectra of valencene **(A)** sample of Aristolochene and **(B)** the NIST library match of Aristolochene

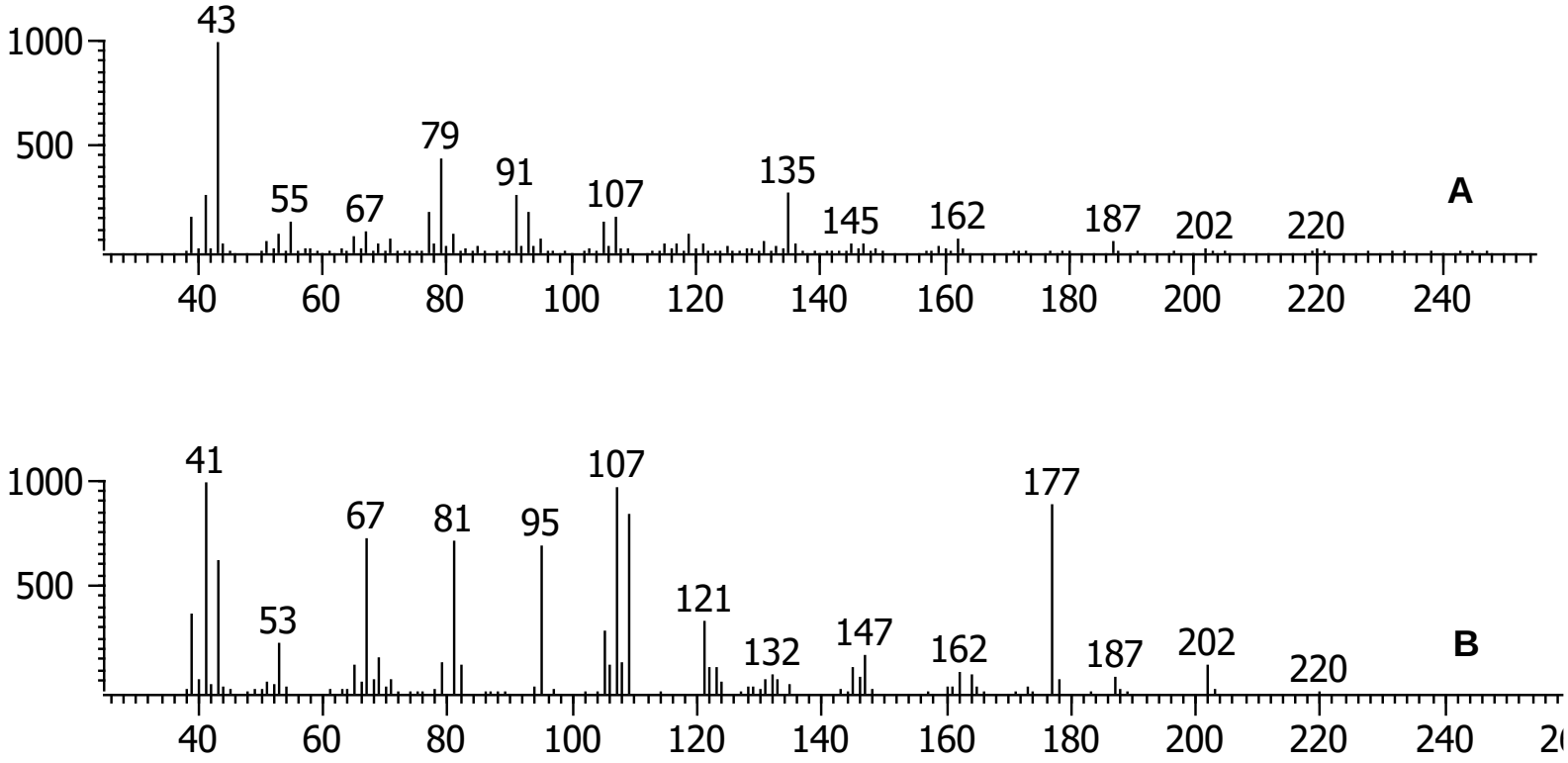


Figure D3: MS spectra of (A) β-nootkatol and (B) α-nootkatol

APPENDIX E: CALIBRATION CURVES

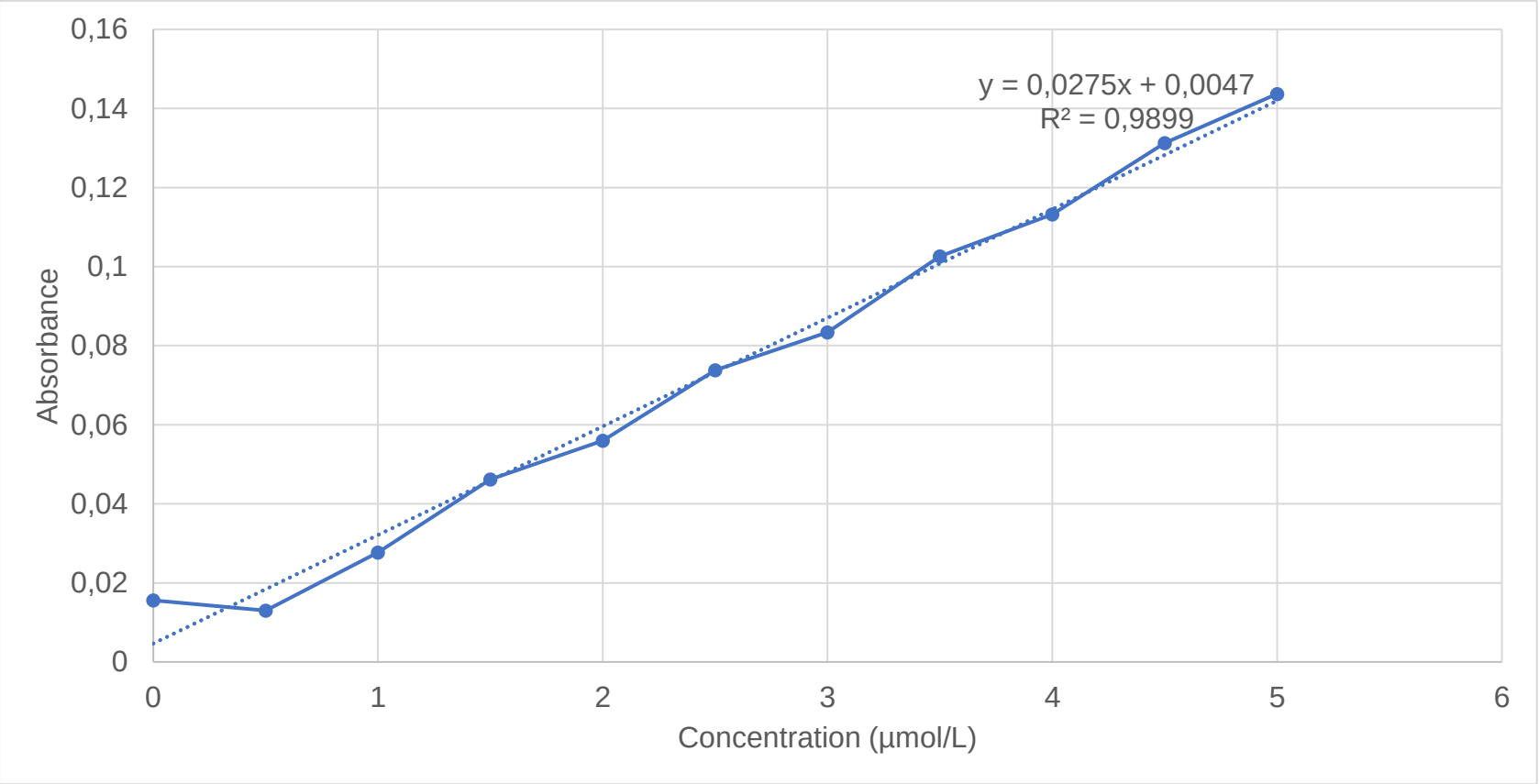


Figure E1: UV-VIS calibration curve of Methylene Blue at 664 nm.

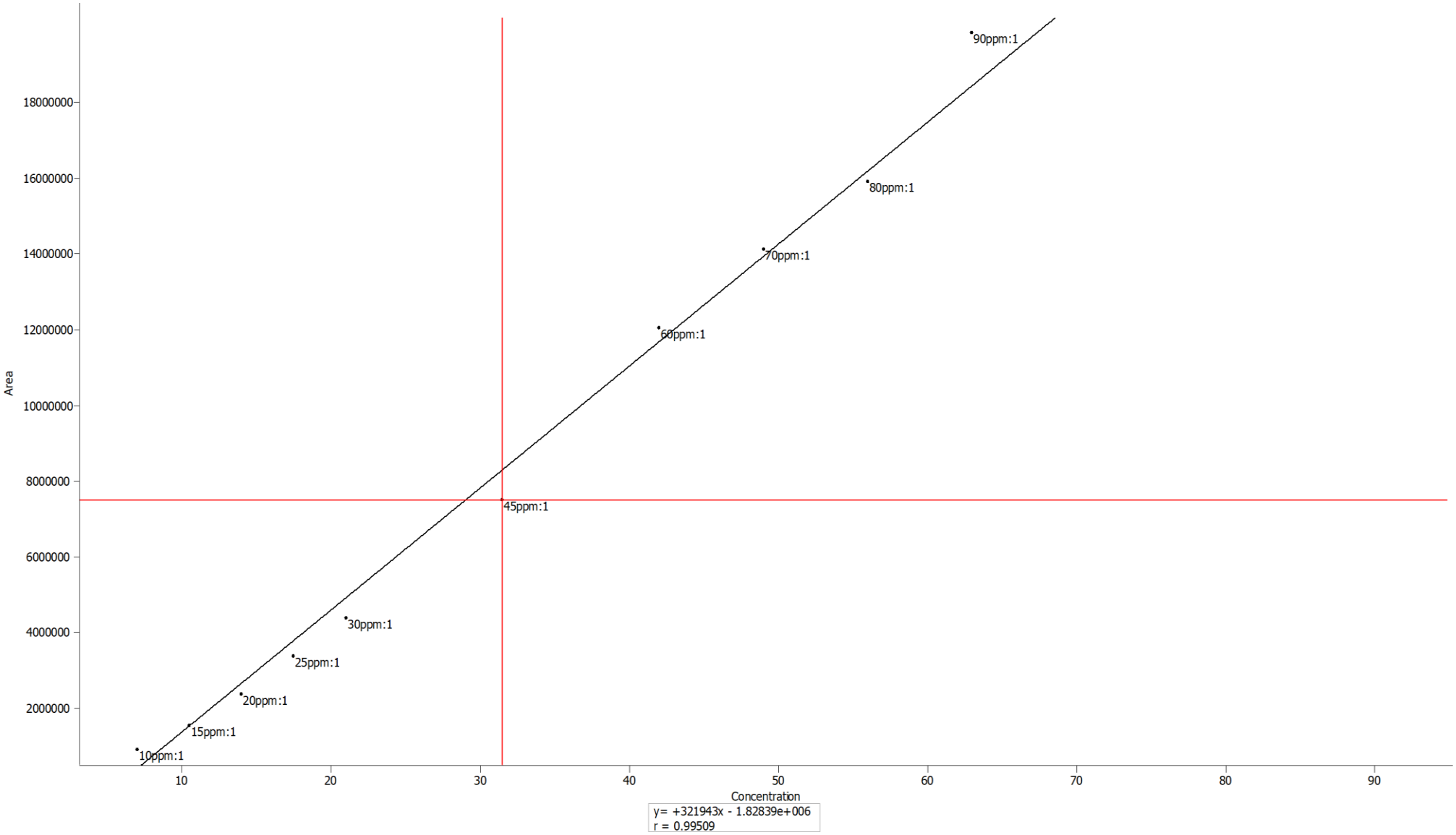


Figure E2: GC calibration curve of VL

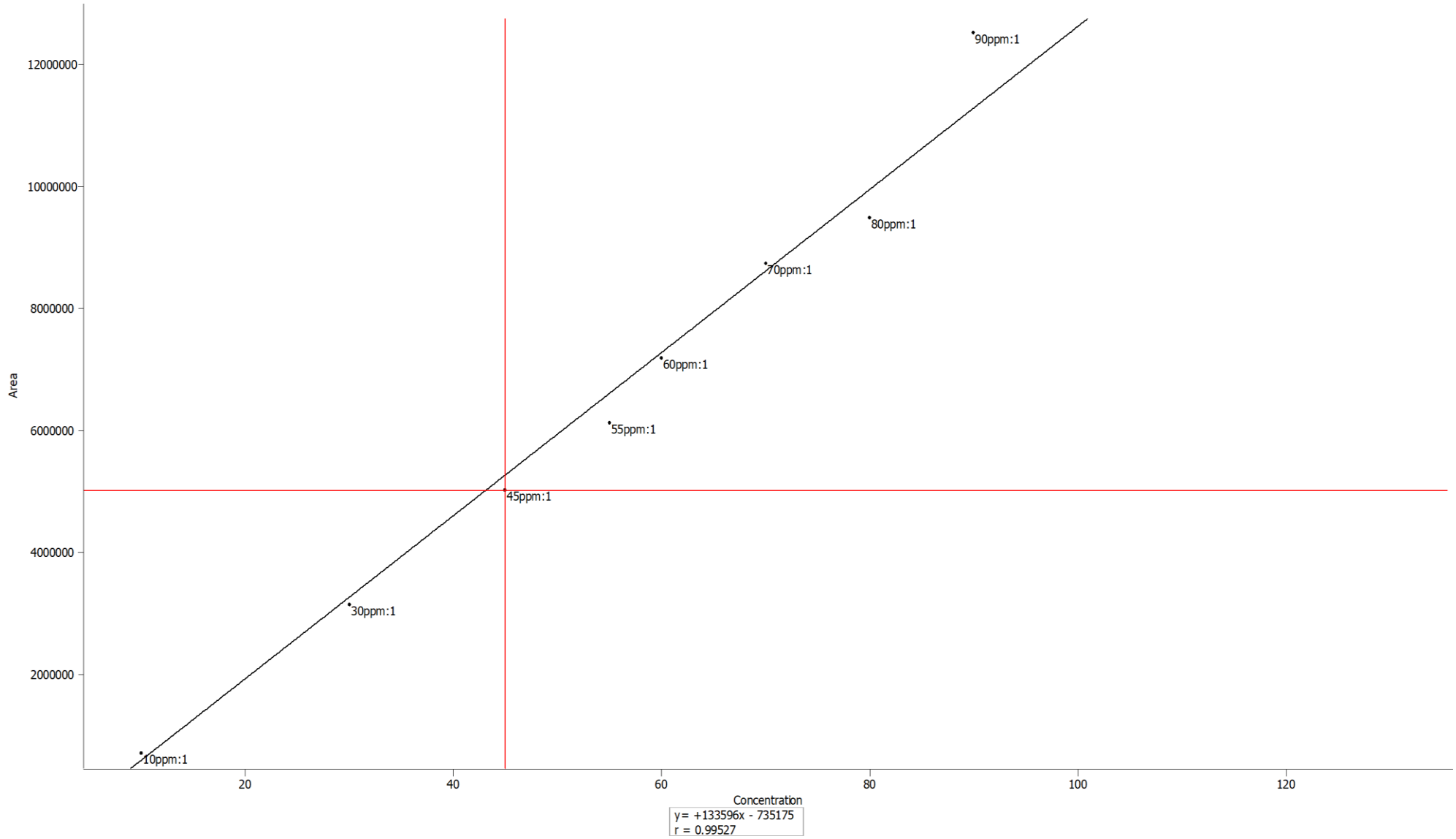


Figure E3: GC calibration curve of NK