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Process development and optimization for biocatalytic production of irones from iris root

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Process development and optimization for biocatalytic production of irones from Iris root

Dissertation submitted for the qualification

Master of Science in Chemistry (full time)

University of Witwatersrand

By

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ABSTRACT

Irones are the pleasantly smelling terpenoids of orris oil used in the fragrance industry that are extracted from the rhizomes of Iris species through a lengthy process. Unfortunately syntheses of irones using chemical methods or Iris rhizome-derived sources have been reported to be long, unsafe and low yielding. These inefficient methods have therefore resulted in a high cost of the orris absolute (an alcohol extract of orris butter) which costs between 40 000 and 70 000 Euros/kg. A promising enzymatic process for irones production with good conversion of the precursors has been reported. However, the processing of the irone source into iridals (irone precursors) requires high temperatures and as a result the safety as well as energy input of the method is affected. Moreover, the prior solvent extraction was reported to affect the quality of the product. The present research aimed to develop a rapid and effective enzymatic process for the production of α- and γ-irones at 2L scale-up, as well as obtaining the final product in the form of orris butter.

During the current studies a method for analysis of the irones samples was developed, and subsequently different solvents were investigated to identify the best sampling method. Thereafter different oxidoreductases were screened to identify the best enzyme source for maximum production of irones. Optimization of temperature, loading of lipoxidase, orris root, oleic acid, and dioxane, the ratio of minerals to irone concentration, incubation period and use of anti-fungal agents were investigated for maximum irones production. Furthermore, different methods to concentrate the irones and to produce the final orris butter product were evaluated. Lastly, testing the effect of purifying the enzyme on the production of irones was investigated.

At laboratory scale the optimum reaction conditions were found to consist of incubating 5 g of fresh homogenised orris root with 20 mL crude soybean lipoxidase (prepared as 1 g soybean flour in 25 mL of 0.01 M borate buffer pH 9.2), with 50 mg manganese chloride and 25 mg ferrous sulphate, 1 mL dioxane and 0.25 mL oleic acid, for 5 days at 37°C in a rotatory shaker incubator. The laboratory scale product could be recovered by using a mixture of equal proportions of acetone and-DMSO for sampling under stringent sterile conditions. At the 700 mL and 2L scale it was determined that with improved agitation and oxygenation of the reaction mixture and subjecting the maturated suspension to Likens-Nickerson distillation resulted in the required orris butter profile. It was further found that purification of the enzyme reduced its ability to efficiently convert the precursors in fresh orris root into irones. Alpha (α-) and gamma (γ-) irones) of the same retention times (3.79 and 3.82) and mass (207 Da) as the α-irone commercial sample were produced at yields around 696 mg irone/kg dry orris root compared with 530 mg irone/kg dry orris root seen with traditionally

processed rhizome. The current research is the first to use crude soybean lipoxidase to oxidize macerated fresh orris root into irones, and the first to identify the importance of minerals in the bioconversion.

DECLARATION

The work I am submitting is my own original work and all sources used in the present study have been acknowledged by means of full references. Furthermore, the present work has not been submitted either in part or in full for any other degree elsewhere or at the University of the Witwatersrand. Moreover, this work has not been previously published.

_____ _____*November 2015* *Ronny Mogege Mohlala*

Signature *Date* *Names and Surname*

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
BSA	Bovine Serum Albumin
C-C	Carbon-Carbon
C-N	Carbon-Nitrogen
C-O	Carbon-Oxygen
CSIR	Council for Scientific and Industrial Research
Cu	Copper
DMSO	Dimethyl Sulphoxide
ee	Enantiomeric excess
EPR	Electron Paramagnetic Resonance
HPLC	High Performance Liquid Chromatography
NRF	National Research Foundation
OD	Optical Density
PEG	Polyethylene glycol
PEP	Phosphoenolpyruvate
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TMA	Trimethyl ammonium
TMOS	Tetramethyl Orthosilicate

SYMBOLS

α- Alpha

β- Beta

γ- Gamma

DEFINITION OF TERMS

Acetylinic: a doubly unsaturated position on a molecular framework, for instance in an alkyne such as acetylene.

Acylases: any of several enzymes that hydrolyse acylated amino acids.

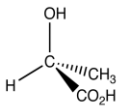
Aminopeptidases: catalyze the cleavage of amino acids from the amino terminus of protein (N-terminus) or peptide substrates.

Aromatic: Relating to or denoting organic compounds containing a planar unsaturated ring of atoms which is stabilized by an interaction of the bonds forming the ring, e.g. benzene and its derivatives.

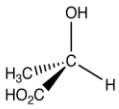
Biocatalysis: is the use of natural catalysts, such as protein enzymes, to perform chemical transformations on organic compounds.

Chiral molecule: is a molecule that is not superimposable on its mirror image.

e.g. 1:



1



mirror image of **1**

Ionones: are aroma compounds found in a variety of essential oils, including rose oil.

Enantioselectivity: the degree to which one enantiomer of a chiral product is preferentially produced in a chemical reaction.

Enzymes: are biological molecules (proteins) that act as catalysts and help complex reactions occur everywhere in life.

Fragrance: the quality or state of having a sweet odour.

Hydratase: any enzyme that catalyzes the addition or removal of the elements of water.

Immobilized enzyme: is an enzyme that is attached to an inert, insoluble material such as calcium alginate.

Iris absolute-Is one of the most expensive of all natural perfume materials obtained by alcoholic extraction of orris butter to separate the fatty acids and their esters.

Odour: a distinctive smell.

Orris butter/ Orris concrete oil (*Buerre d'Iris*) - is the steam distillation product of dried ground rhizomes with yields between 0.2 and 0.3 %.

Racemates: a mixture of equal quantities of two enantiomers, substances whose molecular structures are mirror images of one another

Orris resinoid- is the product of extraction of the comminuted rhizomes with yields of 2.4 to 3.3 % using benzene or 1 to 2 % using petroleum ether.

Terpenes: they are a large and diverse class of organic compounds, produced by a variety of plants, particularly conifers, though also by some insects such as termites or swallowtail butterflies, which emit terpenes from their osmeteria. They are often strong-smelling.

DEDICATION

I dedicate this dissertation to my late Grandparents, Maoto and Mathabatha Mohlala; my late nephews, Kgantsho and Mogau Mohlala; my late father, Malope'a Hlabirwa and my loving Mother, Nogwane'a Hlabirwa.

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DECLARATION OF ADHERENCE TO ETHICAL CONSIDERATIONS

Although the present study did not require ethical approval, where necessary, I have adhered to the research ethics of CSIR found at http://intraweb.csir.co.za/policy/policies.php. Although no experiment was performed at Wits University, the research ethics found at http://www.wits.ac.za/academic/researchsupport/19110/ethics_and_research_integrity.html were read.

CHAPTER 1 INTRODUCTION

1.1 Background

1.1.1 Irones and their origin

Irones are the pleasantly smelling terpenoids of orris oil, important in the fragrance industry, that are extracted from the rhizomes of Iris species (*Iris pallida var. dalmatica, I. germanica* and *I. florentina*) in which they accumulate during storage (Jaenicke and Marner, 1986). A rhizome as illustrated in Figure 1, also termed orris root, is a creeping stem lying horizontally at or beneath the surface of the soil and differing from a root in having scale leaves, bearing leaves or aerial shoots close to its tips, and producing roots from its undersurface (Morales, 2013).

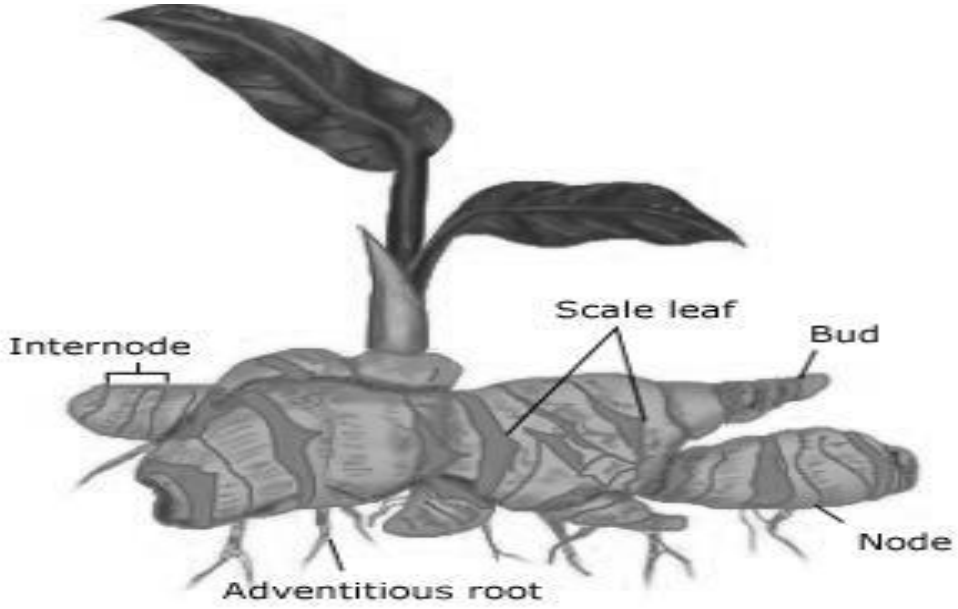


Figure 1-An illustration of a rhizome (Morales, 2013).

In history, orris root was highly prized in the perfume industry. It has been reported that the root develops a pleasant violet-like scent on drying. This scent continues to advance on storage and peaks at approximately three years. Ground orris root was widely used in face powders and other cosmetics until it was noticed it caused allergic reactions. Orris root powder is still used extensively in potpourris, sachets, and pomanders. It even prolongs the scent of the other oils (Van Hevelingen, 1992).

The violet fragrance of orris root is mainly a property of irones. The syntheses of irones and ionones were developed at the time when organic chemists recognised the importance of the violet fragrance in the perfume industry. Subsequently these compounds began to be used in large quantities. Furthermore, certain acetylinic esters possessing violet odour important in compounding

violet type fragrances were also found. Tiemann and Kruger, (1893) researched the compounds in violet flowers responsible for the fragrance. Due to the high cost of violet flowers, they chose to base their investigations on orris flowers which share similar properties. Their hypothesis was that the fragrances of these two flowers must be due to the same compound. They found that a dominant ketone is usually found in orris root oil and it contributes to the aroma. Tiemann and co-workers performed a systematic study of this ketone; determined the structure, and named it irone (Tiemann and Kruger, 1893).

Iris oil was found to contain the following four ketone isomers (Figure 2): (+)-*cis*-α-irone, (+)-*trans*-α-irone, (+)-β-irone, and (+)-*cis*-γ-irone (Rautenstrauch and Ohloff, 1971). It has been established that the distribution of irone isomers and enantiomers in different qualities of iris oils depend upon the botanical species (Krick *et al.,* 1984). The average composition (Galfe *et al.,* 1981) of an iris butter prepared from *Iris germanica* is the following: 0.91% of (+)-*trans*-α-irone (96% enantiomeric excess (ee)), 61.48% of (–)-*cis*-α-irone (82% ee), 0.71% of β-irone and 37.60 % of (–)-*cis*-γ-irone (38% ee). Conversely, when *Iris pallida* rhizomes are used, the corresponding iris butter contains 4.16% of (+)-*trans*-α-irone (98% ee), 34.46% of (+)-*cis*-α-irone (ee = 66% ee), 0.16% of β-irone and 61.26% of (+)-*cis*-γ-irone (96%).

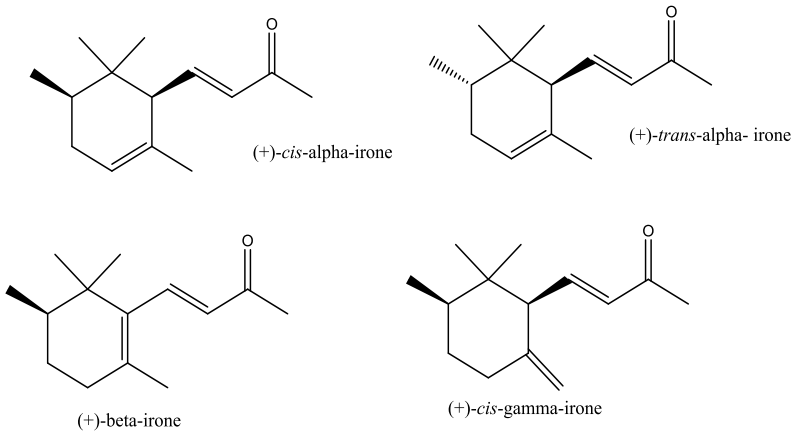


Figure 2-Four isomers of orris oil (Rautenstrauch and Ohloff, 1971).

Iris absolute, produced by alcoholic extraction of orris butter, is one of the most expensive products and it is mainly used in luxury perfumes, such as *Chanel No. 19* (1970) and *So pretty* by Cartier (1995). Nature provides us with most of the best fragrances and flavours, and for decades researchers have been trying to produce these typically aromatic compounds using chemical methods to supply commercial needs. Most of these compounds are chiral and some of them illustrate different odour properties according to their ultimate stereomer configuration (Brenna *et al.,* 2003a) and stereochemistry plays a major role in odour perception (Enberger and Hopp, 1988). Irones synthesis has been reported to pose a challenge to organic chemists (Brenna *et al.,* 2003b) as

the cheapest and most direct synthetic routes were those intended for the preparation of racemates; However, synthetic racemates of irones do not satisfy, from the organoleptic view point, the demand of iris fragrance of contemporary perfumery. Numerous enantioselective syntheses have been stated in literature for enantiomerically enriched irone isomers, but none of them has found practical application (Chapius and Brauchli, 1993, Helminger and Frater, 1989, Lava *et al.,* 2000, Inoue *et al.,* 2000), and hence the natural extract is still utilized in fragrance compositions.

However, Kastner and Maurer (1990) reported the current benchmark method for the conversion of rhizomes to irones, which is very long, troublesome, complicated, expensive and low yielding.

An alternative to the benchmark method utilises nitrite salts for the conversion of iridal precursor (fresh or dry) under mild temperatures and provides an excellent yield. This process utilises a concentrated acid and a complex method to determine irones content in the reaction mixture prior to distillation (Ehret *et al.,* 2001). However, it has been reported that nitrite salt can be toxic in high amounts for human and animals (Sowers *et al.,* 2004).

The biogenesis of irones by oxidative degradation of the iridal-containing extract of rhizomes has been investigated. This involves oxidizing the iridal containing lipid extract of the iris rhizomes with potassium permanganate ($KMnO_4$) in an organic solvent; this is characterized by a laborious procedure for extraction of the precursors and as a result the quality of the final product is too different from the traditionally preferred iris butter or absolute (Ehret *et al.,* 2001; Marner *et al.,* 1990).

Biological routes also exist where the long orris root maturation period is replaced by a microbiological treatment. The bacteria of the family *Enterobacteriacea* in the presence of a plant cell medium was utilised for the bioconversion of powdered iris rhizomes and was found to release irones from their precursors, and after steam distillation produces an iris butter of excellent quality characterized by high irones content.. However, it requires a lot of laborious processing to isolate the strain from iris rhizomes using a conventional dilution method, and it may take up to 20 days just to prepare the biocatalyst (Belcour *et al.,* 1990).

The use of biocatalysis to speed up and simplify the oxidative process using oxidizing enzymes was therefore investigated by researchers. Gil *et al.* (1992) reported an enzymatic and a fungal process for irone production with good conversion of the precursors. However, processing of the rhizome to yield iridals (irone precursors) as reported in Gil's work requires high temperatures, and as a result safety of the method is adversely affected. Moreover, the prior solvent extraction in Gil's study was reported to affect the quality of the product.

The current study seeks to address the challenges mentioned by Gil *et al.,* (1992) on a lipoxygenase based enzymatic route for irones production. In Gil's study the biocatalyst is commercially available purified soybean lipoxidase.

1.1.2 Oxidative biocatalysis: Oxidoreductases

Researchers defined oxidoreductases as a large group of enzymes responsible for catalysing oxidation/reduction reactions involving transfer of two oxygen atoms to a substrate (May, 1979; Phillips and May, 1981). Although there are several subclasses of oxidases, laccases and lipoxidase are the only two of interest in the present research.

1.1.2.1 Laccases

Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductases) are a class of oxidoreductases using two oxygen and multiple copper atoms. Several biological functions have been associated with these enzymes such as immunity and pathogenicity. Laccases have been reported to be non-specific and thus this property makes them suitable to be used widely as biocatalysts in biotechnology (Claus, 2004).

1.1.2.2 Lipoxidase

Lipoxidase (LOX, EC1.13.11.12) is an iron-containing oxido-reductase enzyme, also known as lipoxygenase, which catalyses oxidation/reduction reactions. It catalyses the regio- and stereospecific oxygenation of polyunsaturated fatty acids, their esters and glycerides containing the *cis* double bond system to produce a hydroperoxide. It can be extracted from a range of plants, including a variety of mammals, fish fruits and vegetables (Gökmen *et al.,* 2002; Bisakowski *et al.,* 1998; Iny *et al.,* 1993).

In the present study we modified the patented lipoxidase-based process for irone production with the aim of optimizing the reaction conditions, reducing the process time, and improving the yield of irones in a safe, quality and easy to follow green process.

1.2 Purpose of the study

1.2.1 Hypothesis

Mineral salts are essential for an efficient soybean lipoxidase-mediated production of irones from precursor compounds in orris root.

1.2.2 Aims

Develop an analytical method to separate the different irone isomers and to quantify the different isomers.

Optimise the reaction conditions for maximum biocatalytic production of irones on laboratory scale

Identify the best method for extraction or purification of final product and comparison of the ratio of the isomers to that of commercially available orris butter.

Develop method and assay for purification and immobilization of Soybean lipoxidase

Bench scale runs of the optimized conditions

Compile a protocol for the process

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction

The following review of the literature seeks to provide an in-depth and concise explanation of the discipline of biocatalysis, the enzymes involved and, in particular, soybean lipoxidase as used in the present study, lipoxidase engineering techniques as used in the present study. We will also explore the irone sources, irones *per se* as natural fragrances and various processes for irone production and their limitations as done by other researchers in the field.

2.2 Biocatalysis

Biocatalysis is considered to be advantageous because it uses enzymes (protein catalysts) which have been reported to offer unmatched levels of stereoselectivity and reaction specificity (Fessner and Anthonsen, 2009). This would be of advantage in the synthesis of single enantiomer irones.

For millennia enzymes and microbial cells have been used in the production of important food products and beverages. The first modern applications of biocatalysis were noted some decades ago where a number of enzymes were used to produce valuable products. The examples of enzymes used in this regard were acylases, hydantoinases and aminopeptidases in the production of optically pure amino acids, and the use of nitrite hydratase in the enzymatic production of the bulk chemical acrylamide from acrylonitrile (Drauz *et al.,* 1996; Schmid *et al.,* 2001). Lipase and other enzymes have been recognized to be usable in organic media (Schoemaker *et al.,* 2003; Zaks and Klibanov, 1985) and even in solid phase (Schoemaker *et al.,* 2003) and this has further increased the scope of use of enzymes as catalysts in organic synthesis.

Currently, biocatalysis is seen by both academics and people in industry as a valuable area of research, particularly for the progress of sustainable technologies (green chemistry) for the production of chemicals (Schoemaker *et al.,* 2003) and in the selective and complex synthesis of active ingredients in pharmaceuticals and agrochemicals. A biocatalysis cycle shown in Figure 3 summarises the entire sequence of events in generic biocatalyst development.

2.3 Enzymes as biological catalysts

Enzymes as biological catalysts have been reported to have their own advantages and limitations. High activity, selectivity and specificity are excellent properties of enzymes that allow them to perform complex reactions under benign experimental and environmental conditions (Koeller and Wong, 2001; Wong and Whitesides, 1994).

Efficient catalysis of many chemical reactions has been reported to be delivered by enzymes. Moreover, enzymes also offer high regio- and stereoselectivity, mild reaction conditions and thus low energy consumption. Furthermore, they produce low quantities of by-products; they are biodegradable, preparation on large scale is conceivable through fermentation (microbial enzymes). Reuse is possible (immobilization). They can be designed to a certain extent and if correctly applied they are non-toxic. However, the following disadvantages have been reported for enzymes; instability in aqueous media, probability of inactivation by higher temperatures, at extreme pH-values, in higher salt concentrations and (polar) organic solvents. Moreover, inhibitors such as metal ions, product and substrate have also been reported to cause inactivation of protein molecules. Furthermore, many enzymes depend on co-factors for biocatalysis (Grunwald, 2009).

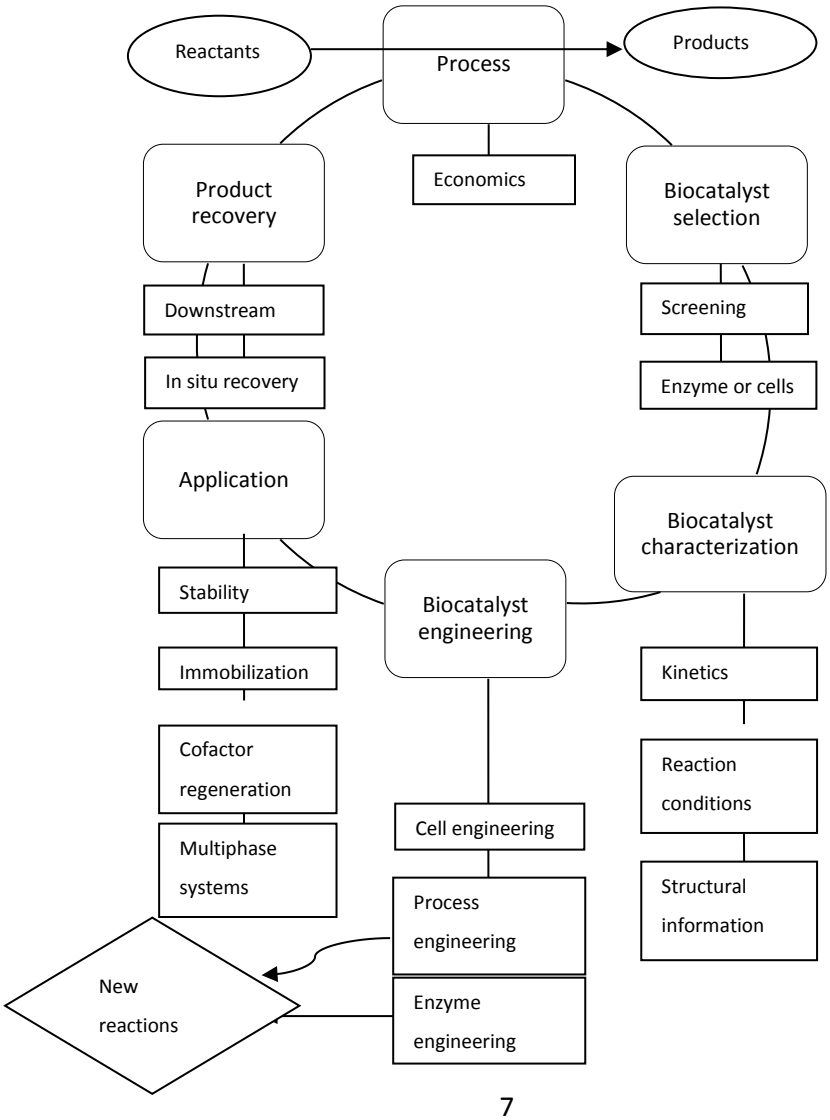


Figure 3-An illustration of the biocatalysis reaction development cycle (Schmid *et al.,* 2001).

2.3.1 Biocatalysis in non-aqueous media

It has been proposed that biocatalysis in ionic liquids has advantages according to the principles of green chemistry when compared with organic solvents (Park and Kazlauskas, 2003b). This is due to lack of vapour pressure in ionic liquids. Moreover, these liquids may not inactivate enzymes, thus making reactions involving polar substrates simpler (Park and Kazlauskas, 2003a; Park and Kazlauskas, 2003b). Higher selectivity, (Kim *et al.,* 2003; Zhao and Malhotra, 2002) faster rates and greater enzyme stability (Hinckley *et al.,* 2002; Kaftzik *et al.,* 2002; Laszlo and Compton, 2002) have also been reported as advantages of biocatalytic conversion in ionic liquids. However, problems in ionic liquids purification and controlling water activity and pH, high viscosity and problems with product isolation have been reported as the limiting factors in the application of biocatalysis in ionic liquids (Berberich *et al.,* 2003; Kim *et al.,* 2003; MacFarlane *et al.,* 2001).

Generally, the ability of enzymes to catalyse reactions is much lower in neat organic solvents compared with their ability to do so in water (Klibanov, 1997). However, determining both the underlying cause and remedies to overcome this effect are starting to attract interest (Schmitke *et al.,* 1996) and it has been reported that there are advantages in using enzymes in organic solvents (Klibanov, 2001). This may include higher solubility of apolar reactants. Although loss of native structure and activity has been reported when some enzymes are used in the presence of particular organic solvents, this is usually in aqueous-organic mixtures and not in absolute organic solvents (Nelson and Cox, 2000). When water is excluded from the reactions enzyme rigidity is increased (Kuntz Jr and Kauzmann, 1974; Rupley and Careri, 1991). Although an enzyme's drive to unfold has been reported in some cases to be high in dry solvents, the lack of protein flexibility in solvents can counteract this, thus allowing various crystalline enzymes to maintain their inherent structures even in anhydrous organic solvents (Fitzpatrick *et al.,* 1993; Gao *et al.,* 1999; Schmitke *et al.,* 1997; Yennawar *et al.,* 1994).

2.3.2 Enzyme engineering

The engineering of enzymes to industrial catalysts has been identified as an important goal and many techniques to improve enzyme features have been developed and implemented in industry. The techniques include the stabilization of enzymes via immobilization, which includes operational stabilization of enzymes on porous supports and rigidification of the enzyme structure by multipoint covalent immobilization (Mateo *et al.,* 2007). Furthermore, Mateo *et al.* (2007) indicated that the

selectivity of enzymes can be modulated via immobilization and that enzyme inhibition can be greatly decreased by these techniques.

2.4 Physiological functions of mineral micronutrients

Metabolic and cellular functions require micronutrients, which are needed for plant growth but are necessary in very minute amounts than those of the primary nutrients such as nitrogen, phosphorus, sulphur and potassium. Plants require certain micronutrients for different purposes, but boron, chloride , copper , iron, manganese , molybdenum nickel , and zinc are considered essential for all higher plants (Yang *et al.,* 2006). The role of these elements is not always carefully considered in biocatalysis reaction development. The elements may be involved in the catalytic active site, but may also moderate activity and stability through interactions elsewhere in the enzyme structure. The following study bases its attention on manganese and iron found in the current study's preliminary experiments to have a potential to improve product formation.

Manganese: Metabolism and development of plants requires this mineral which occurs in 3 oxidation states (II,III and IV) in roughly 35 enzymes isolated from a plant cell (Hebbern *et al.,* 2009). Protection of the cell from degradation by free radicals requires superoxide dismutase containing manganese; The catalytic function of this mineral was reported in two enzymes viz. the oxalate oxidase; and the manganese-containing water splitting system of photosystem II (Barber, 2003). Other examples of the enzymes containing manganese include malic enzyme, isocitrate dehydrogenase, phosphoenolpyruvate (PEP) carboxykinase, and phenylalanine ammonia lyase. Moreover, manganese can be replaced by magnesium in most instances, making its role less specific among the somewhat large group of manganese-activated enzymes, (Marschner and Marschner, 2012). It has been reported that several metabolic pathways requires proteins of this group. Lastly, it has been stated that manganese activation was seen in several enzymes (Hänsch and Mendel, 2009).

Iron: Iron is very important for life. As redox-active metal it is involved in many metabolic pathways. For example, it has been reported that up to 80% of the cellular iron is housed in the chloroplasts, hence is major role in photosynthesis. The type of iron ligand allows for classification of iron-containing proteins into three groups namely: (1) proteins with iron-sulphur clusters (Fe-S), (2) heme-containing proteins, and (3) other iron proteins.

Fe-S proteins: These proteins have crucial functions in the transfer of electrons; they form part of substrate binding sites in enzymes, they occur in iron storage moieties, moreover, they have a role in transcriptional or translational regulation, they can regulate protein structure in the locale of the cluster, and lastly they have been shown to aid in disulphide reduction and sulphur donation (e.g.

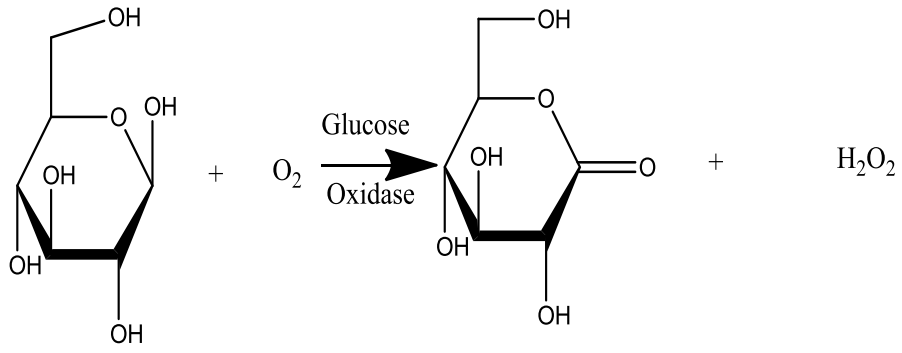
thiorexins). In summation, they serve as enzymes, as electron carriers (e.g. ferrodoxin), and as regulator proteins (e.g. aconitase). The formation of Fe-S cluster occurred quite early in evolution and that let to its conservation later on (Xu and Møller, 2008).

Heme proteins: The cytochromes in photosynthesis and respiration, involved in electron transfer and the globins that bind oxygen are well-known members of this protein group. The oxidative enzymes catalase, peroxidase, and NADPH oxidase, involved in the production and/or scavenging of free radicals, and the large group of cytochrome P450 enzymes are among other examples. Moreover, in plants and microorganisms, these latter facilitate mono-oxygenation reactions in biosynthetic pathways, such as for sterols and many secondary metabolites, though, in animals they catalyse reactions for detoxification of xenobiotics. Furthermore, globins like leghemoglobin are responsible for binding and transportation of oxygen (Cornah *et al.,* 2003).

Non-heme proteins: They bind iron directly. Ferritins are said to be the most prominent of the group. Ferritins are plastidic iron storage proteins which regulate the interaction between iron homeostasis and oxidative stress in *Arabidopsis* (Ravet *et al.,* 2009). Lipoxidase is a non-heme protein.

2.5 Oxidoreductases

Researchers defined oxidoreductases as the large group of enzymes important in oxidation/reduction reactions (May, 1979; Phillips and May, 1981). Two oxygen atoms are necessary for their reaction as shown in Figure 4. The current literature gives basic information on only two oxidoreductases of interest, laccases and lipoxidase.



β-D-Glucose D-Glucono-1, 5-lactone

Figure 4- An illustration of a reaction of oxidoreductases (May, 1979; Phillips and May, 1981).

It was stated that oxidoreductases can be applied in oxyfunctionalization of organic compounds, asymmetric oxyfunctionalization of steroids and other medical drugs, production and modification of polymers, oxidative breakdown of pollutants and the construction of biosensors for use in analytical and clinical environments (May, 1992, 1997; May and Padgette, 1983).

2.5.1 Laccases

Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductases) are oxidoreductases with multiple copper atoms with at most four (4) copper atoms responsible for the protein's catalytic activity, and a molecular oxygen involved in the oxidation of aromatic and non-aromatic compounds through generation of free radicals. These enzymes have a role in immunity and pathogenicity and are known to be non-specific and thus widely applicable as biocatalysts (Claus, 2004).

2.5.1.1 Structure and source

Source: Researchers reported that until recently eukaryotes were the only source of laccases, e.g. fungi, plants, insects (Mayer and Staples, 2002). There is a recent emerging evidence for their occurrence in prokaryotes of proteins with typical features of the multi- copper oxidase enzyme family (Alexandre and Zhulin, 2000).

Structure: They have type I, II, III and IV copper atoms each serving a specific purpose in the oxidation/reduction reaction. Furthermore, extensive description of the positions and compositions for each of the copper atoms in the protein have been carried out. Reports stated that fungal laccases often occur as isoenzymes that undergoes oligomerisation into multimeric complexes. Furthermore, the monomer's molecular mass has been reported to range between 50-100 kDa. Moreover, a carbohydrate moiety (10-45%) linked covalently to these enzymes has been reported to be an important feature with a potential to increase their stability. Figure 5 shows the active site of fungal laccase (Ducros *et al.,* 1998; Ducros *et al.,* 2001).

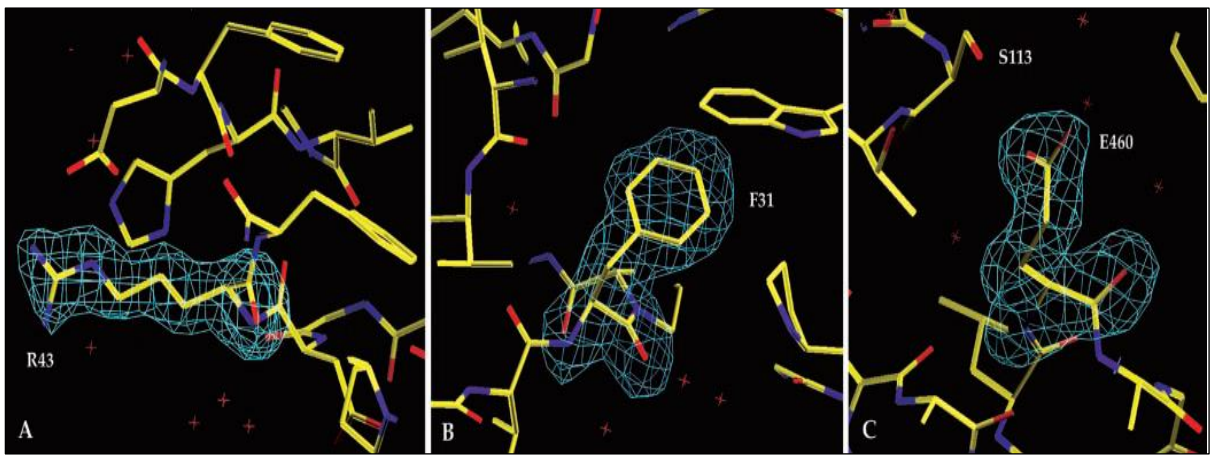


Figure 5- Laccase's active site from the Fungus *Trametes versicolor* at 1.90-Å Resolution Containing a Full Complement of Coppers (Piontek *et al.,* 2002).

2.5.1.2 Substrates and mechanisms of reaction

It was indicated that forceful migration of reducing substrate to molecular oxygen without generation of toxic peroxide intermediates is mediated by several copper centres. (Claus and Filip, 1997; Duran and Esposito, 2000). Central to the above function are four monoelectronic oxidations of the substrate catalysed by the type I copper. The oxidations are followed by the transfer of electrons to the trinuclear cluster, where molecular oxygen is reduced and water is released. The oxidation of substrates generate reactive oxygen species that can undergo non-enzymatic reactions viz:

(i) *Crosss linking monomers:* They carry out oxidation of phenolic compounds and anilines to create radicals that react with each other to form dimers, oligomers or polymers covalently joined by C-C, C-O and C-N bonds. Furthermore, in the case of substituted compounds, partial demethylation and dehalogenation accompanies the reaction. Reports indicate that natural and xenobiotic phenolics or aromatic amines can be bound to the organic humic matrix in soils, and the basis for laccase's potential to detoxify contaminated soils or waste water vests upon these capacities (Duran and Esposito, 2000).

The ligninification process reported in higher plants is constituted in one part by the cross-linking of phenolic precursors by laccasses. Furthermore, in insects, the laccase–catalysed oxidative coupling of catechols with proteins may be involved in sclerotization of the cuticle (Kramer *et al.,* 2001). Cross-linking of protein residues, e.g. tyrosine to form di-tyrosine, in microbes has been discussed as the function of laccases in the assembly of heat and uv-resistant *Bacilus* spores (Hullo *et al.,* 2001).

(ii) *Degradation of polymers:* They mediate degradation of complex natural polymers, such as lignin or humic acids (Claus and Filip, 1998) and results in reactive oxygen species which in turn cleaves covalent bonds to release the monomers. Due to steric hindrance, it might be impossible for the enzymes to come into direct contact with the polymers. Instead, small organic compounds or metals that can also be oxidized and activated by laccases, e.g. veratrylalcohol, 3-hydroxy-anthranilic acid and manganese mediate the radical-catalyzed reactions. In biotechnological processes non-physiological redox mediators are used to increase the oxidation potential of laccases (Claus *et al.,* 2002).

(iii) *Ring cleavage of aromatics:* They are said to mediate degradation of aromatic compounds' rings in several cases. Furthermore, this reaction is reported to be of

biotechnological interest in view of the cleavage of xenobiotics like nitroaromatics and synthetic dyes (Claus *et al.,* 2002; Duran and Esposito, 2000).

2.5.1.3 Applications:

Toxicological studies on laccase from *Myceliophthora thermophile* expressed in *Aspergillus oryzae* revealed that the industrially laccase preparation can be of value in different technical and food applications for process facilitation purposes. It was determined not to be mutagenic by the *Salmonella typhimurium* reverse mutation assay, nor to cause chromosomal abnormalities in cultured human lymphocytes. Lastly, no evidence of inhalation toxicity or skin and eye irritation was reported. Laccases have already been used in food, e.g., stabilization of fruit juices (Brinch and Pedersen, 2002) and dough improvement (WO94/28728, 1994 cited in Brinch and Pedersen, 2002). Recently, they have reports on their application in the prevention of halitosis, as bad breath can be reduced with laccase and a phenol-containing donor in an enzymatic process that removes the volatile sulphur compounds (VSC) responsible (WO99/09143, 1999 cited in Brinch and Pedersen, 2001).

2.5.2 Lipoxidase/lipoxygenase (LOX)

Lipoxidase (LOX, EC1.13.11.12) is an iron-containing oxido-reductase enzyme also known as lipoxygenase, which catalyses oxidation and reduction reactions. In nature it catalyses the regio- and stereo-specific oxygenation of polyunsaturated fatty acids, as well as their esters and glycerides containing *cis* double bonds to produce a hydroperoxide as shown in Figure 6 (Siedow, 1991).

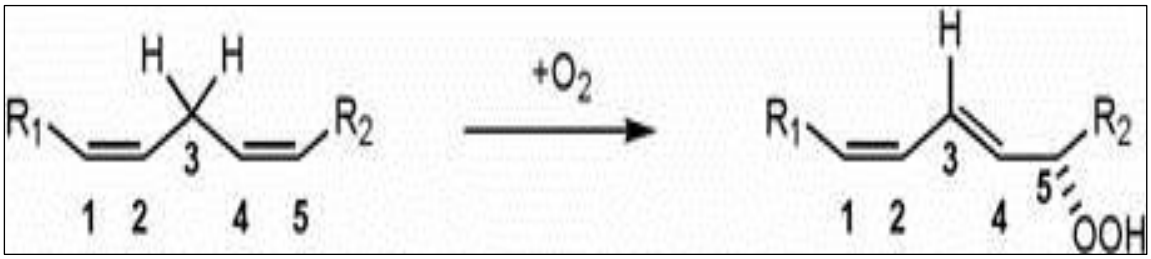


Figure 6-A lipoxygenase-catalysed reaction. One of the two hydrogens of the bis-allylic C-3 methylene group is eliminated from the fatty acid substrate during formation of a hydroperoxide (google images).

The lipoxygenase superfamily:

Lipoxygenase/lipoxidase substrates, the polyunsaturated fatty acids, are essential fatty acids in human. Most bacteria (except cyanobacteria and some marine species (Gerwick and Bernart, 1993)) do not have these substrates while some yeast is without the desaturases necessary for their synthesis. Furthermore, the yeast genome (*Saccharomyces cerevisiae*) and typical prokaryotes do not have laccases and this emanates from the lack of its substrate in these microorganisms. No

definitive account of a lipoxidase has been reported in insects. However, the hydroperoxide derived from arachidonic acid (hydroxyeicosatetraenoic acid, HETE) has been identified in a primitive insect, *Thermobia domestica* (Ragab *et al.,* 1991). Lipoxidase in the unicellular *Chlorella* had been identified (Zimmerman and Vick, 1973) and a partial lipoxygenase cDNA sequence in the data bases from the slime mould *Dictyostelium discoideum*. Multiple lipoxygenases, for example at least 8 identified in the soybean *Glycine max*, are contained in higher plants. There are seven genes that code for the synthesis of lipoxidase proteins in the mouse. Approximately five homologues (and an expressed pseudogene) have been characterized in humans (Boeglin *et al.,* 1998; Krieg *et al.,* 1998; Sun *et al.,* 1997).

Lipoxidase has been extracted from a range of plants, including a variety of fruits and vegetables, as well as mammals and fish (Gökmen *et al.,* 2002: Bisakowski *et al.,* 1998; Iny *et al.,* 1993). Interestingly it has been reported that 200 times more lipoxidase is present in soybeans than wheat. Lipoxidase exists as several isozymes varying significantly in optimum pH, substrate specificity, and end-products (Bisakowski *et al.,* 1998). There have been four distinct isoenzymes, viz. L-1, L-2, and L-3a and b reported in Soybean seeds (Axelrod, 1974; Axelrod *et al.,* 1981), and the behaviour and composition of the latter two are so alike that they might be considered together as a single type, L3. All of the isozymes were found to include a single atom of tightly bound non-heme iron and to have somewhat similar amino acid compositions (Axelrod, 1974; Axelrod *et al.,* 1981; Christopher, 1972). Grounded on the deduced amino acid sequence, L-1 contains 838 amino acid residues and has molecular mass of 94,038 (Shibata *et al.,* 1987). The equivalent values for L-3 are 859 and 96,541 (Yenofsky *et al.,* 1988). L-2 is comprised of 865 residues and has a molecular mass of 97.035 (Shibata *et al.,* 1988). These enzymes are said to require an activation by their Hydroperoxidation product (Haining and Axelrod, 1958).
Moreover, it was reported that no microorganism has yet been identified which produce the enzyme in amounts that are commercially valuable.

The separation of the plant and animal enzymes and formation of several subgroups inside each kingdom is summed as shown in Figure 7. No association between the formation of a particular lipoxygenase product and narrowly related sequences has been identified. In the case of soybean L-1 enzyme (a 15-LOX) and any mammalian 15-lipoxygenase for example, only 25 % identity share exists. There is a 35 % identity share between a 15-LOX, and the two human 15-LOX. By contrast, the close functional homologues across species, forming distinct subgroups share between 70-95 % sequence identity (Brash, 1999).

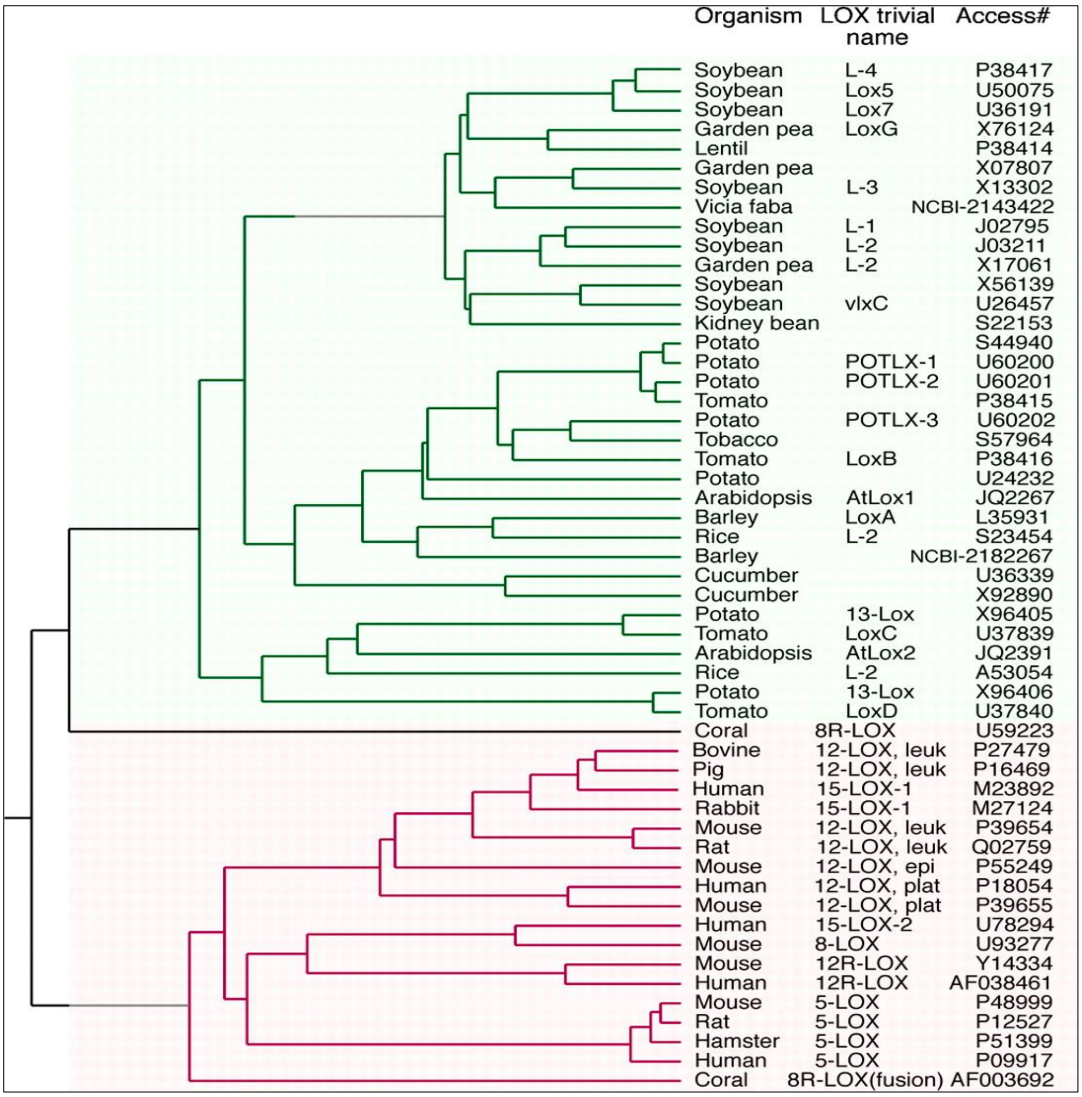


Figure 7- The evolutionary tree of lipoxygenase (adapted from Brash, 1999).

Nomenclature: Lipoxidases are named after the position on the alkane where they oxygenate. Arachidonic acid is oxygenated at carbon 12 by 12-LOX, and when needed, the stereoconfiguration is specified (12R-LOX or 12S-LOX) as shown in Figure 8. The major product in rabbits and humans is 15-HPETE, and hence the enzyme is called a 15-LOX (Yamamoto *et al.,* 1997). Furthermore, the differing chain lengths of the most common substrates of plants (linoleate, linolenate, 18-carbon) and animals (arachidonate, 20-carbon) lead to a plant 13-LOX corresponding to a mammalian 15-LOX; these particular lipoxygenases "count" the substrate carbons from the tail end of the chain, and both react oxygen at the ω-6 position. Some complications do occur, for example, when there is more than one 12-LOX in the same species. However, this can be resolved by, for example, naming the mammalian 12-LOX after their prototypical tissues of their occurrence (hence, the platelet, leukocyte, or epidermal type of 12-LOX). The sequence, catalytic activities, and function of these enzymes differ. Some lipoxygenases lead to formation of a mixture of products, *e.g.* the C-12 and C-15 oxygenation,

catalysed by the mammalian reticulocyte type of lipoxygenase with the relative proportions varying among species (Yamamoto *et al.,* 1997).

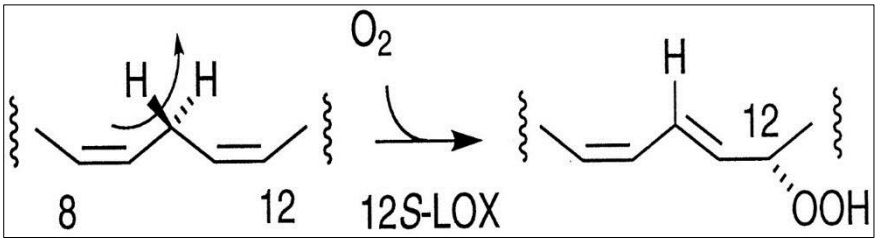


Figure 8-Oxygenation of arachidonic acid at carbon-12 by 12S-LOX (Brash, 1999).

Lipoxidases or lipoxygenases mediate the formation of biological mediators/signalling molecules and modification of membrane structures (peroxidation reactions) and the entire sequence of events is as summarized in Figure 9 (Brash, 1999).

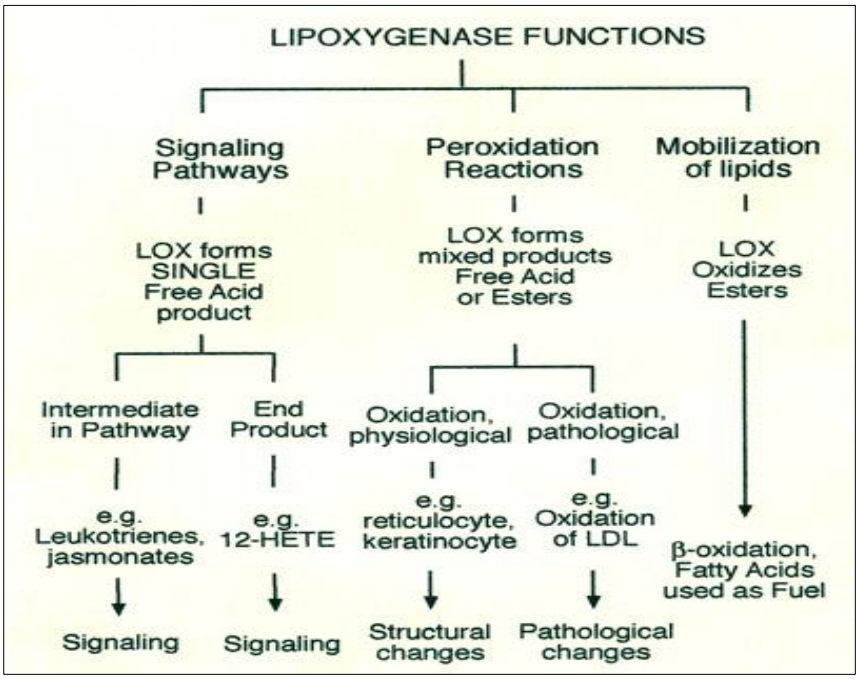


Figure 9-Biological roles of lipoxygenases, adapted (from Brash, 1999).

Lipoxygenase catalysis:

Enzyme structure: A single polypeptide chain with a molecular mass of 75-80 kDa in animals and 94-104 kDa in plants makes up a lipoxygenase protein. This protein has an N-terminal γ-barrel domain as shown in Figure 10 (top panel, white domain) and a larger catalytic domain containing a single atom of non-heme iron. It is the non-heme iron which makes lipoxygenase to appear virtually colourless (Figure 10).

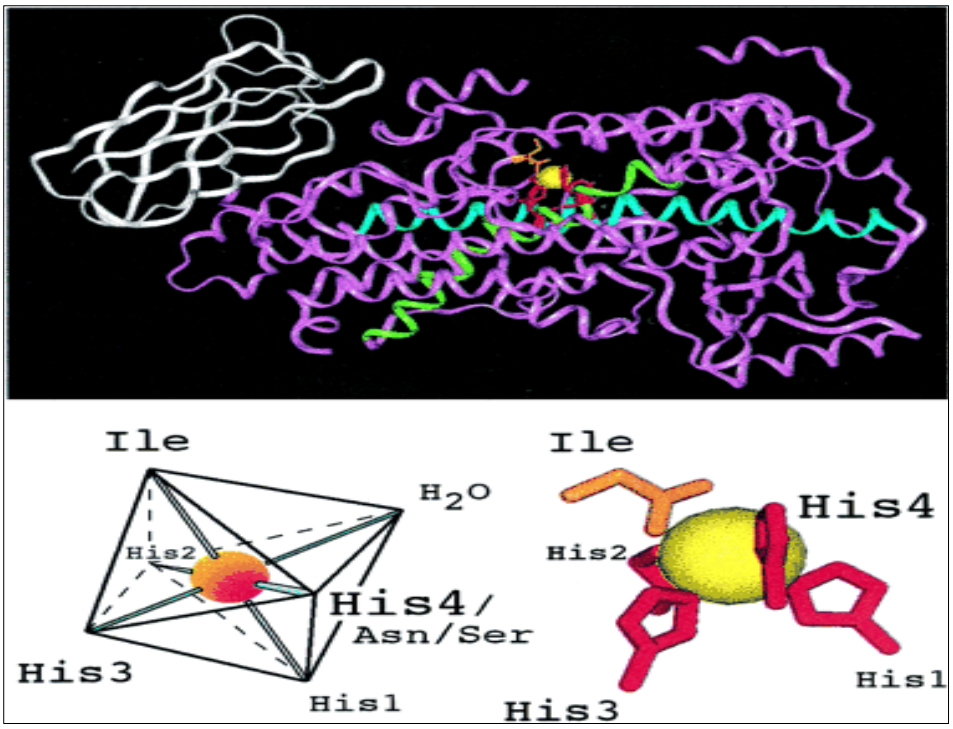


Figure 10- Ligands around the lipoxygenase non-heme iron (Gillmor *et al.,* 1997).

The enzymes usually exist in the ferrous (inactive forms) after isolation requiring prior oxidation to the active ferric enzyme for catalysis (Figure 11). Four available crystal structures have been reported, of which three are of the arachidonate 15-lipoxygeanses, soybean L-1, and rabbit reticulocyte 15-LOX (Boyington *et al.,* 1993; Gillmor *et al.,* 1997; Minor *et al.,* 1993) and the fourth, soybean L-3, is a catalyst of non-specific peroxidation (Skrzypczak-Jankun *et al.,* 1997). No agreement exists on how substrate enters the metal centre or any conclusive information on substrate binding. The apparent access channel for arachidonic acid in this model opens onto the top surface of the protein. The soybean enzymes both possess a large cavity to the right of the iron and an opening to the protein surface on the right hand side (Skrzypczak-Jankun *et al.,* 1997).

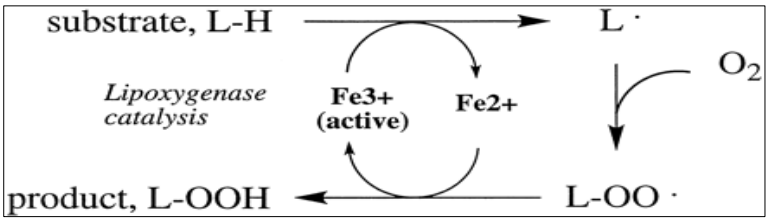


Figure 11- Activation of the inactive lipoxidase (Brash, 1999; Gillmor *et al.,* 1997).

2.5.2.1 Soybean lipoxidase

Of all isoenzymes of soybean lipoxidase studied soybean Lipoxidase-3 (Figure 12) is one good example of such, with its active site having only one non-heme iron. Furthermore, its structure is described as monomeric with a molecular mass approximated at 95 kDa. Moreover, about 839

amino acids comprising of two main domains make up this single polypeptide protein (Fox, 1998; Nelson and Seitz, 1994; Skrzypczak-Jankun *et al.,* 1997). The amino and carboxy terminal domains consist of 146 and 693 amino acids residues respectively. The centre of the carboxy terminal domain is a position for the active site iron. It was reported that the oxygen and nitrogen atoms of certain amino acids such as histidine, isoleucine, asparagine and glutamine are frequently observed ligands in non-heme iron proteins (Fox, 1998; Nelson and Seitz, 1994; Skrzypczak-Jankun *et al.,* 1997; Steczko and Axelrod, 1992).

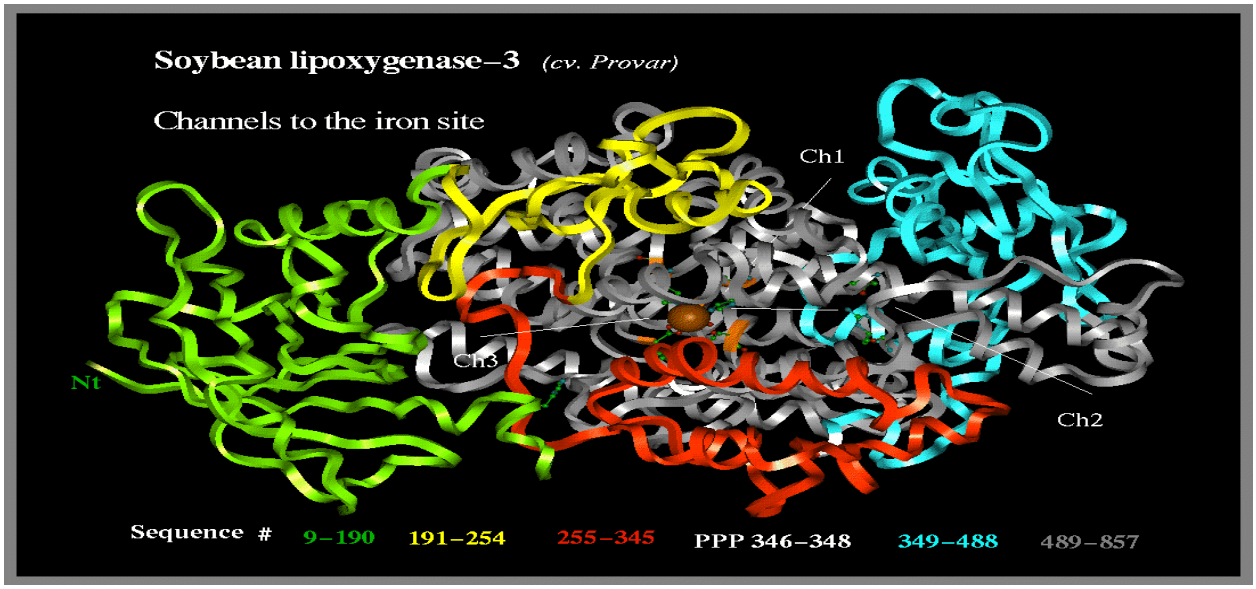


Figure 12-Soybean lipoxygenase isoenzymes L3 (www.soyworld.org1096 × 747Search by image)

2.5.2.2 Substrate specificity for soybean lipoxidase

Several unsaturated fatty acids of varying chain lengths, number and location of double bonds have been tested for the optimal soybean lipoxidase substrate, and it was found that the positions of the double bonds in relation to the carboxyl group did not show any correlation with the rate of peroxidation. Moreover, all the substrates with double bonds at carbon 6 and 9 counting from the methyl group were oxidized at a high rate. Irrespective of the chain length (even or odd), the number and position of double bonds; these acids were good substrates for lipoxidase. Crepineric acid was an exception to all the other fatty acids, even though it was unsaturated at carbon six; the unsaturation was a triple bond and thus it has shown low oxidation rate in comparison. Among the various linoleic acid isomers studied, it became clear that linoleic acid, 9, 12-18:2 is the preferred substrate (Holman *et al.,* 1969).

2.5.2.3 Lipoxidase purification

The diverse applications of lipoxidase in industry (Tukel *et al.,* 2005) makes its purification important. The extract of ground soybeans, the richest source of natural lipoxygenase, contains other protein of

high molecular mass, peptides, oligosaccharides, carbohydrates and various compounds with low molecular mass (Cole, 1993).

Researchers have extracted and purified lipoxygenase from soybean using an aqueous two-phase system. Their purification steps consisted of extraction using 0.2 M sodium acetate buffer pH 4.5, precipitation using PEG 20 000, and two phase extraction using PEG 6000/ammonium sulphate. They found that the activity of soybean lipoxidase (LOX) obtained from the supernatant sample initially increased proportionately with the amount of PEG 20 000. However, this increment was noted up to a 6 % increase in PEG concentration, resulting in a 2.0 fold purification, and any increment beyond this value resulted in a decrease in activity (Lakshmi *et al.* 2009). Generally, other researchers (Mahadevan and Hall, 1992) who have utilized PEG in the initial steps have found similar results as those of Lakshimi *et al.* (2009).

Moreover, Lakshimi *et al.* (2009) identified the advantage of a polymer-salt system over a polymer-polymer phase system; this advantage was associated with low viscosity and shorter time for phase separation. PEG 6000 in ammonium sulphate was found to be the best with 1.57 fold purification and 94 % activity recovery. They also performed SDS PAGE to show that the number of bands is reduced after purification as compared to crude extract.

In a study by Subbaiah *et al.* (2008), defatted horsegram seeds were used as source of lipoxygenase and a thermostable lipoxygenase activity was recovered. Horsegram seeds have been reported to be rich in acidic lipoxygenase activity with 2500 U/gram of flour.

Xinyao *et al.* (2013) stated that recombinant LOX was effectively expressed and secreted by *E. coli* using its endogenous signal peptide. They further reported that when induced with 1 mM isopropyl γ-D-1-thiogalactopyranoside (final concentration) at 20°C for 47 hours; its titre reached 3.89 U/mL. Moreover, Q High Performance and Mono Q5/50GL resins were utilized sequentially to purify LOX to homogeneity so as to characterize its catalytic properties. SDS polyacrylamide gel electrophoresis allowed for an estimation of LOX molecular weight as 70 kDa. The K_M and V_{max} of the recombinant enzyme were 48.9 µM and 0.226 µM/min respectively. Maximum activity of the purified enzyme was exhibited at 25°C and pH 7.5. High Performance Liquid Chromatography (HPLC) analysis of linoleic acid hydroperoxides revealed that the LOX from *P. aeruginosa* falls into the classification of linoleic acid 13(S)-LOX.

Lipoxygenase enzyme activity assays: The substrate solution (linoleic acid dispersed in 0.15 M K_2HPO_4-KH_2PO_4 buffer, pH 7.5) was freshly prepared and aerated with oxygen for 5 minutes before testing. Standard analytical mixture (3 mL) contained 0.3 mM linoleic acid and approximately 1.67

µg/mL enzyme, and one unit of activity was defined as enzyme required to synthesize 1 µmol hydroperoxide per minutes at 25°C (Hughes *et al.,* 1998). The rate of peroxide formation was determined at 234 nm in the UV range using a suitable spectrophotometer (a UV-2450; Shimadzu Co., Kyoto, Japan). Similar assays were used by Diel and Stan, (1978) and Marczy *et al.* (1995).

Suda *et al.* (1995) used a colorimetric method in the visible range: incubating 0.5 mL of 200 mM sodium borate buffer (pH 9.0), 0.1 mL of 100 µM methylene blue, 0.1 mL of 10 mM sodium linoleate substrate; 0-0.3 mL of soybean extract sample, and distilled water to a final volume of 1 mL with the reaction initiated by addition of the soybean extract sample, the absorbance at 660 nm can be recorded with a spectrophotometer (Beckman DU70) for 5 minutes at 25 °C (Suda *et al.,* 1995).

2.5.3 Enzyme Immobilization Methods

2.5.3.1 Covalent binding

This is a conventional, strong and stable method of immobilisation that involves direct attachment of the enzyme and the support material through the covalent linkage as shown in Figure 13 (Wong *et al.,* 2008). Furthermore, the options for support material for enzymes includes polyacrylamide, porous glass, agarose and porous silica (Ghous, 2001). When a process specification does not permit any enzyme in the product, the covalent method of immobilization is preferred. There are two main steps in the covalent binding of the enzyme to the support material such as (1) the activation of the support material and (2) the coupling of the enzyme to the support. Activation is necessary for generating the electrophilic group on the support material, so that the support material reacts with the strong nucleophiles on the proteins (Berna and Batista (2006) cited in Won *et al,* 2005). This type of binding normally occurs between the functional groups in the support matrix and the part enzyme surface that has the targeted amino acid residues.

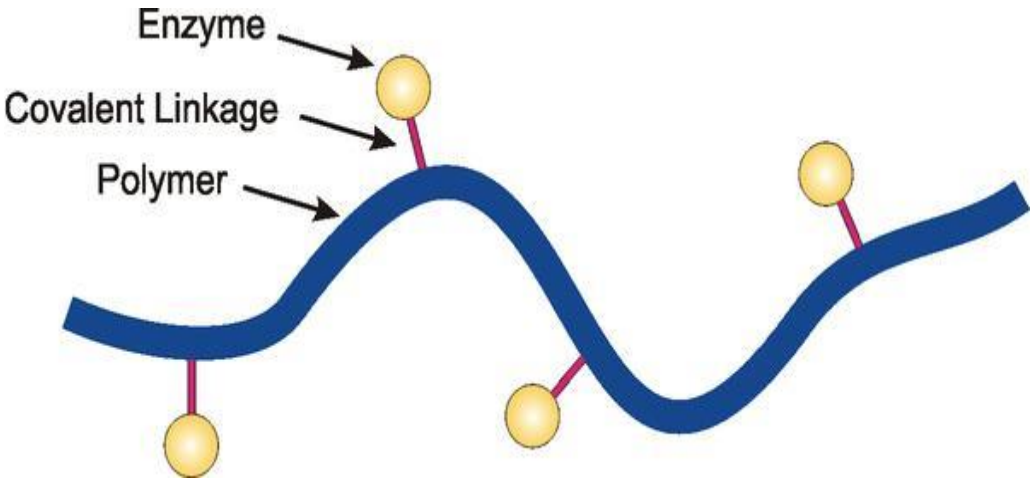


Figure 13-Covalent binding of the enzyme to the support material (www.polychem.mat.ethz.ch601 × 234).

2.5.3.2 Entrapment:

In this immobilization method, enzymes are enclosed in a permeable natural polymeric network which allows the substrate and the products to go through while retaining the enzyme. The entrapment can be done utilizing a gel, fibres or microencapsulation (Bernfeld and Wan, 1963). Moreover, compared to enzyme immobilization entrapment is fast, cheap and mild conditions required for reaction process. Limitation in mass transfer has been reported as the disadvantage of this method of immobilization. The support matrix offers protection of enzymes from microbial contamination, proteins and enzymes in the microenvironment (Riaz *et al.,* 2009). When the enzyme molecules are encapsulated within small spherical semipermeable membranes with a selective controlled permeability the method is called microencapsulation (Rosevear *et al.,* 1987). The large surface area between polymeric material and the enzyme is ensured through this method. Inactivation of enzyme during encapsulation has been documented as the drawback of this method (Bernfeld and Wan, 1963). An example of enzyme entrapment immobilization method is illustrated in Figure 14.

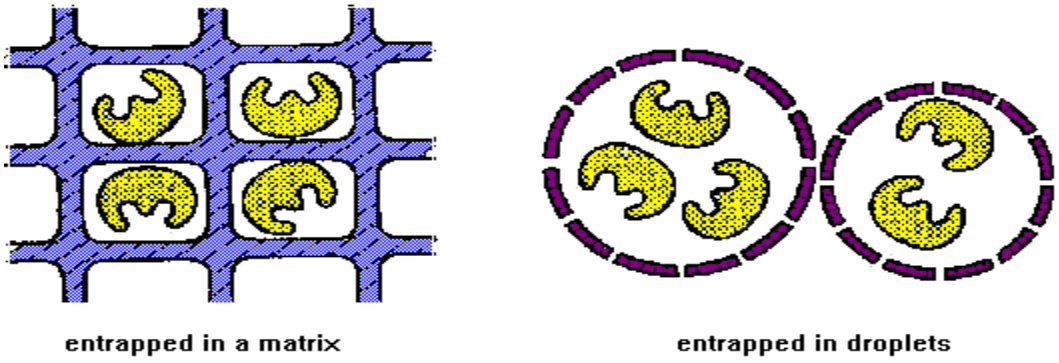


Figure 14-- an illustration of enzyme entrapment immobilization method (www.rpi.edu533 × 214).

2.5.3.3 Adsorption

This is said to be a simple method of enzyme immobilization (Tanyolaç *et al.,* 1998) and the materials used for adsorption include activated charcoal, alumina and resins. This method is cheap and easy, but the shortcoming is a weak binding force between the carrier and the enzyme (Brady and Jordaan, 2009). Furthermore the process of immobilization is reversible. Moreover, the interaction between the enzyme and the surface of the matrix through weak forces is by hydrogen bonds, hydrophobic forces, and Van der Waals forces. The bound, but not distorted enzyme will be formed based on the charges of the matrix and the protein arrangements no reagents are required (Tosa *et al.,* 1967). An illustration of adsorption immobilization method is shown in Figure 15.

2.5.3.4 Ionic binding

Salt linkages can be formed between the enzyme and the support material (typically a commercial ion exchange resin), allowing for bonding called as shown in Figure 15. Changing the temperature, polarity and ionic strength conditions can be used to reverse the non-covalent immobilization process, similar to the principles of ion chromatography (Guisán *et al.,* 1997).

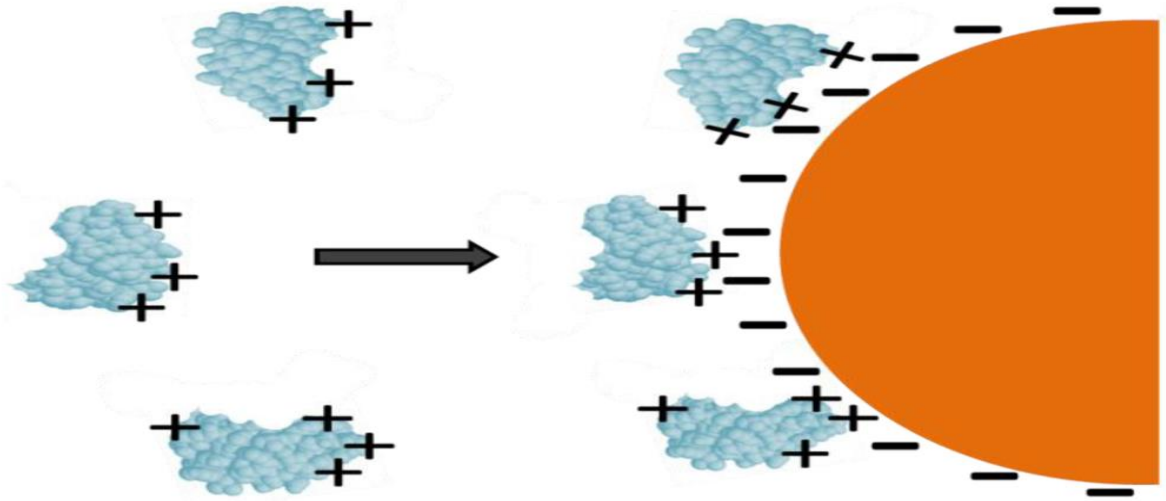


Figure 15- An illustration of ionic binding enzyme immobilization method (www.mdpi.com-1024 × 766)

2.5.3.5 Affinity binding

This method involves immobilization of enzyme to the matrix through specific interactions. The two methods are being followed in affinity immobilization, the first of which is the activation of the support material by coupling an affinity ligand, so that the enzyme will be strongly and specifically bound. However, if this involves the enzyme active site, then there will be no activity. This method offers an advantage in that the enzyme is not exposed to any harsh chemicals conditions (Porath, 1992).

2.5.3.6 Metal linked immobilization

In this type of immobilization, the metal salts are precipitated over the surface of the carriers and it has the potential to bind with the nucleophilic groups on the matrix. The precipitation of the ion on the carrier can be achieved by heating. Moreover, this method is simple and the activity of the immobilized enzymes is relatively high (30-80 %). Lastly, the carrier and the enzyme can be separated by decreasing the pH; hence it is a reversible process that can be used to regenerate the matrix and the enzyme (Yücel, 2011).

2.5.3.7 Immobilization of soybean lipoxidase

Stability of the biocatalyst under more severe conditions employed is required for most bioconversions in industry. Lipoxidase is required for the production of aroma fine chemicals in industry and thus its stabilization on an appropriate support is a critical step as this will allow it to be reused (Laakso, 1982; Parra-Diaz *et al.,* 1993).

Covalent immobilization of purified soybean lipoxygenase (LOX) on four different activated supports have shown that pH optimum in terms of bound activity for the coupling of LOX was 7.5 for CNBr sepharose 4B and Eupergit C, and 8.0 for Fractogel EMD Azlactone and Fractogel EMD Epoxy. Furthermore, it was found that in terms of bound protein and enzyme activity, CNBr sepharose 4B was the best support. Moreover, the shorter immobilization time (2 hours on CNBr sepharose 4B compared to 24 and 72 h on the other supports) was identified as the probable reason for the higher coupling and activity yields. The coupling of LOX on Eupergit C in high ionic strength buffer (0.5 M phosphate buffer), resulted in a high yield in bound activity but poor stability was noted and a complete inactivation after 5 days occurred. Lastly, when the coupling was done in low ionic strength buffers (0.05 M phosphate buffer) there was a low yield in bound activity but high stability (Chikere *et al.,* 2001). Coupling of LOX on oxirane (epoxide) activated acrylic beads in low ionic strength buffers was reported to be associated with poor enzyme activity yield. This represents the major downfall for using low ionic strength buffers in immobilization (del Carmen Pinto *et al.,* 1997; Maguire *et al.,* 1991). It was reported that covalent immobilization of commercially prepared soybean lipoxidase on carbonyldiimidazole activated support requires that lower amounts of the protein be loaded onto the support in order to attain a higher activity. Furthermore, the pH at which coupling occurs was found not to affect the degree of protein loading onto the support and the activity thereafter. Moreover, no enhancement of the coupling yield was noted using dialysis of lipoxygenase before immobilization, indicating that small molecules do not interfere with binding of lipoxidase to its support. It was also found that the reusability of immobilized soybean lipoxidase is better in aqueous buffer than in octane/buffer medium; the activity was retained after seven cycles in the former solution, whereas 40% activity was lost in the latter solution after the same number of cycles. It was noted that 15°C was the optimum temperature for hydroperoxide formation from linoleic acid using the immobilized lipoxygenase. The loss of protein from the support on storage at 5°C was found to be low over a 25 days period with no loss thereafter. Lastly, only about 5% decrease in stability at 5°C was observed over a period of 6 months (Parra-Diaz *et al.,* 1993).

Immobilization of lipoxygenase (LOX) in an alginate-silicate gel matrix is another method developed over the past two decades by researchers; it was found that pH 9.0 in 0.2 M borate buffer are the optimum conditions for sol-gel entrapment of LOX. Furthermore, it was noted that after isolation

and drying under vacuum, the composite gel had a high retention of the protein with good activity of the enzyme and stability at 25°C. Moreover, comparison of activities revealed that the immobilized enzyme activity was lower than that of the free enzyme. An addition of glycerol and borate buffer, or borate buffer saturated with an organic solvent are required for restoration of activity. However, the free enzyme solution loses its activity in less than a day and is not reusable. In contrast, the activity at room temperature is retained by the sol-gel entrapped LOX for at least 25 days and recycling is possible (Hsu *et al.,* 1997).

A high enzyme activity was reported when lipoxygenase (LOX) was inserted into phyllosilicates, an important group of minerals that includes the micas, chlorite, serpentine, talc, and clay minerals. Tetramethyl orthosilicate (TMOS) was hydrolysed to form silicate polymers which were cross-linked with phyllosilicates prior to lipoxygenase insertion. Increased hydrophobicity on the support brought by introduction of alkylamines ensured that charge-charge interaction between the enzyme and the support does not occur. Furthermore, it was stated that, the more the ratio of trimethylammonium (TMA) to TMOS used; the higher the amount of macropores and immobilized enzyme activity. Lastly, it was reported that LOX immobilized in this way is stable on storage and reusable at room temperature (Shen *et al.,* 1998).

2.6 Natural fragrances

Brenna *et al,* (2003a) reported that there are fine flavours and fragrances which are available from nature and that the traditional methods employed by researchers in the past decades involved the usage of chemical routes to extract these aromatic compounds from natural products. Most of the flavours and fragrances are single enantiomers of chiral compounds. Moreover, different odour properties (Amoore, 1982) between enantiomers have been reported. Odour perception of several compounds has been assigned to enantiospecificity of the odour receptors thus making the synthesis of the sole odour active isomer of a certain odorant compulsory (Enberger and Hopp, 1988). Brenna *et al.* (2003a) indicated that most irone isomers exhibit different odour properties when they are in their chiral configuration. We often cannot find enough of them from plants because the plant raw materials contains very low amounts of desired compounds, thus making extraction process very expensive and time consuming. Besides, their production is dependent on the weather, which is difficult to control. Several enantioselective syntheses have been reported in literature for the enantioselective enriched irone isomers, but none of them has found practical application (Chapuis and Brauchli, 1993; Daniel Helmlinger, 1989).

2.7 Irones

Irones are terpenoids with a pleasant smell and are important in perfumery. They can be produced from, iris rhizomes (orris root), and their extracts are called iridals. It has been reported that just after being reaped, the rhizomes are irone-deficient and thus require maturation and oxidation to produce the irones (Marner *et al.,* 1982). Furthermore, most authors approve that the main source of the Irone is *Iris florentina*, *Iris pallida* and *Iris germanica* (Mika, (1991) and Berger, (2007). On the basis of their experiments, Tiemann and Kruger, (1895) drew a conclusion that an irone is a double bond isomer of ionone (rose ketones, another important perfumery compound). The structures of irones and ionones were thoroughly studied and it was found that irones have an additional methyl group at position 5 (Naves, 1947; Ruzicka *et al.,* 1947), which results in their distinct odour. In 1971 Rautenstrauch and Ohloff found that Iris oil contained the following four isomers as illustrated in Figure 16 with (+)-*cis*-α-irone, (+)-β-irone and (+)-*cis*-γ-irone being the first to be identified (Naves, 1947).

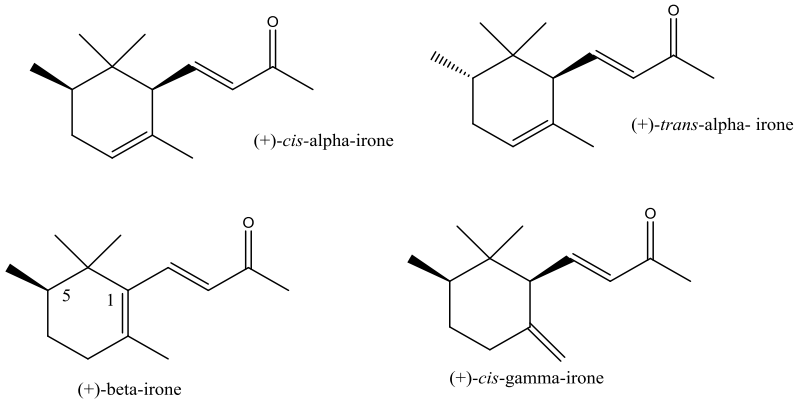


Figure 16- Four isomers of irones (Naves, 1947).

2.8 Iridals and ambrein

The first irones precursors to be isolated were the dihydroirone precursors from *Iris germanica.* Spectroscopic means and an X-ray analysis of the γ-isomer, which crystallized from methanol, allowed for determination of its structure. The compounds are bicyclic triterpernoids (Figure 17). These compounds are said to be closely related to ambrein (Figure 18), however, ring A is cleaved between C2 and C3 and the methyl group from the bridge C6 is shifted to carbon 11. The cyclization of the terminal double bond of squalene by addition of a methyl group instead of a proton, explains why the dihydroirone- instead of the dihydroionone-moeity is formed. Furthermore, in *I. pallida* the α-irone precursor (Figure 19 no. 1) and in traces the C26 deoxy derivative (Figure 19 no. 2), were found, whereas *I. florentina* contained the gamma isomer (Figure 19 no. 3). Thus, the two main components (Figure 19 no.1 and 3) and the dihydroirone precursors (Figure 17 no.1 and 2) differ not

only in the additional double bond but also C26 oxidation. A recent investigation on the bark of *I. germanica* rhizomes discovered in addition to the above mentioned iridals, the existence of the C26 deoxy compound (Figure 19 no. 4) and the irone precursor (Figure 19 no. 5), isomeric at the acrolein moiety (Marner, 1997). Bicchi *et al.* (1993) stated that extracted iridals and related esters possess a molecular weight in the range 450-900 Da which makes high performance liquid chromatography (HPLC) the analytical technique of choice for their analysis in a crude *Iris* extract. They reported the results of the analysis of *I. pallida* extracts carried out by HPLC coupled through a particle beam interface to a mass spectrometer in varying ionization modes. At least, fourteen iridals and fatty acid iridal esters were characterized in the *Iris pallida* rhizome extracts under investigation. A table outlining the characteristics of these iridals and related esters is provided in the Appendix. *Cis*-α and trans-α-irone as well as *cis*-γ-irone are formed by oxidative breakdown of some of the bicyclic iridals contained in the rhizomes of *I. pallida*, in particular iripallidal (no. 12 in the table) and iriflorental (13 on the table).

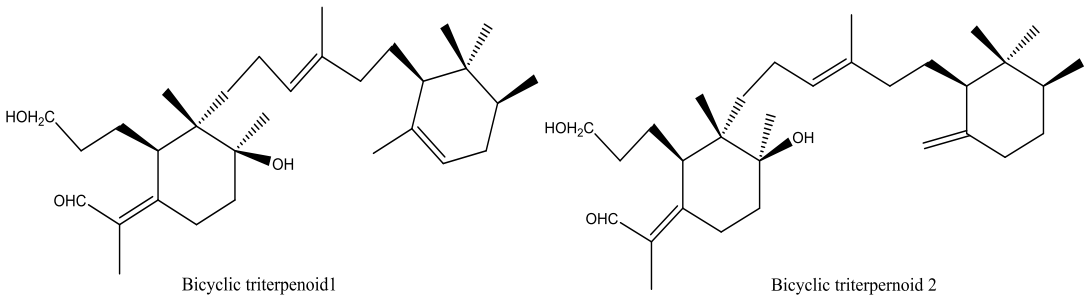


Figure 17- Iridals, bicyclic triterpernoids (Marner, 1997).

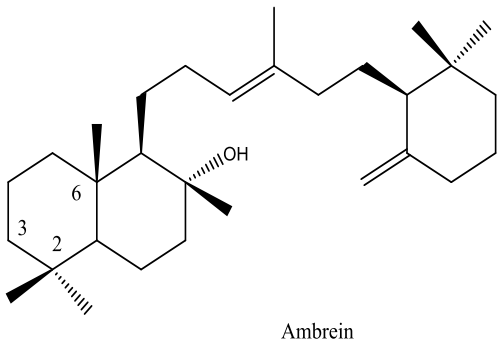


Figure 18- The structure of ambrein (Marner, 1997).

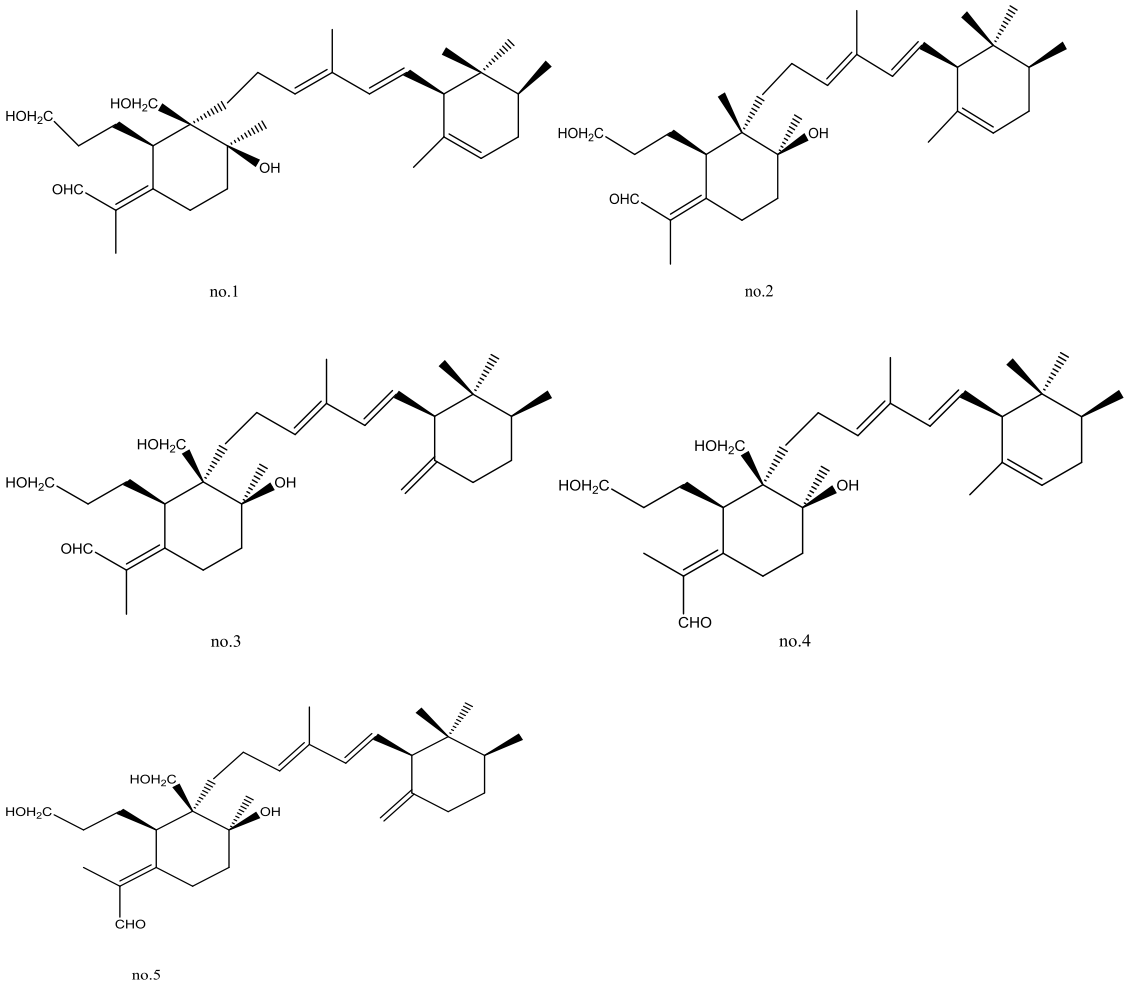


Figure 19- α-irone precursor (1) and C26 deoxy derivative from *Iris pallida* (2) are shown; gamma irone precursor from *Iris florentia* (3), C26 deoxy compound from *Iris germanica* and its isomer (4 and 5 respectively) (Marner, 1997).

2.9 Production of essential oil in the perfume industry

In the fragrance manufacturing, contemporary essential oil production is done by extraction, utilizing volatile solvents like petroleum ether and hexane. The chief benefit of extraction over distillation is that consistent temperature (usually 50°C) can be sustained during the process. As a result, extracted oils have a natural odour that is unmatched by distilled oils, which may have suffered chemical changes through high temperatures. This feature is of sizable importance to the perfume industry; however, the established distillation method is of minor cost compared to the extraction procedure. One of the distillation techniques employed to isolate essentials oils is hydrodistillation. In this method, the aromatic plant material is packed in a still and a sufficient quantity of water is added and brought to boil; alternatively, live steam is inserted into the plant charge. Due to the impact of hot water and steam, the essential oil is released from the oil glands in the plant tissue. The vapour mixture of water and oil is condensed by indirect cooling with water. From the condenser, distillate flows into a separator, where oil separates automatically from the distillate water.

2.10 Screening for the presence of irone ($C_{14}H_{22}$-O) in the rootstocks of the chosen iris species.

Pavol, 2012 reported that when separated by HPLC two irone peaks similar in retention time to those of the irone standard were detected in the samples of rootstocks of distinguished iris species; the retention times for the two peaks shown by the irone standard were 23.403 and 23.624 minutes respectively on HPLC; the method applied to achieve these is summarized below. Material preparation was as such: rootstocks of distinguished iris species were cleaned of substrate, washed, sliced and freeze-dried; the freeze-dried material was allowed to stand for 7 days after which it was soaked in HCl-acidified methanol (1:50, Penta, Czech Republic); the flasks with samples were incubated for 9 hours (3 times for 3 hours with 30 minutes break) into the water bath to provide optimum conditions for the solutions; for every 1 part of the freeze-dried rootstocks used, 3 parts of the acidified methanol was added.

The pure extract was obtained via filtration and evaporation of methanol under reduced pressure, and then diluted to a volume of 10 mL and measured by HPLC with a diode array (DAD) detector at 254 nm. The retention time of the samples was then compared with the retention time of the standard irone solution, irone-mixture of irones, from the Merck Company (Pavol, 2012).

2.11 Synthesis of irones

2.11.1 Process for the treatment of orris root

It has been reported that treatment of fresh orris roots by washing, blanching, cooling, comminuting (reducing to small particles) and incubation steps can cause an increase in the irone content in the roots more quickly than it can be achieved when conventional methods viz. treating the fresh root with ionizing radiation and refluxing the dried root before bioconversion. Comminuting is reported to cause disruption to the cell structure of the plant material and thus initiating a wound response. The wound response stimulates the release of enzymes responsible for conversion of compounds found in the root to irones. Interestingly, no specific degree of comminuting is required as long as it can be enough to cause the wound response (Canhoto *et al.,* 2009).

2.11.2 The current commercial process for irone production

In a study by Brenna *et al.* (2003a) it has been reported that irones are abundantly used compounds in the perfume industry. They are characterized by a pleasant smell and their synthesis has been reported to be very difficult for organic chemists. The current commercial process reported by Kastner and Maurer (1990) on the conversion of rhizomes to *cis*-and *trans*-α-irones, β-irones and *cis*-γ-irones is very long (Figure 20), troublesome, complicated, and low yielding. As a result of this

slow and non-effective method the price of the final product is expensive. In this method, there is an initial storage of rhizomes for up to 3 years in a dry aerated environment. Subsequently the rhizomes are homogenized (comminution) and then incubated with dilute sulphuric acid before they undergo steam distillation to produce a light yellow to brown-yellow solid mass with a violet-like odour known as Orris butter. The Orris butter has a number of definitive properties (Kastner and Maurer, 1990). At 38 – 50°C the solid melts to a yellow to yellow-brown liquid. The acid number is 175– 235, correlated with an acid content of 71 –95 % (calculated as myristic acid); ester number: 4 – 35; ketone content (calculated as irone): 9 – 20 %; soluble with ethanol in all quantities at 50°C (Pavol, 2012). Canhoto *et al.,* (2009) stated that liquid chromatography-mass spectrometry (LC-MS) is useful for analysis of irones and other constituents of orris butter. Critical to the present study is the requirement to improve such a method and consequently reduce the high pricing of the final product. The main components of the oils are *cis*-*γ* irone (usually 30 –40 %) and *cis*-α-Irone (usually 20 – 30 %) which are also responsible for the typical odour.

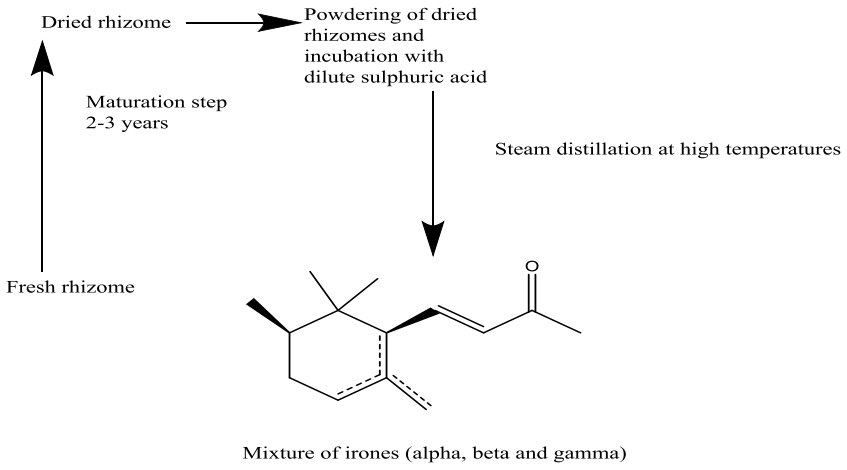


Figure 20-The benchmark method for irone production (Kastner and Maurer, 1990).

2.11.3 Organic Synthesis of irones

Irones have long been synthesized by synthetic chemical means. One example is the transformation of α-pinene to 2-(2,2,3-trimethyl cyclobutyl)-hepta-2,4-dien-6-one was accomplished, and the product further subjected to ring opening and concurrent cyclisation, which resulted in the formation of a mixture of irones as illustrated in Figure 21 (Eschinazi, 1959, 1961).

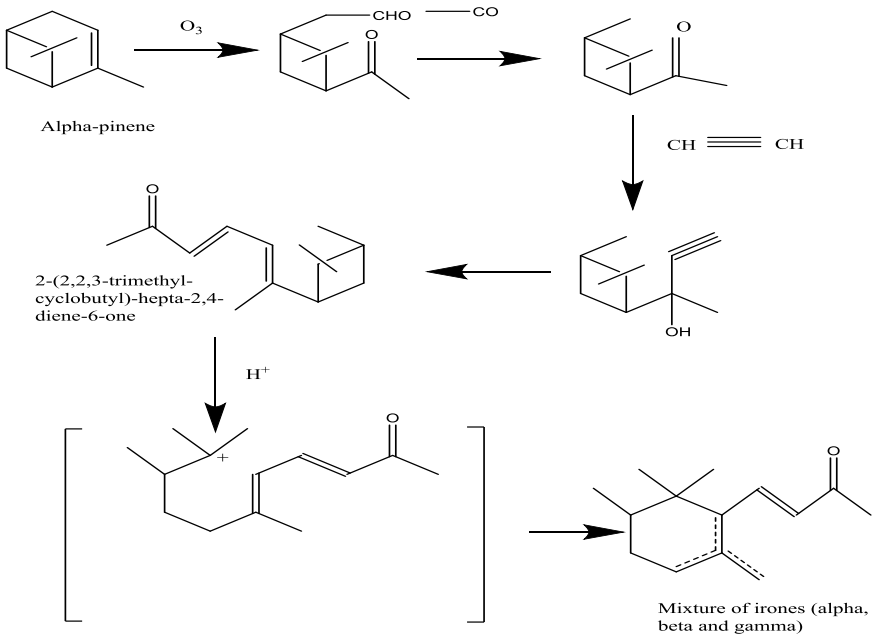


Figure 21-The synthesis of irones from α-pinene (Eschinazi, 1959, 1961).

There have been several other attempts to synthesise these compounds and one of them was the acid catalysed cyclisation of 9-methylpseudoionone; derived from 5,6-dimethyl-hept-5-en-2-one (Ishihara *et al.,* 1974). An attempt to reach irones through Friedel-craft type methylation at the double bond of geraniol, citral and pseudoionone was unsuccessful (Barton and Mousseron-Canet, 1960). A stereoselective synthesis of β, γ-*cis* and γ-*trans* irone isomers through cyclic sulphone intermediates was also reported (Torri *et al.,* 1990). Several other chemical syntheses were reported by Chemists around the globe (Rautenstrauch and Ohloff, 1971; Yoshikoshi *et al.,* 1982). However, these new methods of synthesis developed were still somewhat complex and chemical intensive (Eswari, 1992).

2.11.4 Biosynthesis of irones from iridals, extracts of orris root.

The biosynthesis of irones occurs via the degradative oxidation of methylated bicyclic triterpernoids called cycloiridals that, together with monocyclic iridals, are present in the rhizomes of various species of iris (Jaenicke and Marner, 1986; Krick *et al.,* 1983). The transfer of the methyl group to the terminal double bond of an open-chain iridal and subsequent cyclization leads to the formation of a mixture of three regioisomers α-, β-, and γ-irones as shown in Figure 22.

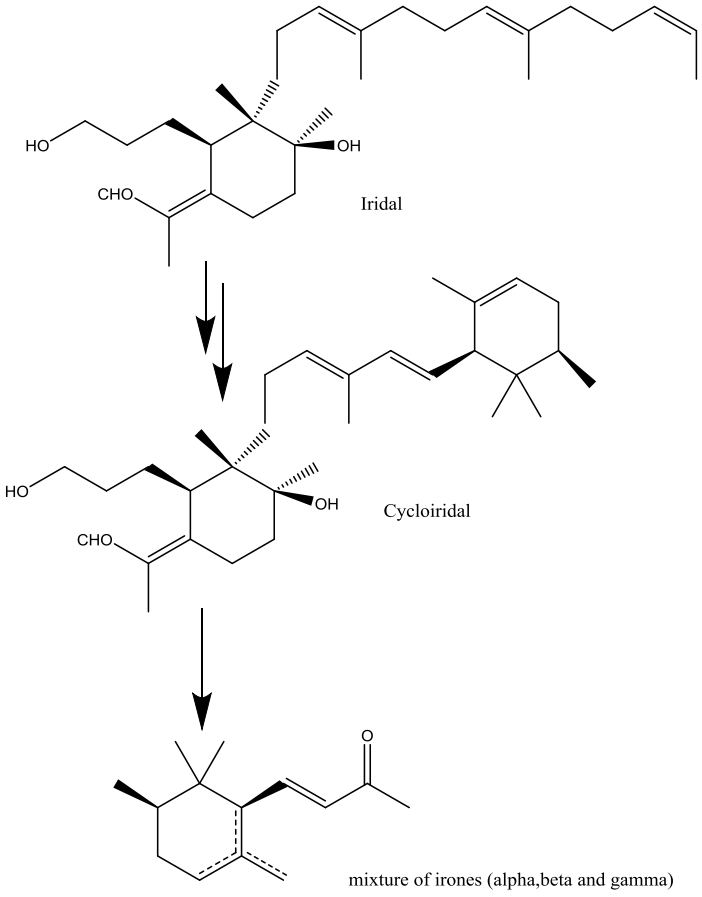


Figure 22- Biogenesis of irones from iridals (Brenna *et al.,* 2008).

2.11.5 Production of irones from fresh iris rhizomes by treatment with nitrite salts

So far the most time consuming step in the extraction of natural irones from the orris root is the maturation period which takes around 3 years. This maturation step can be accelerated by treating orris root (and other sources of irone precursor) with nitrite salts down to a mere 24-30 hours. It is not known as to which compounds or mixture of such compounds that when treated with the nitrite, lead to irones. The preferred conditions for the process are a nitrite salt concentration ranging between 0.5-1 g/L, a 1:5 to 1: 20 ratio by weight of iris rhizome substrate to medium, a pH between 2 and 3 and a temperature near ambient are. The yield is in the relatively high range of 1.2-1.4 g/kg of dry rhizomes (Ehret *et al.,* 2001), but the toxicity of high concentrations of nitrite salt on both human and animals, (Sowers *et al.,* 2004) means this method has a serious drawback.

2.11.6 Isolation of irones during separation of essential oils

Irones can be produced during a process to separate essential oils from orris root. At least two major steps have been outlined: (i) steam distillation of the dry orris root by the use of a distillation vessel or (ii) extraction by the use of an extraction vessel. A mixture consisting of an essential oil and at least one hydrophilic phase is produced by this step. Following these, contact of the mixture with a hydrophobic adsorbent in an adsorption vessel allows for retention of the oil. Subsequently the

hydrophilic phase not adsorbed is recycled back into the steam distillation vessel and the essential oils are desorbed from the adsorbent. However, this process involves relatively complex procedures and specialized equipment such as solid phase extraction column packed with 200 g (dry weight) of 40 µm spherical C18-coated silica (Ennelin and Jumppanen, 2002; Jumppanen and Ennelin, 2004).

2.11.7 Enzymatic process for obtaining irones from irone precursors

In 1992, the company Elf Aquitainic patented an enzymatic process to obtain irones from iridals. The terpene precursor was extracted with alcohol from crushed rhizomes for 48 hours at 80°C and this extract was then submitted to enzymatic oxidation for 48-60 hours at 30°C (Figure 23). This was done in the presence of a surfactant and using either an isolated soybean lipoxidase, or horseradish peroxidase in the presence of hydrogen peroxide. After 48 to 60 hours irones were recovered by steam distillation. The yield using the two different enzymes was respectively 1.8 and 0.50 g/kg of dry weight of rhizomes (Gil *et al.,* 1992). It should be noted that a mixture of irones are formed by the above process, however, for simplicity only (+)-*cis*-α -irone is shown in Figure 23.

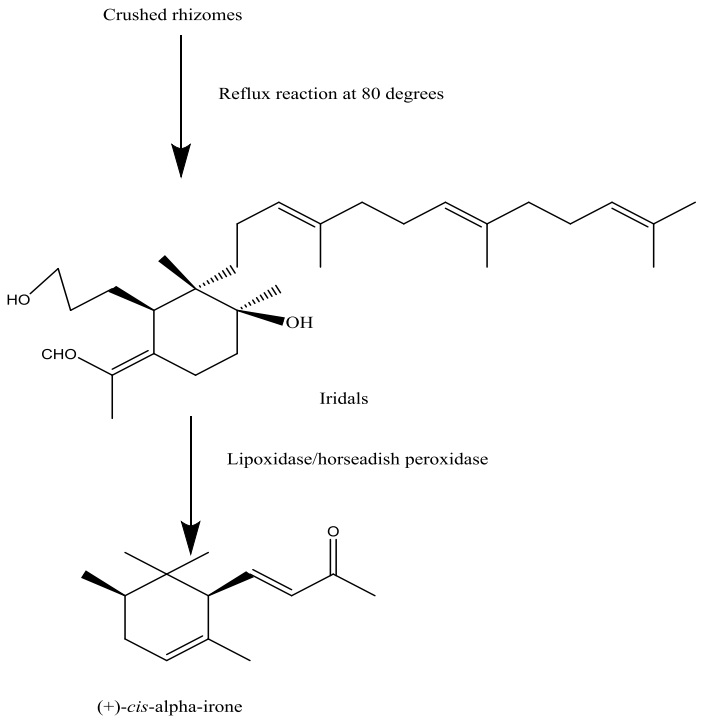


Figure 23-Synthesis of irones from iridals extracted from crushed rhizomes (Gil *et al.,* 1992).

The rapidity, high yields of the lipoxygenase process and its lack of toxic reagents, make this a potentially preferred process. However, certain aspects of the process could be improved to make it commercially viable. These include; elimination of the step required for extraction of the irones precursor molecules, processing of the substrate and enzyme prior to bioconversion, finding the best solvent(s) for sampling of the irones, optimisation of lipoxygenase to orris root profile, pH, incubation time, temperature, the concentrations of oleic acid, dioxane and minerals, and the method for isolation of the final product.

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Sodium chloride (analytical reagent), calcium chloride dihydrate and ethanol absolute from Minema; sodium acetate trihydrate, manganese chloride, 99 % purity, di-potassium hydrogen orthophosphate (K_2HPO_4), 99 % purity, imidazole for synthesis ≥ 99 %, dichloromethane (DCM), 99.5 % purity, ferrous sulphate $7H_20$, 98-105 % purity, disodium hydrogen orthophosphate, Na_2HPO_4 (anhydrous) 98 % and sodium hydroxide pellet, 98 % from Merck Chemicals (Pty) Ltd; sodium dodecyl sulphate (SDS), 98.5 %, oleic acid, 96 % purity, potassium di-hydrogen orthophosphate (KH_2PO_4) Trizma® base, 99.9 % purity, glutaraldehyde solution, cupric sulphate anhydrous and mineral oil from Sigma-Aldrich; polyethylene glycol (PEG) 20 000 and zinc sulphate from Saarchem supplied by Merck Chemicals (Pty) Ltd, boric acid powder (analytical reagent) from Rochelle Chemicals; PEG 6000 from FLUKA; bovine serum albumin (BSA) standard (2 mg/mL) from Pierce; Quick Start™ Bradford 1 × Dye Reagent from Biorad; diethyl ether (HPLC), 99.5 % assay, propan-2-ol (isopropanol) HPLC, 99.8 % assay, acetonitrile (ACN) HPLC, 99.9 % assay, 1,4 dioxane (analytical reagent), 99.8 %, acetone, 99.8 %, dimethylsulphoxide (DMSO), 99.5 %, methanol 99.9 %, from Lab-Scan analytical sciences.

3.1.2 Equipment

Coffee grinder from Platinum (Quality and Style), Gilson pipettes from Lasec/SA, pH meter from Mettler Toledo, syringe filters-Clarinert™ from Angela Technologies, 2 mL crimp neck vial, 32 × 12 mm clear glass from Angela Technologies, via Separations, Heraeus Pico 17 centrifuge from Thermo-Scientific, Rotavapor RII from Butchi Switzerland, weighing balance BP 21005 from Sartorius, weighing balance WAS220/X from RADWAG®, DU® 800 Spectrophotometer from Beckman-Coulter™, Allegra™ X-22R Centrifuge, dry bath, from Optima Scientific, gene disruptor vortex mixer from Scientific industries (Pty) Ltd, orbital shaker incubator from MRC, Oven from Lasec/SA, Waring commercial blender from Lasec/SA, waterbath from Labhouse Scientific, Avanti™ J-25I centrifuge from Beckman Coulter, Power Wave HT from Biotek, Likens-Nickerson distillation apparatus from Glass world Pretoria and UPLC high-definition quadrupole time-of-flight MS instrument (UPLC-qTOF SYNAPT G1 HDMS system, Waters, Manchester, UK).

3.1.3 Other consumables

Fresh and partially dried orris root from Clive Teubes cc. The partially dried orris was received as thinly sliced while the fresh root was received as iris rhizome whose leaves were cut on arrival leaving only the bottom part with the node, internode, scale leaf, bud and adventitious root.

Soybean flour was obtained from Impilo foods; Gold Star instant yeast, Suberase (laccase) enzyme was supplied by Novo Nordisk A/S Bagsvaerd, Denmark (produced by submerged fermentation of *Aspergillus oryzae*, containing a gene originating from *Myceliophthora thermophila*).

3.2 Methods

3.2.1 Analytical Method

LC-MS analysis:

The extracts were evaluated on an ultra-performance liquid chromatography (UPLC) high-definition quadrupole time-of-flight MS instrument (UPLC-qTOF SYNAPT G1 HDMS system, Waters, Manchester, UK) fitted with an Acquity BEH C8 column (2.1 x 150 mm, 1.7 µm; Waters Corporation). A dual solvent system consisting of eluent A: 0.1% formic acid in water and B: 0.1% formic acid in methanol (Romil Chemistry, UK) was used at a uniform column temperature of 70°C. A 10 min gradient method at a constant flow rate of 0.4 mL/min eluent was used for separation of compounds, and the conditions were: 60% B over 0.0-1.0 min, 60-95% B over 1.1-6.0 min and 95% B over 6.1-7.0 min. Thereafter, the column was returned to initial conditions at 8 min and allowed to equilibrate for 2 minutes. Chromatographic separation was monitored using a photodiode array (PDA) detector with a scanning range set between 200-500 nm, 1.2 nm bandwidth resolution and a sampling rate of 20 points/sec.

Quadrupole time-of-flight mass spectrometry (Q-TOF-MS):

Post-PDA detection, the metabolites were further detected with the aid of a SYNAPT G1 high definition mass spectrometer operating in positive ionization mode. The MS conditions were as follows: capillary voltage of 4.0 kV, sample cone voltage of 25 V, extraction cone voltage of 4 V, MCP detector voltage of 1600 V, source temperature of 120°C, desolvation temperature of 450°C, cone gas flow of 50 L/h, desolvation gas flow of 550 L/h, *m/z* range of 100-800, scan time of 0.2 sec, interscan delay of 0.02 sec, mode set as centroid, lockmass set as leucine enkephalin (556.2771 Da), lockmass flow rate of 0.1 mL/min, and mass accuracy window of 0.5 Da. High purity nitrogen gas was used as desolvation, cone and collision gas. The software used to control the hyphenated system was MassLynx Ver. 4.1 (SCN 704).

A gas chromatography method was also developed (see Appendix), but without analytical standards, could not be used to determine compound identity and concentration.

3.2.2 Sample extraction methods

Most authors agree that the main source of the irone is *Iris florentina*, *Iris pallida* and *Iris germanica* (Mika 1991 and Berger 2007 cited in Pavol, 2012), but other sources also mentioned different Iris species as a possible source of irone. In the present study, *Iris pallida* had been used on the basis of availability.

3.2.2.1 Processing of the orris roots prior to extraction

Two rhizome samples, *Iris pallida* were obtained from Teubes cc. as fresh and partially dried orris roots as indicated in details in section 3.1.3. It is already known that the irone content of Iris species peaks after three years of storage (Marner *et al.,* 1982; Van Hevelingen, 1992).

As a start, 50 g of each of the two rhizome samples were taken. The partially dried orris root was not washed on arrival because it was free of dust particles as it was pre-sliced and washed before delivery. The partially dried orris root was homogenized twice without addition of water by using a normal house blender. The fresh orris root was washed with distilled water before being commuted and homogenized as the dried.

3.2.2.2 Standard method for extraction of iridals (irone precursors)

The method of extraction was modified form of that reported by Gil *et al.,* (1991). Following homogenization, 500 mL of ethanol was added to a 1 L round bottom flask containing processed root sample (50 g) and refluxed at 83°C for 20 hours with stirring and cooling using running water. The solid materials were removed by vacuum filtration using Whatman no.1 filter paper and the ethanol was removed by evaporation under reduced pressure. Two hundred and fifty (250) mL of dichloromethane was added to the filtered solids and the reaction mixture was refluxed again for 20 hours at 40°C with stirring and cooling using running water. The solid material was once again removed by filtration using Whatman no.1 filter papers and dichloromethane removed by evaporation under reduced pressure. The products were analysed by LC-MS.

3.2.3.1 Standard biocatalysis reaction

The following reaction was used as the base case and control reaction, this method was a modification of that of Gil *et al.,* (1992). A sodium borate buffer (0.01 M) pH 9.2 was made up. A mass of 0.5 g of the extracts prepared previously containing the terpene precursors was mixed with 4.5 mL of soybean lipoxidase, 1 g soybean flour in 25 mL borate buffer (0.01 M pH 9.2), 0.5 mL dioxane, 0.25 mL oleic acid and a spatula tip of manganese chloride and ferrous sulphate. The reaction was allowed to occur at 30°C with stirring for up to 72 hours and air was blown through the reaction mixture.

For sample preparation, 1 mL samples produced via the above biocatalysis reaction were taken at 24 hour intervals (0, 24, 48 and 72 hours). To the samples 0.4 mg sodium chloride and 1 mL diethyl ether were added. The layers were separated by centrifugation for 2 minutes at 13000 rpm in a microcentrifuge. The diethyl ether layer was removed by pipette and submitted for analysis.

3.2.5 Optimisation experiments

3.2.5.1 Evaluating solvents for sampling of irones

To 1 mL samples diethyl ether, aqueous buffers or an acetone-DMSO combined solution were tested to determine the most efficient method for extracting the active compounds for analysis:

Method 1: To the samples, 0.4 mg sodium chloride and 1 mL diethyl ether were added. The layers were separated by centrifugation for 2 minutes at 13000 rpm in a microcentrifuge. The diethyl ether layer was submitted for analysis.

Method 2: The samples were mixed by vortexing for 30 seconds with 0.01 M sodium borate buffer (pH 9.2) or 0.5 M phosphate buffer (pH 7.15), centrifuged at 13 000 rpm for 2 minutes in a microcentrifuge and the supernatants were taken for analysis.

Method 3 (ideal method): The samples were centrifuged at 13 000 rpm for 2 minutes and the supernatants (aqueous phase) were discarded. The solids were suspended in the combined solution of 0.5 mL acetone and 0.5 mL DMSO, vortexed for 30 seconds and then centrifuged under the same conditions as above and the supernatants were taken for LC-MS analysis.

3.2.5.2 Iridal breakdown per time and Enzyme selection

As the bioconversion reaction involves an oxidation reaction, different oxidoreductase enzymes were tested for feasibility. Preliminary experiments have shown the difficulty in weighing the irone precursors (iridals) prior to setting up biocatalysis reactions due to their sticky oily nature. In order to overcome this problem the bioconversion of fresh orris root to irones without prior extraction of iridals was attempted. Three oxidoreductase biocatalysts, soybean lipoxidase, laccase (suberase) and whole cells of *Saccharomyces cerevisiae* (which are rich in a broad range of oxidoreductases), were tested for the bioconversion. Due to the scarcity of the fresh root at particular times in the year, subsequent experiments were done with the partially dried orris root to determine the optimum parameters for the bioconversion. These optimised parameters were then tested on the fresh root for comparison.

Enzymes: Crude lipoxidase from soybean was prepared by dissolving 1 g of soybean flour in 25 mL of borate buffer (0.01 M, pH 9.2) by slowly stirring overnight at 4°C. The slurry was then centrifuged at

4000 rpm for 5 minutes at 4°C before collecting the supernatant containing the lipoxidase (enough lipoxidase to be used in all reactions was prepared). The laccase (Suberase) derived from *Myceliophthora thermophila* and produced in a recombinant host (*Aspergillus oryzae*) was supplied by Novo Nordisk A/S Bagsvaerd, Denmark.

Saccharomyces cerevisiae preparation: 1 g of dried instant yeast (Gold star) and a teaspoon of sugar were dissolved in 25 mL of 0.5 M phosphate buffer pH 7.15 in a 50 mL Schott bottle. The solution was mixed and stirred for approximately 30 minutes at 30°C. The solids were removed by centrifugation at 13 000 rpms for 2 minutes and the supernatant was collected.

Reaction procedure: 5 g of the root (homogenized with the Waring blender) was equally divided among three round bottom flasks (50 mL each) correctly labelled. To the first flask, 25 mL of crude soybean lipoxidase supernatant, 0.25 mL oleic acid, 0.5 mL dioxane, a spatula tip each of manganese chloride and ferrous sulphate were added and the sample was mixed by stirring. To the second flask, 18 mL of 0.5 M potassium phosphate buffer pH 7.15 and 2 mL of laccase (suberase) were added and the sample was mixed by stirring. To the third flask, 20 mL of the *Saccharomyces cerevisiae* enzyme was added and the sample was mixed by stirring. All the conversion reactions were allowed to proceed at 30°C for 0, 12, 24, 48 and 72 hours with stirring and slowly blowing air into the reaction as a source of oxygen. The soybean-lipoxidase samples were later analysed as both full scan and at iridals mass (450-900 Da) in order to evaluate their dissociation over time. Bicch *et al.* (1993) reported High Performance Liquid Chromatographic-Particle Beam-Mass Spectral analysis of iridals from *I. pallida* rhizomes. He mentioned that the iridal mass is in the range 450-900 Da.

3.2.5.3 Reaction optimisation

Standard reaction preparation procedure: The partially dried orris root (note: this matured root already contains some irones) was blanched for selected times (as stated below) at 60°C before homogenization. The reactions (5 g blanched (3 min) and homogenized root, 0.5 mL dioxane, 0.25 mL oleic acid, spatula tips of ferrous sulphate and manganese chloride and 1g soybean flour in 25 mL borate buffer as indicated in section 3.2.5.2) were prepared in triplicate in autoclaved 250 mL Erlenmeyer flasks and were run at 30°C and 180 rpms in an orbital shaker incubator for 72 hours with daily sampling starting a time 0.

Optimisation began with separate evaluation of the influence of loading each of the following parameters to the standard reaction as indicated in section 3.2.5.3: 2.5 g orris root, either 0.5 g or 2 g lipoxidase in 25 mL of borate buffer (0.01 M pH 9.2), 0.5 mL oleic acid and 1 mL dioxane on the process at both 30 °C and 37 °C

Optimisation continued with determination of the best ratio of salts wherein the effect of loading different Mn: a Fe ratio on production of irones was determined. Soybean lipoxidase may be activated by manganese chloride and ferrous sulphate. The standard reaction at 37°C as previously described was used with modifications: The effect of a 2:1 (50 mg: 25 mg), 1:1 (25 mg: 25 mg), and 1:2 (25mg: 50 mg) ratio of manganese chloride to ferrous sulphate were evaluated. Also tested was the effect of using either manganese chloride, ferrous sulphate, or no minerals (control). The best results on the dried root were tested later tested on the fresh root and were compared with results obtained when using other minerals as follows: $CuSO_4$, $CaCl_2$ and $ZnSO_4$ were tested individually in amounts of 25 mg each or in combination as follows: 25 mg: 25 mg, 50 mg: 25 mg or 25 mg: 50 mg ratios of $CuSO_4$: $CaCl_2$. The reactions were allowed to proceed for 5 days with sampling done at day 0, 1, 3, and 5.

Lastly, Optimisation of blanching times and selection of antifungal agents was carried out. In previous experiments fungal growth occurred beyond three days of incubation, and hence inclusion of some antifungal agents was evaluated.

The experiment was varied by testing the effect of: blanching the root for between 3 and 5 minutes and adding 100 µl of either acetonitrile (99 %), 5 % ethanol, 0.68 mg/mL imidazole, SDS or Isopropanol (99%) or 100 µl SDS (final concentration 1 %). The effect of imidazole was further evaluated by testing the use of imidazole concentrations of 0.34 mg/mL and 1.36 mg/mL.

3.2.6 Comparison of fresh root to dried root irone productivity.

Best reactions previously conducted on dry root were tested on fresh root for comparison. The blanching time and temperature (3 min at 60°C), salts ratio (50 mg $MnCl_2$ and 25 mg $FeSO_4$), oleic acid (0.25 mL) orris root (5 g) and crude lipoxidase (1 g soybean flour in 25 mL borate buffer) were kept constant. The effects of using either 1 mL dioxane, 100 µL isopropanol (90 %) and 100 µL of 0.68 mg/mL imidazole buffer on the process were investigated for each root.

As a benchmark for irone formation, one of the methods reported in literature by Courtois *et al.,* (1998) to have resulted in good conversion was reproduced and compared with the optimized lipoxidase method. The main findings of the study were that treating the fresh orris root (after distilled water washing and homogenization) with about 0.5 g/L to about 1 g/L of sodium nitrite resulted in 1.84 g irones/kg dry rhizomes and that steam distillation of the maturated mixture yielded 1.85 g orris butter, yield 0.37 % /fresh rhizomes or 0.92 % /dry rhizomes.

Optimized method: 5 g of fresh orris root (washed, peeled and blanched for 3 minutes at 60°C) was incubated with 25 mL of crude lipoxidase suspension, 50 mg of $MnCl_2$, 25 mg $FeSO_4$, 0.25 mL oleic acid and 1 mL dioxane for 5 days at 37°C with agitation at 200 rpm and sampling at day 0, 1, 3 and 5.

0.7 L Scale-up reactions:

A set of 700 mL reactions were performed in 2 L sterile reactors. Lipoxidase was prepared as such 28 g soya flour was stirred overnight each in 700 mL borate buffer (0.01 M, pH 9.2), i.e. at the same flour to buffer ratio as in the small scale reactions); solid particles were removed by centrifugation at 4000 rpm, 4°C for 5 minutes. Fresh Orris root was washed with sterile water and sprayed with 70% ethanol; and subsequently homogenised and blanched for 5 minutes at 60°C. The homogenised root, enzyme, 14 ml dioxane, 14 ml oleic acid, 1.4 g $MnCl_2$ and 0.7g Fe_2SO_4 were added to reactor; reactions were run at 37°C and 300 rpm for 3 days. The influence of oxygen on the reaction was determined by providing oxygen (100 %) to one reactor, while the other was sealed to exclude oxygen. Samples were taken at 0 hour and every 24 hours.

2L scale up reactions: The optimized method was further tested at 2 L bench scale, in addition the pH of the reaction mixture was measured and found to be 5.2, and it was adjusted to 6 and 7 for comparison. The reactions were allowed to proceed for 24 hours with aeration and improved agitation. The samples were taken at 0 hour and every 3 hours for 24 hours.

3.2.7 Comparison of process extraction methods

Solvent extraction: The maturated mixtures of orris root (partially dried or fresh) previously subjected to lipoxidase oxidation were subjected to solvent extraction in a 1:5 (50 mL maturated mixture: 250 mL solvent) ratio. The solvents employed in the extraction were hexane (99 %), ethyl acetate (98 %), or petroleum ether 40°C-60°C.

After mixing of each maturated mixture with the solvent, the equilibration process was accelerated by vigorous shaking of the separating funnel for about 2 minutes subsequent to separation, the initial organic phase was set aside and then re-extraction was performed. The extracts were combined and washed with a solution of ultrapure water to remove water soluble impurities.

Steam distillation and solvent extraction: Steam distillation has been reported to concentrate the irones (Jumppanen *et al.,* (2001) and Courtois *et al.,* (1998)). Hence the maturated mixtures of orris root previously subject to enzymatic oxidation were steam distilled for 3 hours and then subjected to solvent extraction as indicated above. The vapour was then condensed to a liquid (the distillate). In this instance, steam was used to lower the distillation temperature of high boiling organic compounds that are immiscible with water. In the process, steam was charged to the matrix

to volatilise the hydrophobic liquid and carry it across to a chilled condenser for subsequent liquefaction and separation from water (See Appendix for apparatus).

Likens-Nickerson technique: This technique is sometimes called simultaneous distillation; it was designed by Likens and Nickerson as an original device for analysis of hop oil. This one-step isolation-concentration of flavour constituents permitted a dramatic period saving over the separated operation and, due to their continuous recycling, a great decrease of treated volumes of liquids (Likens and Nickerson, 1964).

In this technique, oxygenated sample from above was centrifuged to remove buffer. Prior to the procedure, the entire system (Figure 24) was purged with nitrogen (N_2, 2-3 mL/min) for 5 minutes. Nitrogen was introduced at area H. Orris root was added to apparatus (vessel A) with 400 ml fresh water. Dichloromethane was added stepwise to vessel B (30 ml initially, 25 ml after 1 hour and 20 ml after 2 hours. The oil bath temperature for the root was 135 - 140°C and for dichloromethane was approximately 75°C. Both flasks were stirred. As the contents of flask A started boiling a distillation-extraction of the irones to form orris butter extract occurred. The result was mixing of the sample vapour (from flasks A) and the solvent vapour (reasonably pure from flask B) which condensed at area C by means of a cold finger maintained at -0.4°C by a chiller. Upon condensation at area C, the two liquid layers separated by density gradient (the DCM layer settled at the bottom while the aqueous layer took the top position) before returning to their respective flasks. The entire steam distillation-solvent extraction procedure was carried out under a 2 mL/min nitrogen flow. The steam distillation was carried for 3 hours and the dichloromethane extract was collected from flask B (Figure 24 and Appendix).

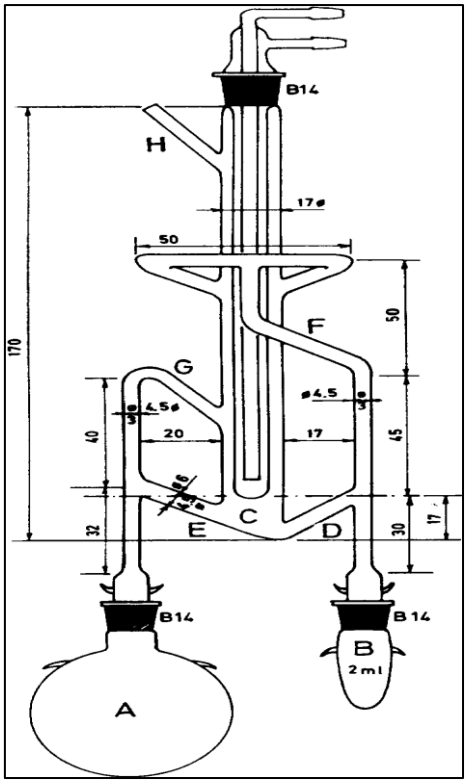


Figure 24-Likens-Nickerson distillation unit adapted from (Bouseta and Collin*, 1995). A-the vessel for putting the sample, B-the vessel for placing the organic solvent (diethyl ether), C-the condensation area, H- the opening for nitrogen aeration, Inlet and outlet are for inward and outward movement of water between the condenser and the chiller (Chaintreau, 2001).

A volume of 1 mL of the DCM extract was taken for LC-MS analysis as is. The sampling method for the steam distillate of irone mixture as reported by Canhoto *et al.,* (2009) was reproduced by drawing about 5 mL of the same extract, extracting it with about 100 mL of HPLC water in a 39°C water bath, vigorously shaken for separation, and 1 mL of the organic layer was taken for LC-MS analysis.

3.2.8 Purification of soybean lipoxidase

Purification method:

Although there are several methods reported on lipoxygenase isolation, the present study adapted the extraction and purification of soybean lipoxygenase using Aqueous Two-Phase System by Lakshimi *et al.* (2009), because it is easy and cheap to use. About 10 grams of soybean flour was dissolved in 100 mL of 0.2 M sodium acetate buffer pH 4.5, the solution was stirred for 1 hour at 4°C. The slurry was centrifuged for 5 minutes at 4000 rpm at 4°C. The pH of the supernatant was adjusted to 6.8 using 2 M NaOH. Approximately 74 mL of the supernatant was recovered. About 1 mL sample was taken from the supernatant for protein concentration and enzyme activity determination.

PEG 20 000 (5 %) was added to the extract and the solution was stirred for 10 minutes and allowed to settle for 20 minutes at 4°C. The solution was then centrifuged at 4 000 rpms for 5 minutes at 4°C and approximately 63 mL supernatant was recovered for soybean lipoxidase respectively. Sample (1 mL) was taken from the supernatant containing LOX for protein concentration and enzyme activity.

PEG 6000/ammonium sulphate was prepared as follows: 75.99 g PEG 6000 and 26.4 g ammonium sulphate was dissolved in 300 mL deionized water and mixed thoroughly by stirring. The extract was subjected to two phase separation with predetermined amount of PEG 6000/ ammonium sulphate in deionized water such that the extract was maintained at 30 % (v/v). The solutions were stirred for 1 hour and were allowed to stand until phase separation. Samples (1 mL) from the top and bottom layers were taken for protein concentration and enzyme activity respectively. This procedure was reproduced and 50 mL of each purification fraction was stored at -20 °C.

Preparation of BSA standard curve: A BSA standard (2 mg/mL) was used to prepare accumulative amounts of BSA in the range 0.1 to 0.5 mg/mL as designated in Table 1:

Table 1- Preparation of different concentrations of Bovine Serum Albumin (BSA)

Final BSA concentration (mg/mL)	Volume of BSA standard (2 mg/mL) (µL)	Volume of deionized water (µL)
0.1	10	190
0.2	20	180
0.3	30	170
0.4	40	160
0.5	50	150

For the Bradford assay, triplicate 10 µl volumes of the BSA dilution in were mixed gently with 240 µL of Bradford reagent. The mixture was allowed to stand for 5 minutes at room temperature and then absorbance was read against the blank at 595 nm on a plate reader. From these, a standard curve of absorbance against increasing BSA concentration was plotted as indicated in the Appendix.

Determination of protein concentration

A volume of 10 µl of purified LOX was introduced into a well (300 µL) of a micro-titre plate in triplicate. To each well 240 µl of Bradford reagent was added and the solution was mixed and allowed to stand for 5 minutes at room temperature. The protein concentration was extrapolated from the standard curve prepared by the method above (see Appendix for the curve).

Enzyme activity assay:

Preparation of the substrate: 2.1 mg of linoleic acid sodium salt (Sigma-Aldrich) was dissolved in 25 mL 0.15 M phosphate buffer pH 7.5, the substrate solution was saturated with oxygen for approximately 5 minutes. The substrate was stored at -20°C and used immediately after being defrosted, while still cold.

The activity was determined using a UV-assay against a blank sample. A 30 × dilution of the enzyme was achieved by adding 58 µl of deionized water to 2 µL of the enzyme (1/30 dilution). For a reaction, 15 µL of the enzyme dilution was added in triplicate to quartz cuvettes of 1 cm path length. The reaction was initiated by addition of 985 µl of the substrate, linoleic acid (0.084 mg/mL). Enzyme activity calculations were done as follows:

Gibian *et al.* (1986) determined the molar coefficient of extinction for 13-hyroperoxy-*cis*-*trans*-9, 11-octadecadienoate ($C_{18}H_{32}O_4$, 312 Da) from average of values obtained from three independent methods to be 23 000 $M^{-1}.CM^{-1}$, such value was used in the current research. This fatty acid is a product of oxygenation of linoleic acid 18:2(9, 12) by soybean lipoxygenase at carbon 13. This oxidation causes a shift of carbon 12 double bond to carbon 11.

$$Concentration\ of\ linoleic\ acid\ peroxide\ formed\ (M) = \frac{\frac{\Delta Abs}{\Delta time}}{(\varepsilon \times l)}$$

ΔAbs = change in absorbance, Δt = change in time, ε= molar extinction coefficient (M^{-1} cm^{-1}), and l = path length (cm). The concentration in mol/L/min was then converted to µmol/mL/min. In this case the mL refers to the volume (1 mL) in the reaction tube. Therefore, the amount (µmoles) of fatty acid produced/minute in the total reaction was calculated as thus; µmoles of fatty acid/min = (1 ml total volume) x µmoles/min/ml to get total µmoles of fatty acid generated in µmoles/min. The 1 ml of total reaction volume contained only 0.015 ml of enzyme which was generated by a 1/30 dilution containing [X] mg/mL of total enzyme before it was diluted. Therefore, [X] (mg/mL) of enzyme before dilution x (0.015 mL) x (1/30) = [X] mg lipoxidase used in the assay. Knowing the amount of fatty acid produced/min and the protein concentration expressed in milligrams, the specific activity of our sample was calculated. Specific activity = µmoles fatty acid produced per min /mg lipoxidase.

Enzyme kinetics: 6.72 mg/mL linoleic acid salt stock was prepared from a 99% salt purchased from Sigma-Aldrich. Increasing concentrations of linoleic acid were prepared from the stock solution as indicated in Table 2. Triplicate samples of the substrate were added first into quartz cuvettes (1 cm path length) and the reaction was initiated by addition of the enzyme whose concentration was kept constant at 0.12 mg/mL. The reaction rates were determined over a period of 5 minutes by the UV assay at a wavelength of 234 nm against a suitable control in quartz cuvettes of 1 cm path length.

The assay was performed at a pH of 7.5 and a temperature of 25°C. The KM and Vmax of soybean lipoxidase were determined from the Lineweaver-Burk plot represent by the following equation: $\frac{1}{V} = \frac{Km}{Vmax[s]} + 1/Vmax$ which is a known method for plotting the data in a linear fashion. However, this plot has a disadvantage of giving too much weight to points on the graph at lower substrate concentration. An alternative method, Eadie-Hofstee plot with some advantage over the Lineweaver-Burk plot, (equation: $V = -Km(\frac{V}{[S]}) + Vmax$) was used. Although, the use of V on both axis causes noticeable errors in the measured reaction rate and consequently the calculations for Vmax and Km subsequently become less accurate. Another method of plotting a linear relationship is through utilization of Hanes-Wolf plot with the equation: $\frac{[S]}{V} = \frac{1}{Vmax}\ [s] + \frac{Km}{Vmax}$

Despite eliminating some of the previously mentioned errors, this method suffers from exaggeration of any inaccurate measures in substrate concentration as substrate concentration is used on both axes. A separate blank was prepared for each substrate concentration.

Table 2: Reaction cocktail for determining the effect of linoleic acid sodium salt concentration on the activity of soybean lipoxidase

Linoleic acid dilution concentrations (mg/mL)	Linoleic acid dilution volume (µL)	Soybean lipoxidase volume (µL)	0.15 M sodium phosphate buffer pH 7.5 (µl)
0.015	986	15	-
0.021	985	15	-
0.032	985	15	-
0.042	985	15	-
0.062	985	15	-
0.084	985	15	-
0.092	985	15	-
0.168	985	15	-
0.336	985	15	-
0.48	985	15	-
Blank	985	-	15

Dialysis of purified soybean lipoxidase:

A 66 cm length of Pierce SnakeSkin™ Pleated Dialysis Tubing, MW cut-off 3500 to 10000 daltons (Thermo Scientific), was hydrated with distilled water and then drained and a clasp applied to the lower end. To this was added 100 mL of the purified soybean lipoxidase, and sealed with a clamp.

The now hydrated tubing was placed in a glass beaker with 600 mL 0.001 M sodium borate buffer pH 9.2 while stirring.

The buffer was discarded and replaced every three hours (this was repeated twice). After the second buffer change the dialysis tube was allowed to stay overnight. The volume of the tube doubled due to influx of water from the buffer. The excess water was removed by placing the tube in a container with PEG 6000 to reduce the volume. Approximately 94 mL of Soybean lipoxidase was recovered and 1 mL was taken for protein concentration and enzyme activity.

Testing the effect of pH on purified soybean lipoxidase activity:

Different buffers were prepared as shown in Table 3. The pH optimum of purified soybean lipoxidase was determined using the UV assay. The temperature, substrate and enzyme concentrations were kept constant at 25 °C, 2.1 mg/25 mL and 0.12 mg/mL respectively. For a reaction, 15 µl of the enzyme was added in triplicate to quartz cuvettes of 1 cm path length. The reaction was initiated by addition of 985 µl of the substrate (linoleic acid in 0.15 M phosphate buffer). The reaction rates at each pH were then determined in triplicate over a period of 120 seconds against suitable blank samples. Lipoxidase temperature optimum was determined by comparing activities at 5, 25, 30, 37, 50, 60, 90 and 100 °C.

Table 3-Preparations of different buffer pH for use in determining the effect of pH on soybean lipoxidase activity

pH	Volume of 1 M KOH (mL) [a]	Volume of 2 M KCl (mL) [b]
2.0	0.5	24.35
3.0	1.8	24.05
4.4	2.8	23.60
6.1	4.2	22.65
7.2	5.5	21.50
8.4	6.3	20.85
9.1	7.0	20.50
10.0	7.8	20.05

[a, b] in the table above represents the volume of 1 M KOH and 2 M KCL to be added to 25.0 mL of a stock solution, which is 0.008 M in CH_3COOH, H_3BO_3, followed by dilution of the mixture to 100 mL using deionized water to give a buffer of the desired pH at a final ionic strength of 0.5 M (Jordan, 1980). The pH values were verified and adjusted where necessary.

Testing the effect of purified lipoxidase on the process:

Purified lipoxidase: 25 mL of each of the purified soybean lipoxidase fractions (crude, PEG 20 00 phase and PEG 6 000 bottom phase) was used to oxidize the precursors in orris root. Bioconversion reactions; for the reactions, 5 g fresh orris root (blanched for 3 minutes at 60°C, homogenized using

a coffee grinder and transferred to Erlenmeyer flasks). To each flask 25 mL of lipoxidase fraction, 0.25 mL oleic acid, 0.5 mL dioxane, 25 mg of ferrous sulphate and 50 mg of manganese chloride were added. The pH of the mixture was measured and found to be 6.1 and reactions were permitted to proceed for 5 days at 37°C in rotatory incubator shaker with sampling at day 0, 1, 3 and 5.

3.2.9 Immobilization of soybean lipoxidase

Immobilization:

Entrapment in alginate gel:

Sodium alginate (30 g) was dissolved in 1 litre of distilled water to make a 3% solution. Approximately 1 mL of partially purified soybean lipoxidase was mixed with 10 ml of 3% (wt.) sodium alginate solution. The beads were formed by dripping the polymer solution from a height of approximately 20 cm into an excess (100 mL) of stirred 0.2 M $CaCl_2$ solution with a syringe and a needle at room temperature. The bead sizes were controlled by pump pressure and the needle gauge. The beads were left in the calcium solution to cure for 0.5-3 hours. The emulsion was centrifuged at 4500 rpm for 10 minutes at 4°C to recover the pellet. The pellet in the form of beads was washed once with distilled water and was kept in the cold room.

Immobilization of soybean LOX on a porous resin:

Preparation of particles (standard method):

This method forms “dendrispheres”, now available commercially under the trade name Resyn (Jordan *et al.,* 2009). Five (5) mL mineral oil (Sigma) was added with 0.05 ml of nonoxynol and 0.2 ml of glutaraldehyde (20% (m/v) from a 25% solution grade II to form Emulsion A. Emulsion B was prepared by adding 5 mL of mineral oil with 0.05 ml of nonoxynol and 0.200 mL of PEI pH 9 10% (v/v); take into account that it is already 50%. Each emulsion was vortexed for 30 seconds.

Emulsion A was added first into a beaker and stirring was started; Emulsion B was added to the stirring solution in a single dose to permit the polymer cross-linking reaction at 700 rpm for 60 minutes. The emulsion was then centrifuged at 4500 rpm for ten minutes to recover the particulates formed. The pellet was resuspended and washed with 30 ml x 5 with distilled water in order to remove the mineral oil, nonoxynol and unreacted glutaraldehyde and PEI. The final supernatant was clear and the pellet was resuspended in about 4 mL of 50 mM phosphate buffer, pH 7.

Immobilisation:

Purified lipoxidase (1 mL, at 0.1 mg/mL protein), was added to 0.5 ml of dendrispheres and rotated on a rotator for 2 hours at 4°C. The reaction was stopped by centrifugation and washing the dendrispheres at least 5 times with 50 mM phosphate buffer, pH 7. A solution of 5 mg/mL BSA was added to block any unreacted sites and rotated for another 1 hour. The dendrispheres were collected by centrifugation and washed at least 5 times with water.

Determination of the protein bound:

Prior to incubation with each support, the amount of protein in the purified lipoxidase was measured by Bradford assay; after coupling was complete, each enzyme-support complex was separated from the buffer and the mixture washed as described in the immobilization procedure. The volume of the combined supernatants recovered after each immobilization and washing was measured, and the amount of recovered protein determined; the estimate of the protein bound to each support was determined from the difference between the amount of added and recovered protein.

Activity Measurements: Enzyme activity assay and kinetics were done as in earlier assay for purified lipoxidase. Table 4 shows the reaction cocktail for the kinetics of dendrispheres-immobilized soybean lipoxidase.

Table 4- Reaction cocktail for determining the kinetics of soybean lipoxidase immobilized on dendrispheres

Linoleic acid concentration (mg/mL)	Volume of LOX (µL)	Volume of linoleic acid dilution (µL)
0.02	15	985
0.04	15	985
0.06	15	985
0.08	15	985
1	15	985
1.2	15	985
1.4	15	985
1.6	15	985
1.8	15	985
2.0	15	985
2.2	15	985

The concentration of linoleic acid was varied as explained in the assay; that of dendrispheres- immobilized LOX was kept constant at 0.1 mg/mL.

*A mass of 0.15 g was used in the case of immobilized lipoxidase.

Effect of pH on immobilized soybean lipoxidase activity: this was determined as with free enzyme using 15 µL or 0.15 g of dendrispheres or alginate-entrapped lipoxygenase. Linoleic acid substrate

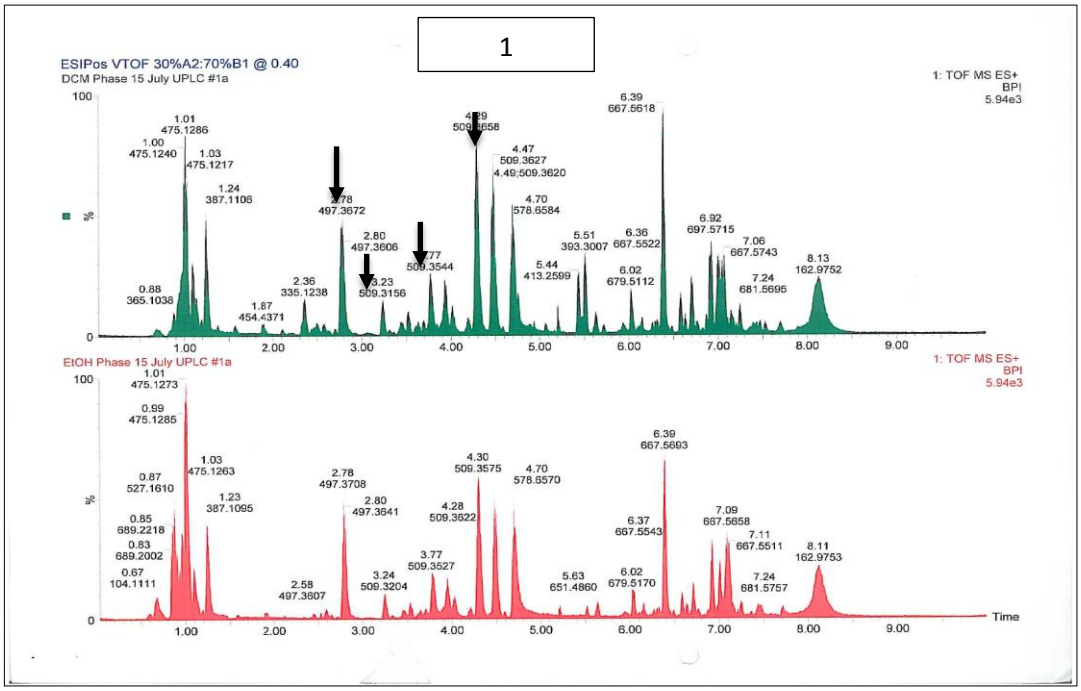
was prepared as in the purification methods. Enzyme activity was measured as initial rate of product (hydroperoxide formation) by an increase in absorbance at 234 nm using a spectrophotometer. Also identified was lipoxidase optimum temperature amongst 5, 25, 30, 37, 50, 60, 90 and 100 °C.

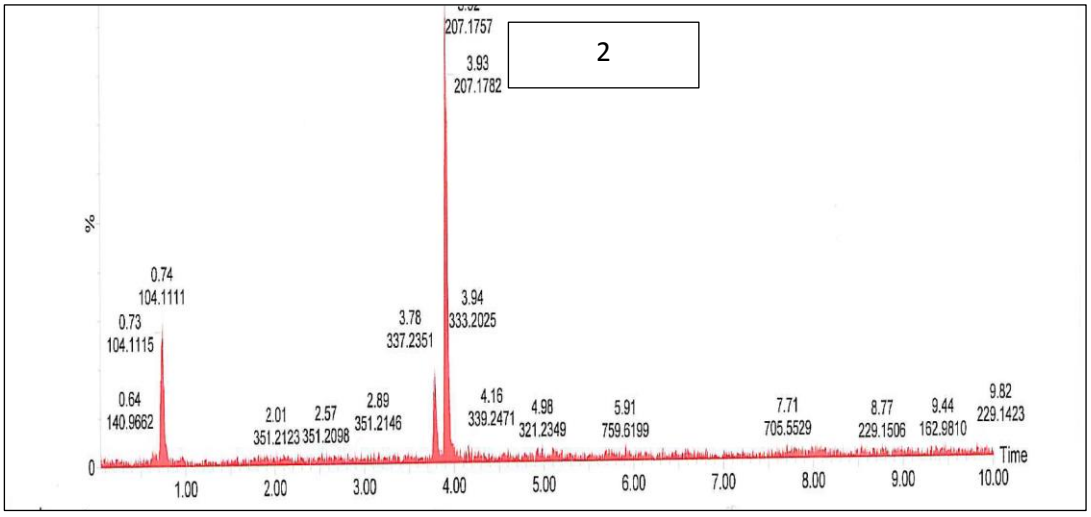
CHAPTER 4 RESULTS AND DISCUSSION

4.1 Analytical method development

GC and LC-MS analysis of the orris root samples were tested and compared; LC-MS analysis was identified as the best method to compare the bioconversion yields. The latter method offered similar extracted mass chromatograms at a similar retention time as the α-irone standard sample of unknown concentration, and offered better certainty based on the ability to confirm compounds definitively based on their masses. Due to unavailability of irone analytical standards, the calibration curve (necessary for extrapolation of estimate irones concentrations) was constructed using the commercial α-irone sample. The calibration curve was constructed as integrated peak areas versus increasing α-irone concentration (see Appendix). In cases where comparative irone production graphs were plotted, the results were expressed simply as integrated peak areas. The irones concentrations (mg/kg orris root) for selected results were stated in the discussion and such were calculated from the straight line formula ($Y = 17239.9X$) obtained from the calibration curve, where y is integrated peak area, 17239.9 is the gradient and x is irones concentration. Because in most cases we used 5 g orris root per 25 mL reaction which is equivalent to 0.2 g per mL, the irone concentrations in mg/mL were divided by 0.2 g/mL to obtain irone concentrations in mg/g orris root. This was followed by conversion of mg/g to mg/kg.

Figure 25 presents extracted mass chromatogram showing the iridals (Mass 450-900 Da) - extracted from fresh orris root (1). Lipoxidase oxidation of the extracts produced irones (Mass 207 Da) in the samples analyzed as acetone-DMSO layer (2) or (309 Da) in the samples analysed as diethyl ether (3) layer. The 309 Da obtained in the diethyl ether layer instead of 207 Da was suspected to be due the presence of impurities. However, no irones were detected from the borate buffer layer (4).





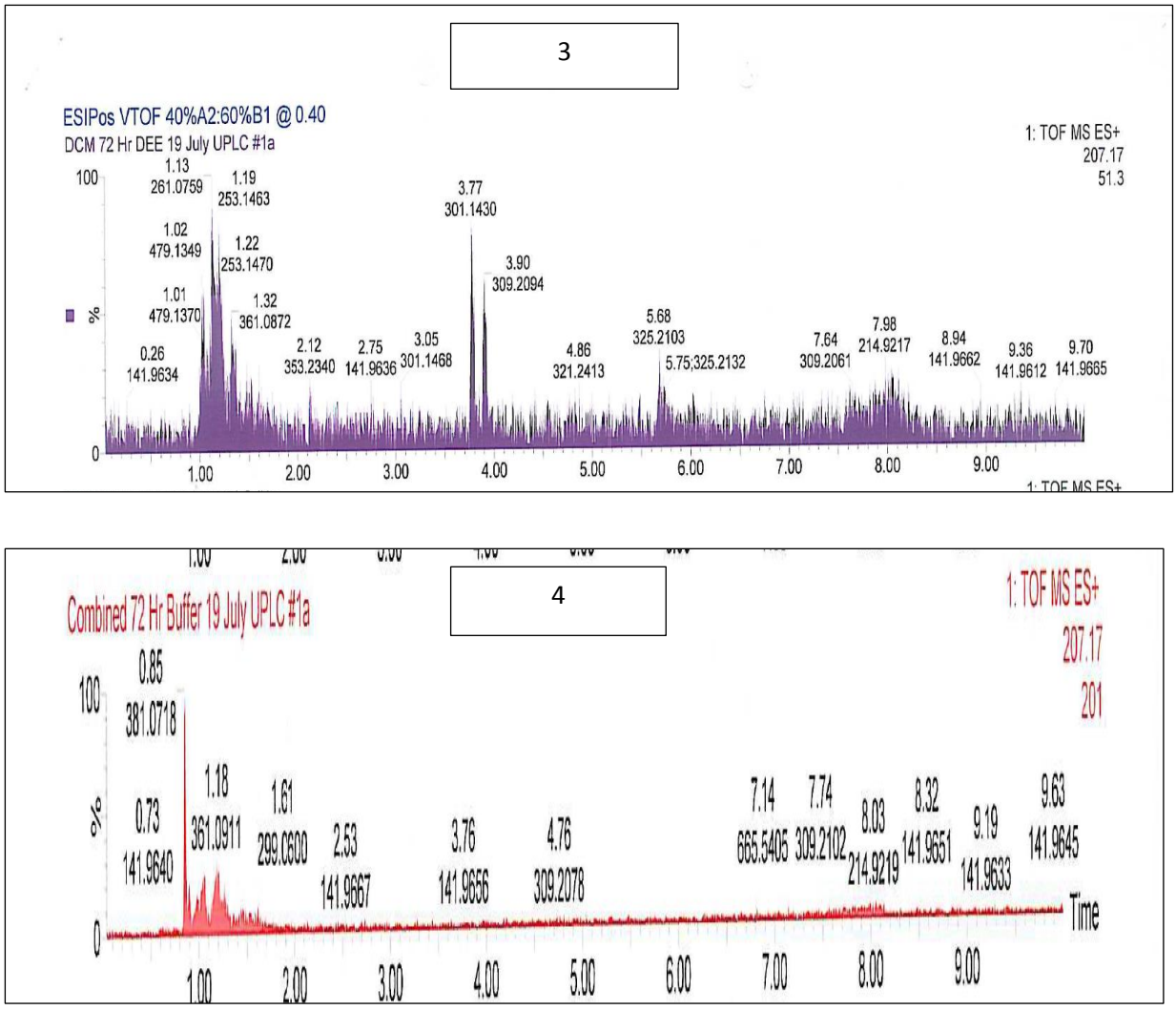


Figure 25-Illustration of extracted mass chromatograms for fresh orris root extract (top left) and irones (mass 207 Da) sampled with either acetone-DMSO (top right), diethyl ether (bottom left) or borate buffer layer (bottom right). Fresh orris root extract of mass closer to 474 and 528 (arrows) corresponding to compounds 9 and 10 (see table 1 appendix).

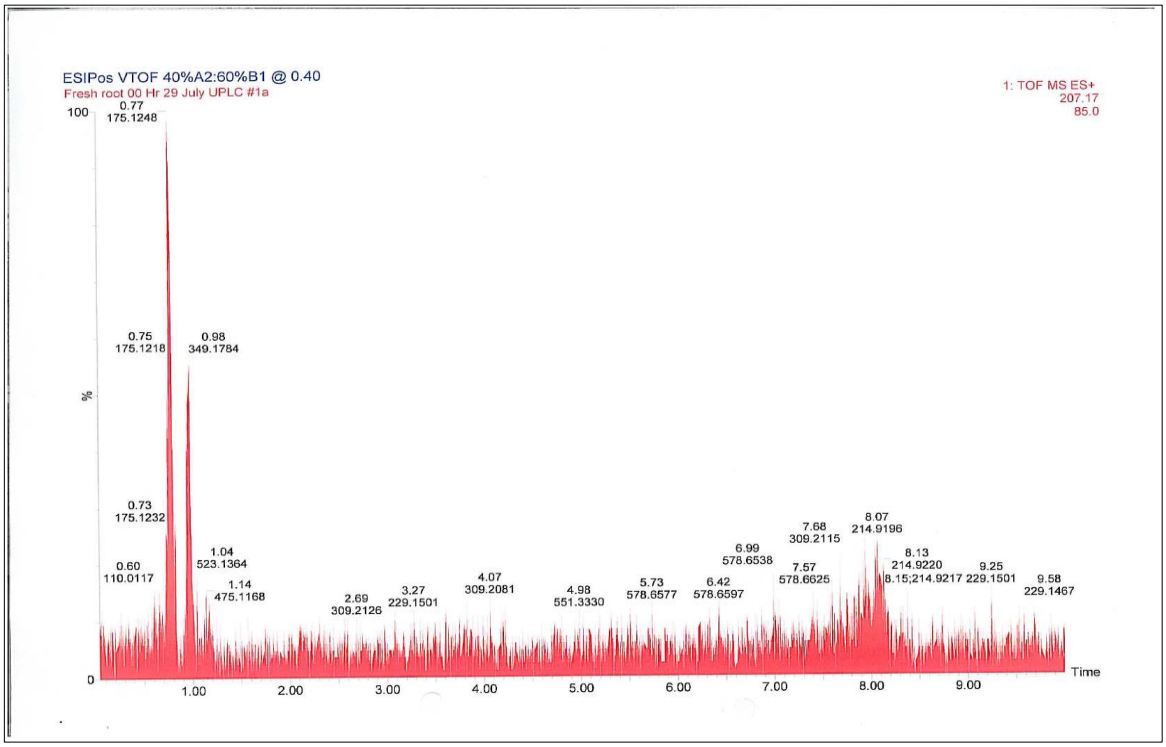
Fresh root does not contain irones according to the literature (which we confirmed), while matured dried root contains between 0.2 and 3 mg/kg irones. Due to the low quantities of the desired products, the extracted mass chromatograms were needed to identify the irones. When the maturated irone suspension was mixed with diethyl ether, centrifuged and the ether layer analysed, we obtained peaks at 3.79 and 3.82 minutes, which correlate with the standard sample's retention times for γ- and α-irone(see Appendix). It should be noted that because we only had α-irone commercial sample we assumed that the peak eluting at 3.79 minutes was that of γ-irone based on information obtained from literature (Rautenstrauch and Ohloff, 1971). We could not confirm whether the above irone isomers are *cis* or *trans.* Production of all irone isomers and separation in to *cis*- and *trans*-isomers requires chemical treatment of alpha-irone commercial sample and such was not an interest in the present study. Production of beta-irone requires chemical treatment of the alpha-irone which was not of interest in the present study.

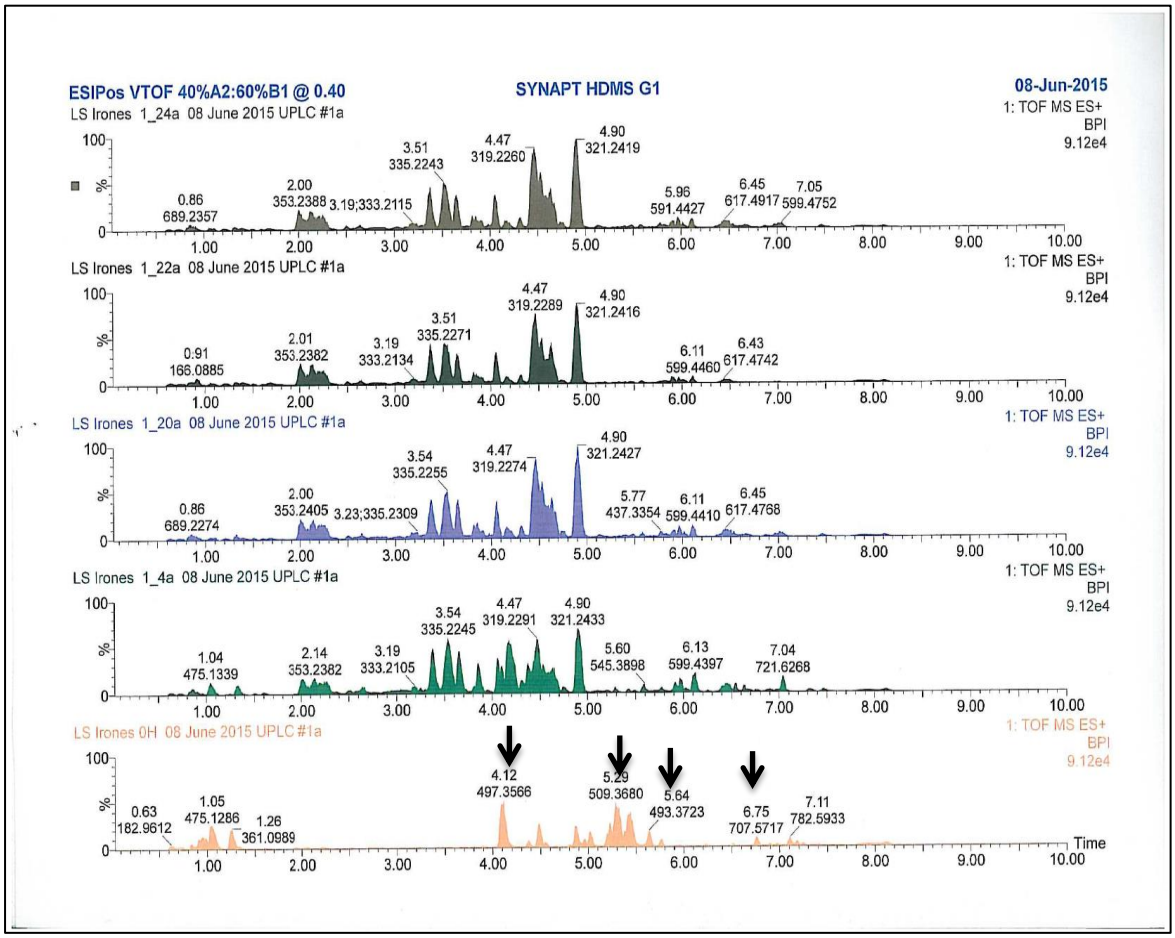
When the suspension was simply centrifuged without addition of any solvent no irones were detected in the aqueous layer. When it was first centrifuged, aqueous layer discarded and solids suspended in a combined solution of equal proportions of Acetone-DMSO more compounds were present in the analysis and the peak heights were slightly higher than in the diethyl ether, indicating a higher concentration of the extracted compounds.

4.2 Iridal dissociation over time and biocatalyst selection

This involved the evaluation of, three oxidoreductases which are soybean lipoxidase, laccase (suberase) and *Saccharomyces cerevisiae*, for the bioconversion of fresh orris root iridals to irones without prior extraction of iridals. The following Figures represent the LC-MS chromatograms for maturated irone mixtures. Due to the complex nature of the full spectrum and the low concentration of irone in the total sample, a scan was done specifically looking for the mass of 207 Daltons to confirm that it is irones (γ- and α-) which are eluted at 3.79 and 3.83 minutes Such chromatograms were compared with that of a commercial α-irone sample (see Appendix).

In the case of determination of dissociation of fresh orris root iridals over time, both full scan and extracted mass chromatograms were done precisely looking for the iridal masses in the range 450 and 900 Da as stated in Bicchi *et al.* (1993). Iridal masses closer to 470, 474, 486 and 684 Da corresponding to compounds 7, 9, 12 and 14 (see table I in the Appendix) were identified by LC-MS and their degradation by lipoxidase over time was monitored (Figure 26). The exact masses identified as sodium adduct were 493.3723, 497.3566, 509.3680 and 707.5717 Da. We probably could have obtained exact masses as Bicchi *et al.* (1993) if we used iridals isolated by their method.


ESIPos VTOF 40%A2:60%B1 @ 0.40
Fresh root 00 Hr 29 July UPLC #1a
1: TOF MS ES+
207.17
85.0
0.77 175.1248
0.75 175.1218
0.98 349.1784
0.73 175.1232
0.60 110.0117
1.04 523.1364
1.14 475.1168
2.69 309.2126
3.27 229.1501
4.07 309.2081
4.98 551.3330
5.73 578.6577
6.42 578.6597
6.99 578.6538
7.57 578.6625
7.68 309.2115
8.07 214.9196
8.13 214.9220
8.15;214.9217
9.25 229.1501
9.58 229.1467
%
Time


ESIPos VTOF 40%A2:60%B1 @ 0.40
SYNAPT HDMS G1
08-Jun-2015
LS Irones 1_24a 08 June 2015 UPLC #1a
1: TOF MS ES+
BPI
9.12e4
0.86 689.2357
2.00 353.2388
3.19;333.2115
3.51 335.2243
4.47 319.2260
4.90 321.2419
5.96 591.4427
6.45 617.4917
7.05 599.4752
LS Irones 1_22a 08 June 2015 UPLC #1a
0.91 166.0885
2.01 353.2382
3.19 333.2134
3.51 335.2271
4.47 319.2289
4.90 321.2416
6.11 599.4460
6.43 617.4742
LS Irones 1_20a 08 June 2015 UPLC #1a
0.86 689.2274
2.00 353.2405
3.23;335.2309
3.54 335.2255
4.47 319.2274
4.90 321.2427
5.77 437.3354
6.11 599.4410
6.45 617.4768
LS Irones 1_4a 08 June 2015 UPLC #1a
1.04 475.1339
2.14 353.2382
3.19 333.2105
3.54 335.2245
4.47 319.2291
4.90 321.2433
5.60 545.3898
6.13 599.4397
7.04 721.6268
LS Irones 0H 08 June 2015 UPLC #1a
0.63 182.9612
1.05 475.1286
1.26 361.0989
4.12 497.3566
5.29 509.3680
5.64 493.3723
6.75 707.5717
7.11 782.5933
%
Time

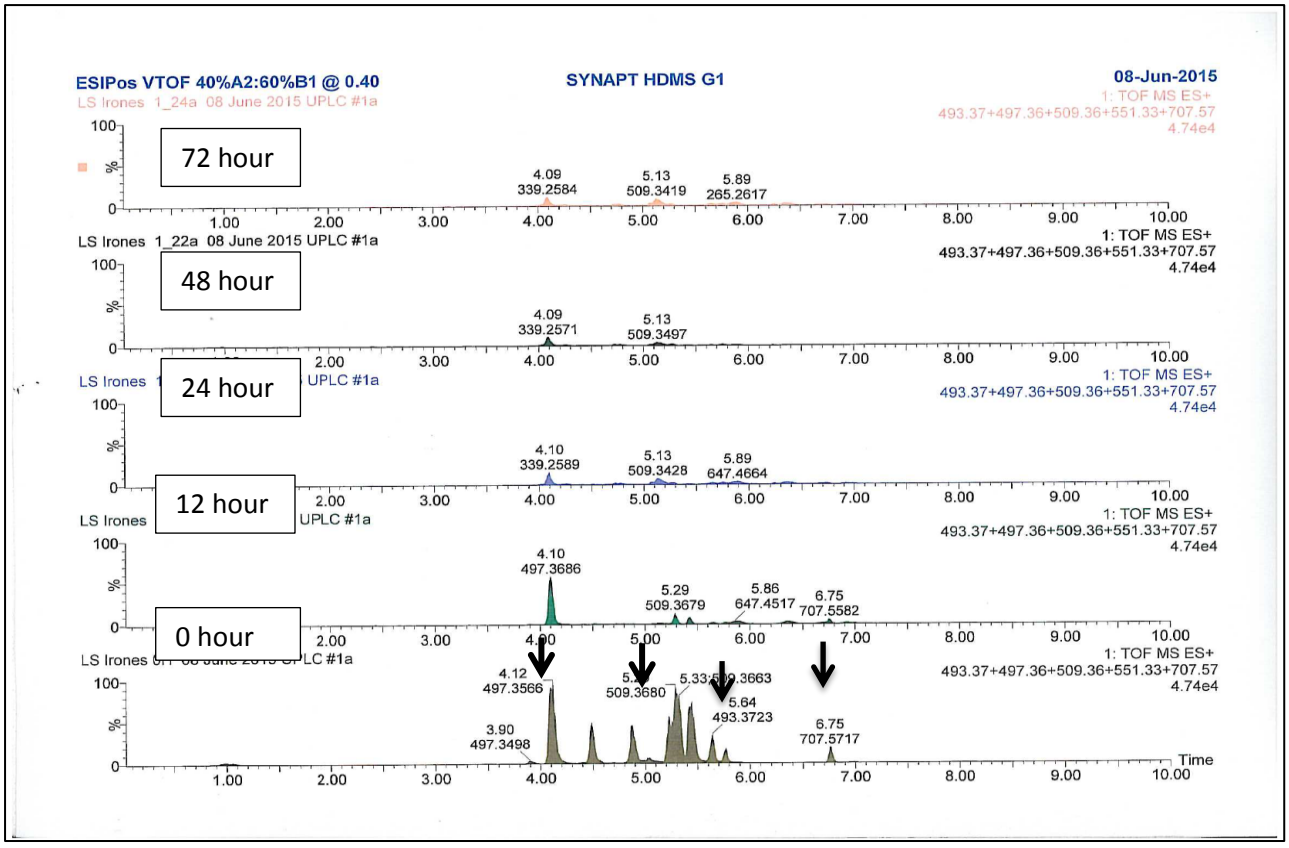


Figure 26-The chromatogram at 207 Daltons showing macerated fresh orris root extract (top). No irones can be observed. A Full scan and an extracted mass chromatogram at 493.3723, 497.3566, 509.3680 and 707.5717 Da showing the conversion of iridals over time (top right and bottom).

The fresh orris starting material (top left) had no irones (as defined as peaks of mass 207 Da detected at 3.79 and 3.82 minutes). Both the full scan (top right) and extracted mass chromatograms (bottom left) shows that the iridals are disappearing with time, suggesting a successful lipoxidase-driven oxidation of fresh orris root iridals over time. The results showing the extracted mass chromatogram (ESI+ mass of 207 Da) for γ- and α- irones biocatalytically generated from fresh orris root and analysed by LC-MS are presented in Figure 27.

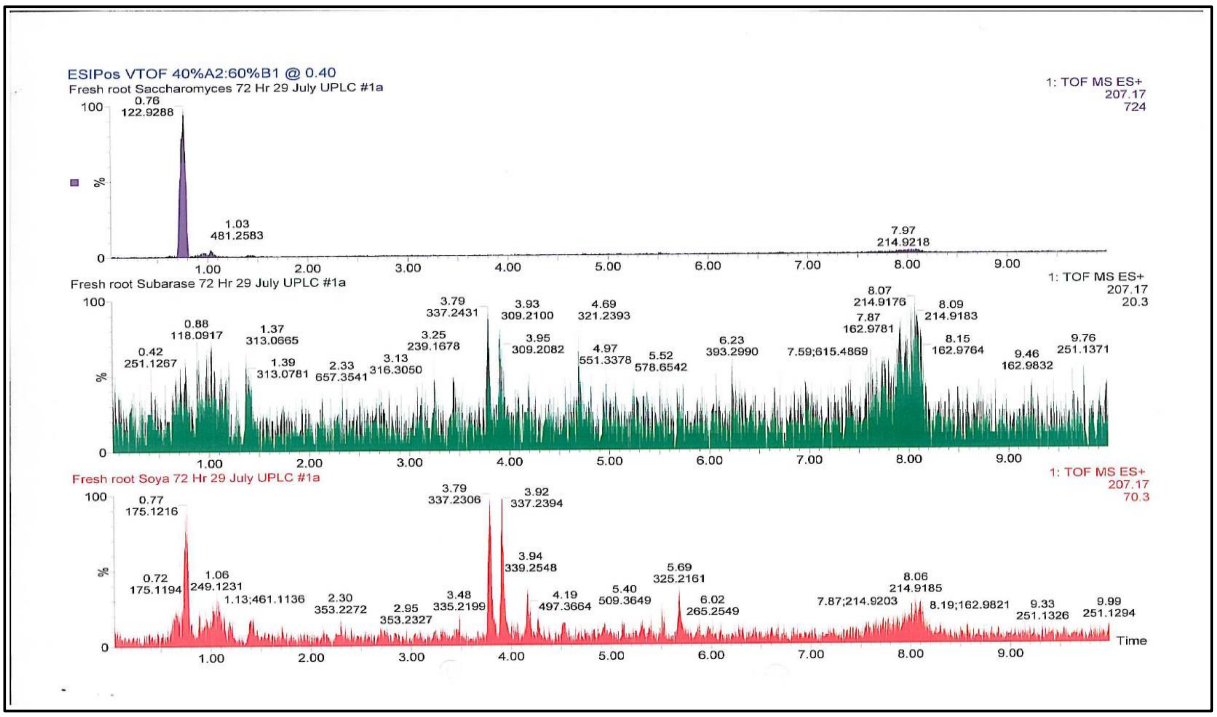


Figure 27-The extracted mass (207 Da) LC-MS chromatograms of irone samples at 72 h using different oxidoreductase biocatalysts; *Saccharomyces cerevisiae* (top), Suberase (middle) and Soybean lipoxidase (bottom). γ- and α-irone peaks elutes at 3.79 and 3.82 minutes respectively.

This study is the first to prove that Suberase can oxidize iridals in fresh orris root to generate irones. However, Soybean lipoxidase produced the highest concentration of the two irones on comparison based on the peak intensity, while *Saccharomyces cerevisiae* was unable to release irones from orris root.

4.3 Optimization of small scale reactions:

4.3.1 Reaction vessel configuration

Several experiments aimed at optimising the reaction conditions were carried out in 50 mL reaction vessels with continuous supply of air, and with temperature control using circulating water baths. Although we were able to produce irones by this method the yields were low and highly variable.

Standardisation of the size of the orris root particles was attempted whereby the root was first washed with distilled water, blanched for 3 minutes at 60°C, homogenized using a blender then further homogenized using a coffee grinder before being sieved to ensure uniform particle size was tried. However, this was not found to be the cause of the variability. The use of a more dilute orris root suspension was evaluated, but with no success and resulted in low product formation. Increased aeration also yielded no benefits.

An alternative was to change the reaction vessel configuration. Hence the use of Erlenmeyer flasks agitated on a rotatory shaker incubator was explored. This resulted in far more reproducible reactions, and was used in future small scale experiments.

4.3.2 Optimisation of reaction conditions:

The following experiments investigated the concentrations of the reaction solution (oleic acid, dioxane, and orris root and soybean lipoxidase) to determine the optimum conditions for the production of α- and γ- irones. The reactions were performed at a temperature of 30°C and 37°C and agitated at 200 rpm. Sampling was at time 0 hours and then daily for 72 hours initially, it was later done at day 0, 1 and then every two days for 5 days.

Precursor: In the first experiment, the effect of varying the orris root loading (from 2.5 to 5.0 g per 25 mL total reaction volume) was evaluated (Figure 28).

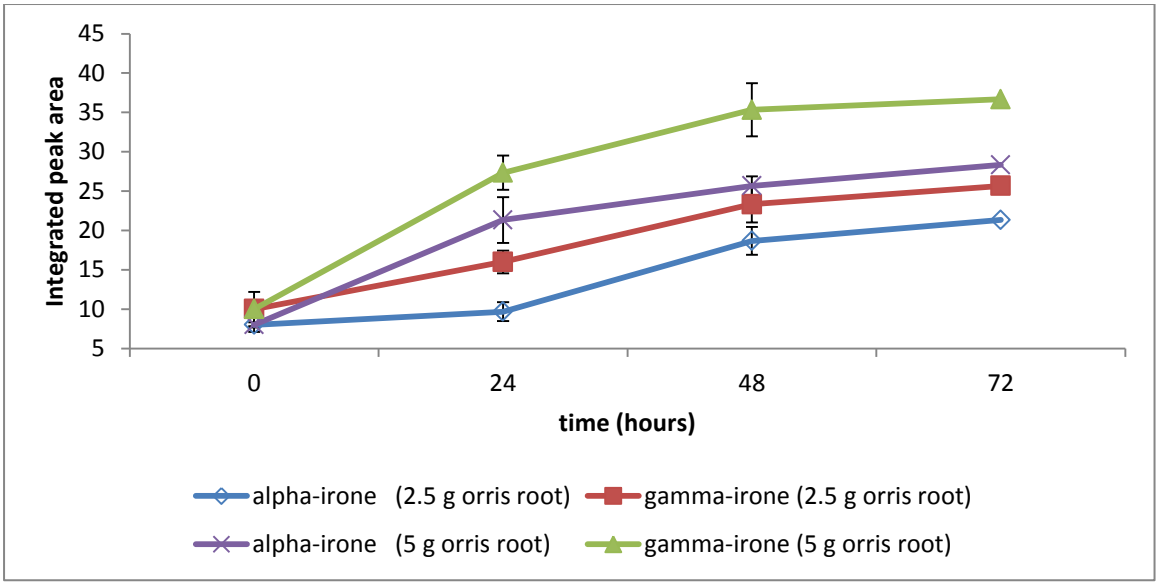


Figure 28-The change in irone production on incubation of either 0.25 g or 5g of orris root with soybean lipoxidase for 72 hours at 30°C. The error bars represent mean ±SD of three replicate samples (3n).

Amount of α- and γ-irones increased with time when orris root was loaded as either 2.5 g or 5.0 g per 25 mL total reaction volume. Thus, when 2.5 g orris root was tested, the estimated concentrations of α- and γ- irones were 6.1 and 7.5 mg /kg dry orris root at 72 hours. Amounts obtained when 5 g of the root was used were 8.1 and 11 mg /kg dry orris root at the same time.

Enzyme (catalyst): As illustrated in Figure 29, the experiment investigated the result of loading crude soybean lipoxidase suspension prepared as 0.5 g, 1 g or 2 g soybean flour in 25 mL borate buffer (0.01 M, pH 9.2) on α- and γ- irone production at 72 hours.

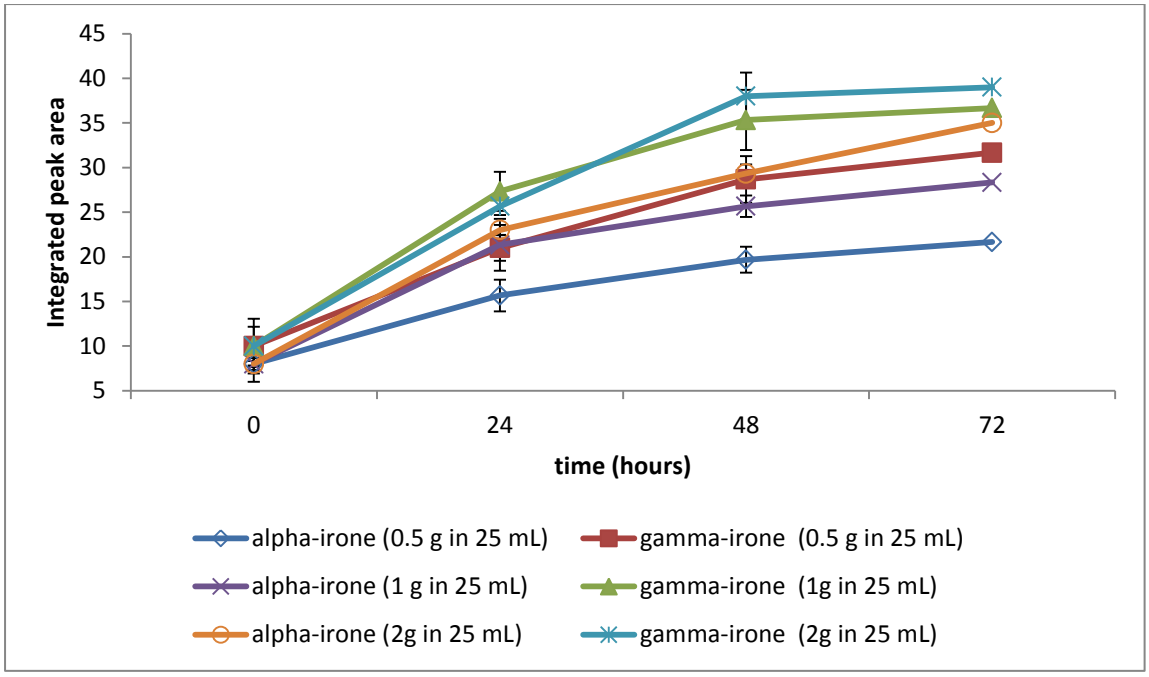


Figure 29-The change in irone production on incubation of 5 g orris root with lipoxidase extracted from either 0.5 g, 1 g or 2 g soybean flour suspended in 25 mL borate buffer for 72 hours at at 30 °C. The error bars represent mean ±SD of three replicate samples (3n).

Alpha- and gamma- irones accumulated with incubation period for all different lipoxidase enzyme suspensions. At 72 hours 6.4 and 9.3, mg of α- and γ-irones per kg dry orris root was generated by crude lipoxidase prepared using 0.5 g soybean flour extracted in 25 mL borate buffer. Using 1 g soybean flour this increased to 8.1 and 10.7 mg of alpha- and gamma- irones per kg dry orris root, and at 2 g soybean flour 10.2 and 11.3 mg of alpha- and gamma- irones per kg dry orris root. Thus a higher yield was achieved with lipoxidase enzyme extracted from 2 g soybean flour, though this suspension did not double the yield.

Enzyme substrate: Oleic acid is one of the reaction components involved in the enzymatic oxidation of orris root to irones as reported by Gil *et al.,* (1992). It serves as the substrate for the enzyme which results in the generation of the hydroperoxide required for oxidation the irone precursors. Altering the standard reaction by adding an increased volume (0.5 mL) of oleic acid yielded 8.1 and 8.4 mg irones /kg dry orris root for α- and γ-irones at 72 hours when compared with 8.1 and 10.7 mg irones/kg dry orris root generated when only 0.25 mL oleic acid was added. Hence, surprisingly, addition of the lower volume of 0.25 mL oleic offers a slightly better conversion of dry orris root iridals to irones (Figure 30). It can therefore be assumed that the unsaturated fatty acid is present in excess.

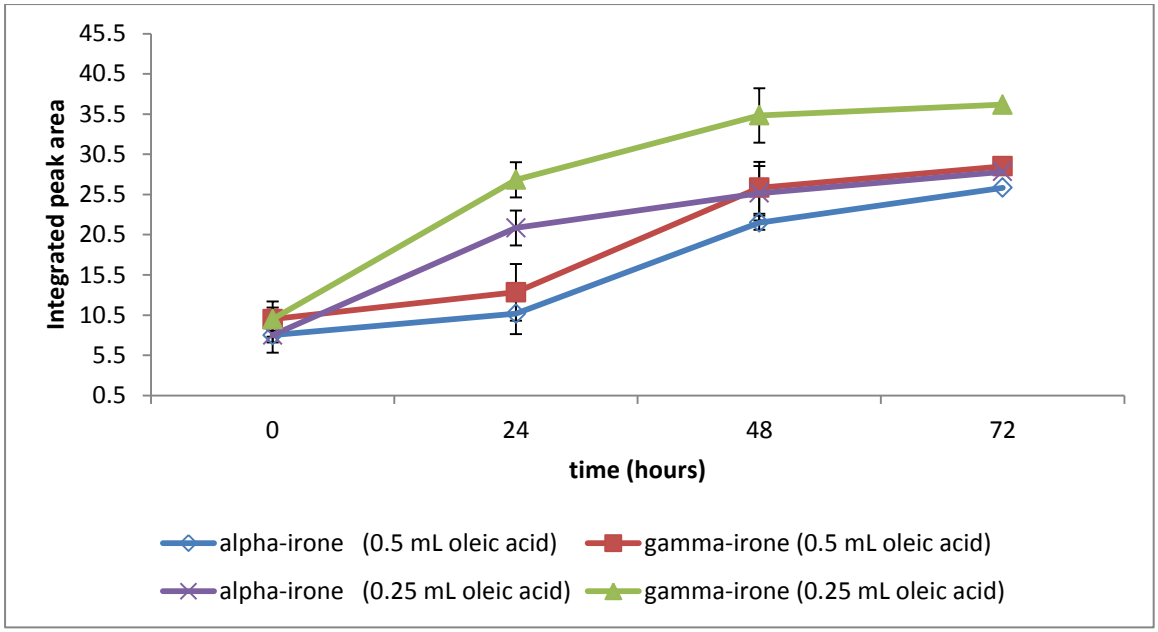


Figure 30- The change in irone production on incubation of 5 g of orris root with 0.25 mL or 0.5 mL oleic acid for 72 hours at 30°C. The error bars represent mean ±SD of three replicate samples (3n).

Co-solvent: Dioxane is one of the additives required in the lipoxidase-catalysed oxidation of orris root to irones as reported in Gil *et al.,* (1992). Dioxane in this case is used as a co-solvent which improves the dispersibility of the hydrophobic molecules in the aqueous medium without denaturing the enzyme. The maximum amounts for produced irones (α- and γ-) were 10.7 and 11.9 or 8.1 and 10.7 mg/kg dry orris root for the reactions which utilized either 1 mL or 0.5 mL dioxane. Hence increasing the dioxane volume generated a higher concentration of irones, indicating a solubility limitation (Figure 31).

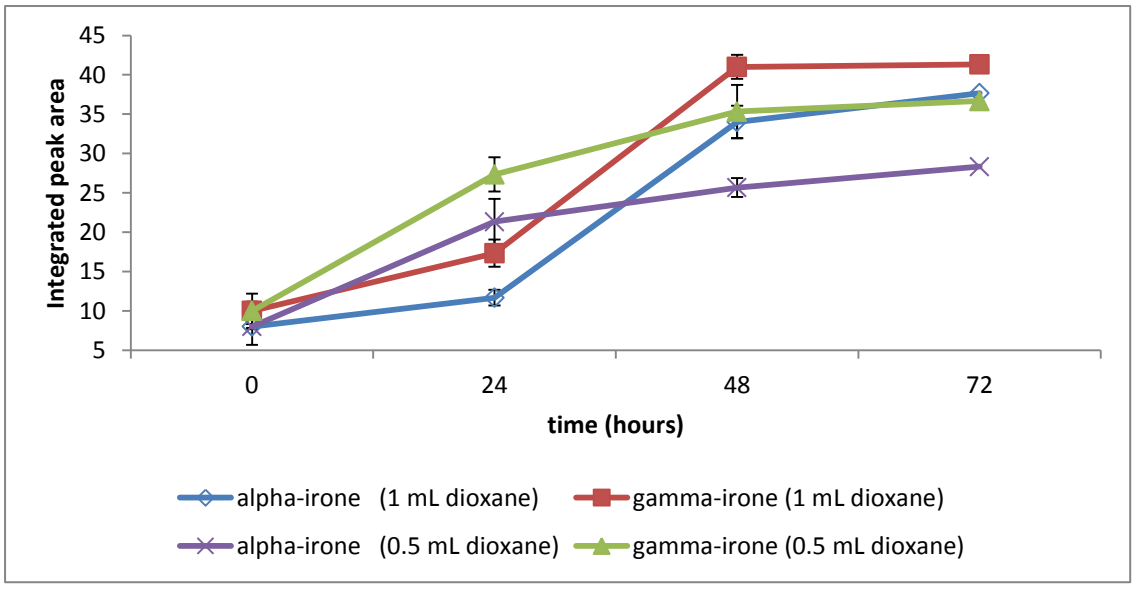


Figure 31- The change in irone production on incubation of 5 g of orris root with either 0.5 mL or 1 mL dioxane at 30°C. The error bars represent mean ±SD of three replicate samples (3n).

Temperature: The influence of temperature was considered, and a comparison between the standard reaction at 30°C and 37°C on the formation of irones is shown in Figure 32. Standard reaction at 37°C offered 23 and 32 mg irones/dry orris root at 72 hours compared to 8 and 11 mg/kg orris root for the reaction at 30° C. It became apparent from this observation that temperature is an important parameter in the present bioconversion reaction.

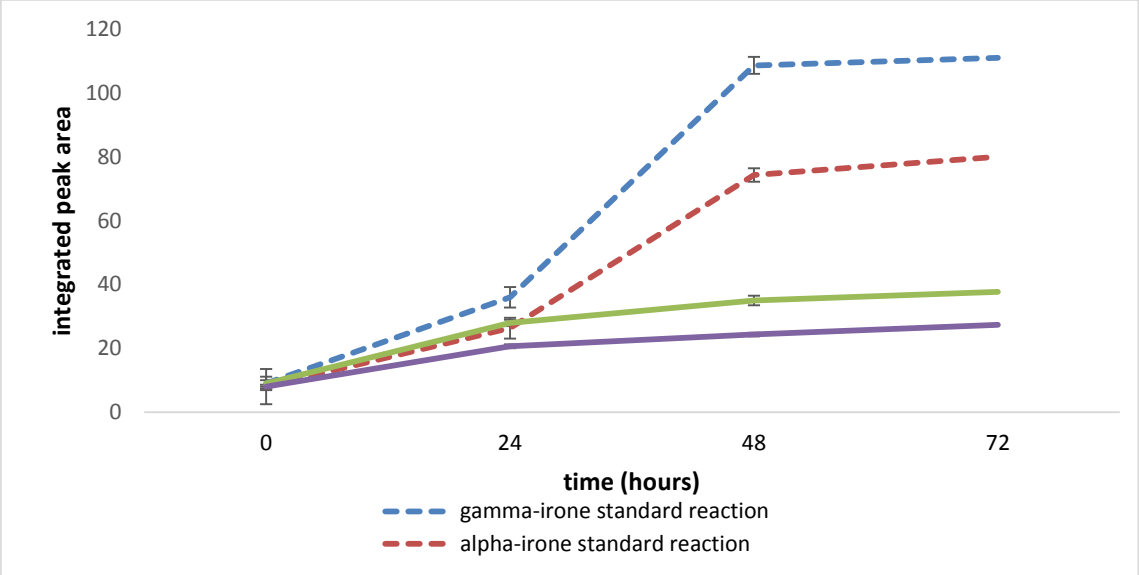


Figure 32- A comparison between standard reactions on the amount of irones generated over a 72 hour period at 30 (solid lines) and 37 °C (dotted lines) on incubation of 5 g of orris root. The error bars represent mean ±SD of three replicate samples (3n).

The dramatic increase in yield with increased reaction temperature may result in limitation of some of the reactants, and hence optimisation was repeated at the higher temperature. An investigation was carried out to single out the effect of using 2.5 g (instead of 5 g) of orris root on the peak areas of irones formed. The reactions were carried out at 30°C and 37°C and a comparison (Figure 33) was made.

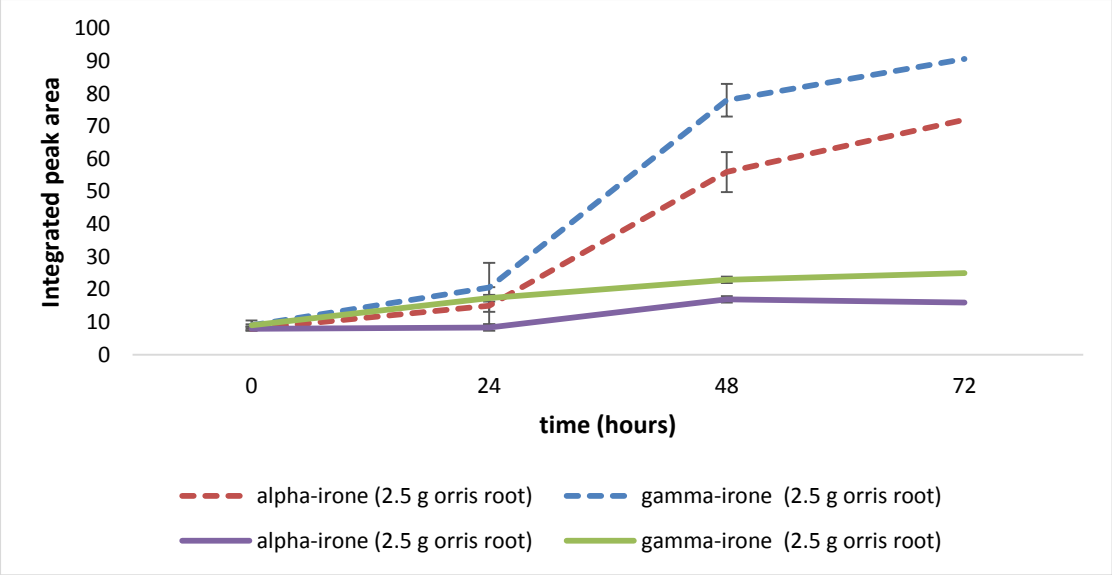


Figure 33- A comparison between loading of 2.5 g (instead of 5 g) orris root at 30°C (solid lines) and 37°C (dotted lines) on the peak areas of irones generated over a 72 hour period. The error bars represent mean ±SD of three replicate samples (3n).

At 72 hours the α-irone was concentration was 4.6 while that for γ-irone was 7.3 mg/kg dry orris root for the reaction conducted at 30°C related to 21 and 26.4 mg/kg dry orris root for the reaction conducted at 37°C. It can be inferred from this observation that; the use of 2.5 g orris root, and the higher temperature (37°C) improves product formation per mass of orris root, and will offer economic advantages.

Different soybean lipoxidase suspensions viz. extracts of 0.5 g and 2 g soybean flour in 25 mL borate buffer at 30°C were compared with similar suspensions at 37°C for effectiveness in improving the formation of α- and γ-irones and the results of the contrast are presented in Figure 34.

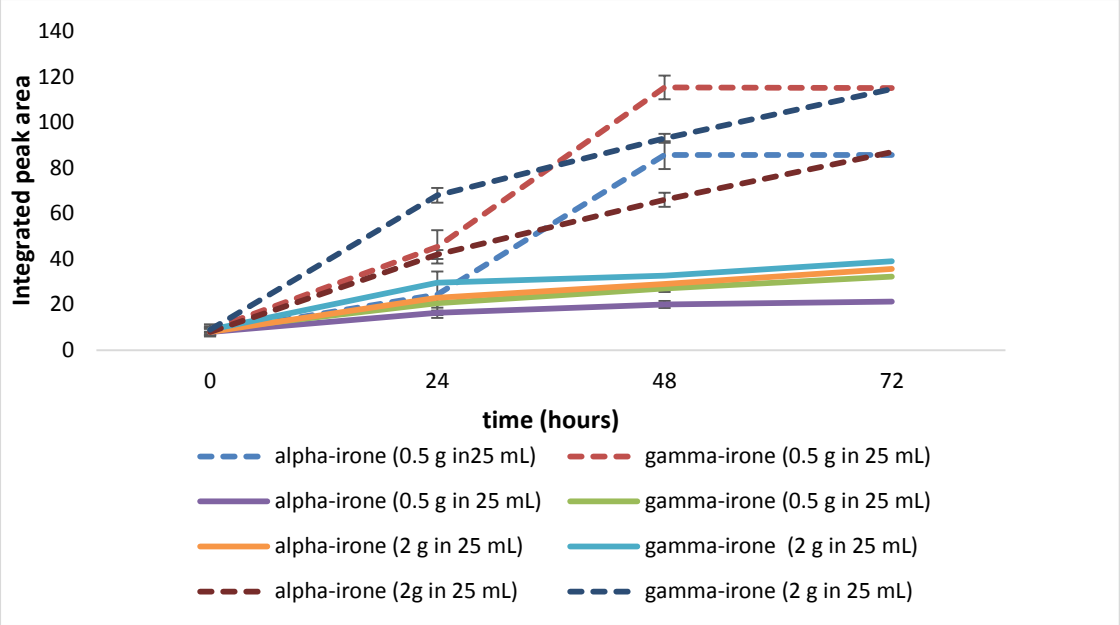


Figure 34- The use of 0.5 g and 2 g soybean lipoxidase suspension in 25 mL borate buffer on the process at 30° C (solid lines) and 37° (dotted lines). The error bars represent mean ±SD of three replicate samples (3n).

Lipoxidase extracted from 0.5 g soybean flour produced 25 and 33.4 mg/kg dry orris root (α- and γ-irones) at 72 hours for reactions carried out at 37°C compared with 25.2 and 33.4 mg/kg dry orris root produced by the enzyme extracted from 2 g of the same flour at a matching temperature. Both lipoxidase enzyme suspensions produced one-fourth of the irone concentrations achieved at 37°C when tested at 30°C. Due to minute variance in irone production seen when either 0.5 g, 1 g or 2 g soybean flour per 25 mL borate buffer lipoxidase suspension was tested at 37°C; it follows that, either 0.5 g or 1 g flour per 25 mL borate buffer will be the ideal suspensions at 37°C for economic reasons. It also implies that the enzyme was again not a limiting factor in these reactions.

Hence, during this reaction optimisation process, temperature had the largest effect. For completeness, experiments on variation of oleic acid and dioxane addition, previously performed only at 30°C were repeated at 37°C (Figure 35).

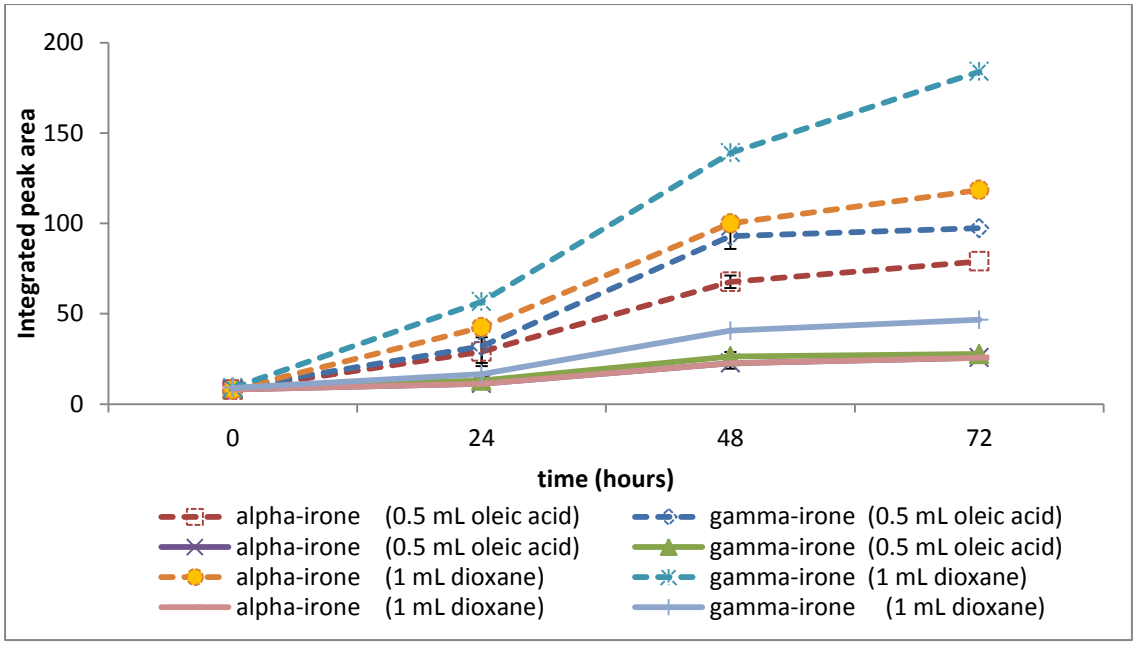


Figure 35- A comparison between the effect of increased oleic acid (0.5 mL) and dioxane (1 mL) on the reaction at 30 °C (solid lines) and 37 °C (dotted lines). The error bars represent mean ±SD of three replicate samples (3n).

As portrayed in Figure 35, for addition of 1 mL dioxane that at 37°C, irones concentrations (α- and γ-) reached 34.5 and 53 mg/kg dry orris root, compared to only 10.4 and 13.6 mg/kg dry orris root produced at 30°C, again pointing to a solubility limitation in spite of the increased reaction temperature. The use of 1 mL oleic acid on the process had a much smaller influence at 72 h. Amounts of 23 and 28.1 mg/kg dry orris root for 37°C reactions and 7.5 and 8.1 mg/kg dry orris root for 30°C reactions were generated. It follows from these findings that 1 mL dioxane and a temperature of 37°C can be utilized for concentrated production of irones at laboratory scale.

4.4 Testing the influence of salts on the process.

The current test investigated the influence of various ratios of manganese chloride to ferrous sulphate ($MnCl_2$: $FeSO_4$ at 1:0, 0:1, 1:1, 2:1 and 1:2) on the production of irones (Figure 36) with sampling at 0, 1, 3, and 5 days. In the preceding tests unmeasured quantities (spatula tips) of minerals were utilized, so here the quantities needed to be more precise to provide a reliable comparison.

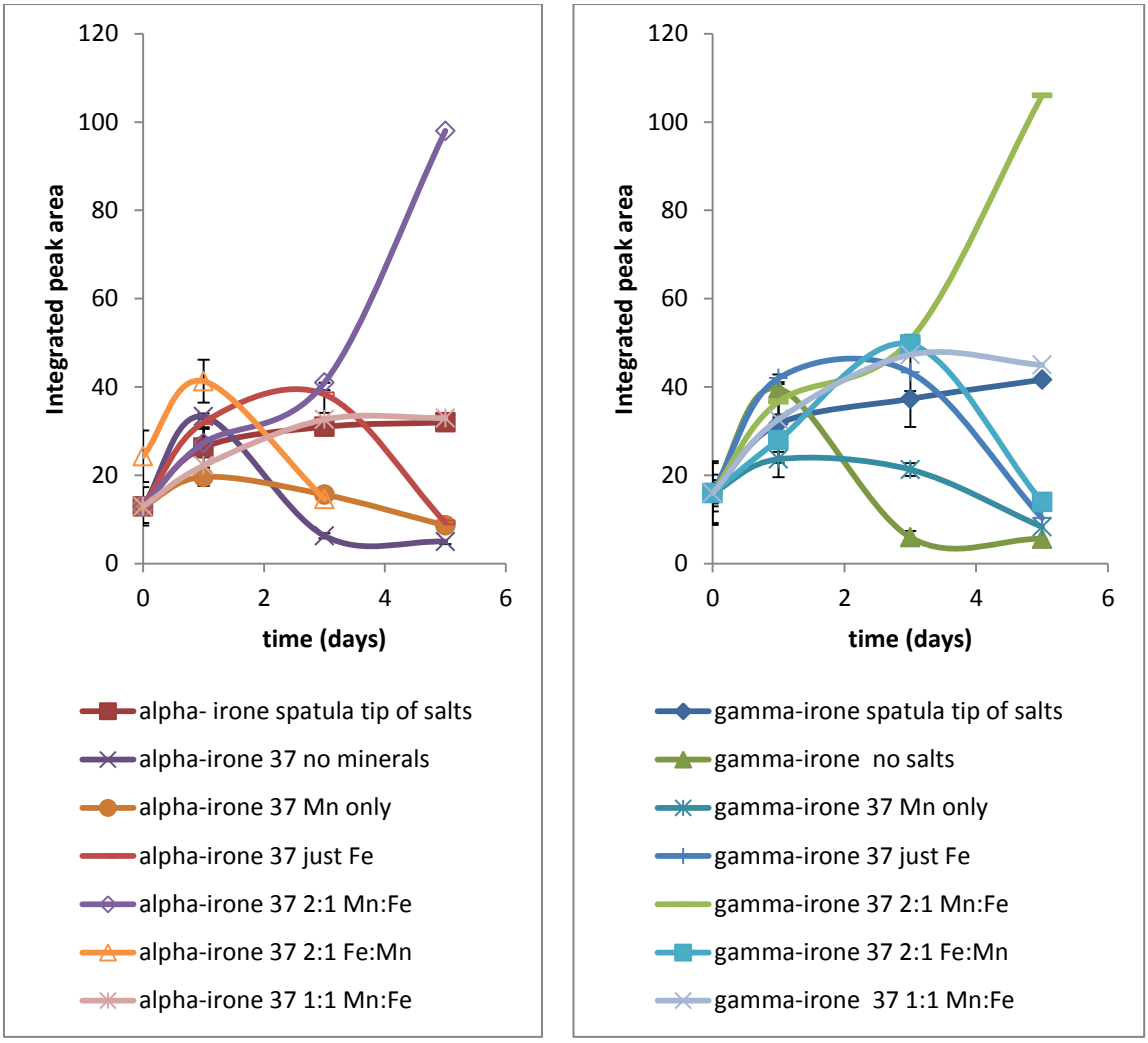


Figure 36-Comparison among the effect of spatula tip, no minerals, Mn only, just ferrous, 1:1, 1:2 and 2:1 Mn: Fe ratio on the amount of irones generated over a 120 hour period. Spatula tip of salts- a spatula tip of $FeSO_4$ and $MnCl_2$ was added; no minerals-no salt was added; Mn only- 25 mg of $MnCl_2$ was added; just ferrous-25 mg of $FeSO_4$ was added; 2:1 Mn:Fe- 50 mg and 25mg ($MnCl_2$ and $FeSO_4$) were added; 1:2 Mn:Fe- 25 mg and 50 mg $MnCl_2$ and $FeSO_4$ were added; 1:1 Mn:Fe- 25 mg each of $MnCl_2$ and $FeSO_4$ was added. Curves a) and b) - shows α- and γ-irones peak areas per time. Sampling was done at day 0, 1, 3 and 5 with the peak area at time 0 subtracted to obtain the area at each sampling point. The error bars represent mean ±SD of three replicate samples (3n).

As can be seen in Figure 36 that a combination of manganese and iron salts were necessary, with $MnCl_2$ in excess. Under these conditions a final yield of 28 and 31 mg (α- and γ-) irones /kg dry orris root were produced.

4.5 Optimisation of blanching times and testing of antifungal agents:

Blanching was aimed at inactivating the orris root enzymes (e.g. tyrosinase) to prevent colour formation by tannins (which could get into the final product and also have a negative effect on extraction). Moreover, the previous experiments revealed that some fungal growth occurred, which may retard product formation, the aim of this study was to determine if blanching the root for an extended time or blanching the root and then adding antifungals can retard the noted fungal

growth. Moreover, it has been reported that blanching the root at 60°C for 3-5 minutes before the bioconversion improves the concentration of irones (Canhoto *et al.,* 2009).

The following experiment explored the influence of prior blanching the root for 3, 4 or 5 minutes at 60°C on the lipoxidase-mediated oxidation of orris root to irones carried out at 37°C, the comparison of which is depicted in Figure 37. It appears that the blanching time had no significant difference in the amount of the generated irone isomers.

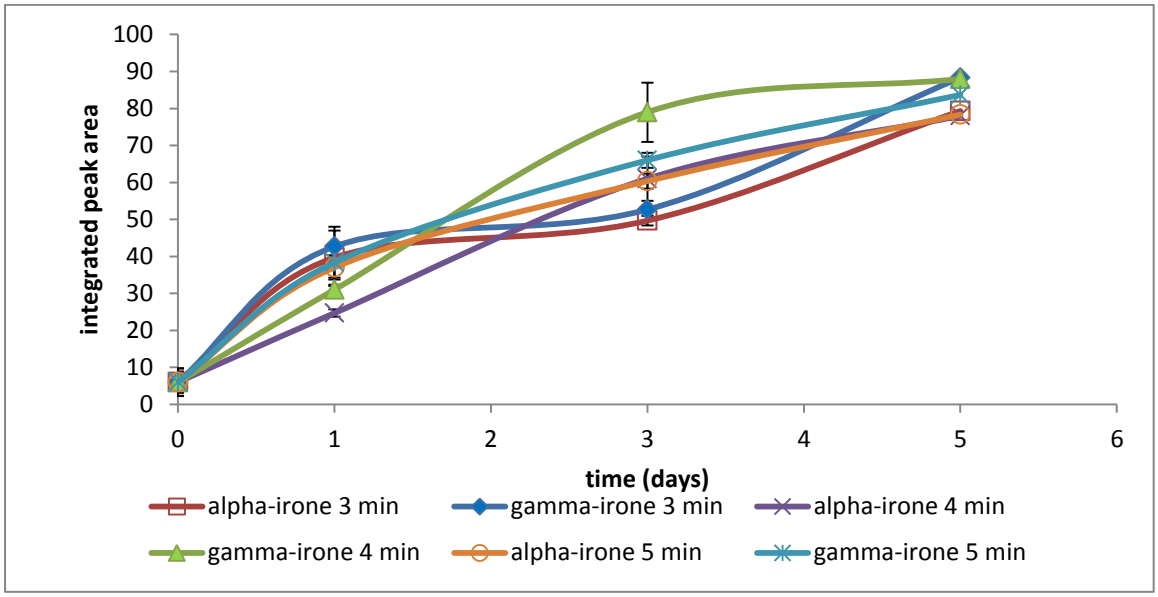


Figure 37-The effect of blanching the root (for 3 to 5 min) prior to irone production. The error bars represent mean ±SD of three replicate samples (3n).

Addition of 100 µL of 99 % acetonitrile or 70 % ethanol, or 0.68 mg/mL imidazole buffer (pH 6.8) to the standard reaction in section 3.2.5.7, was tested to identify the best chemical in retarding fungal growth (Figure 38).

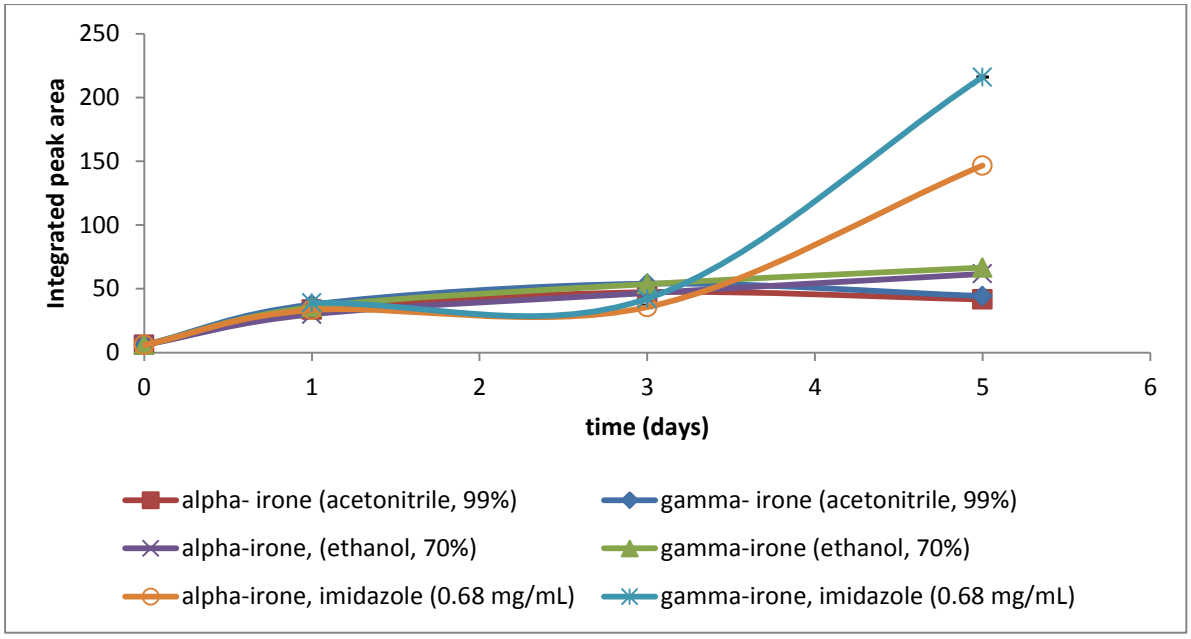


Figure 38-The effect of adding either acetonitrile, ethanol or imidazole on the production of irones.

Imidazole buffer addition resulted in partial disappearance of the fungi from the flasks, improvement in the production of irones compared to the addition of similar volumes of either 70 % ethanol or 99 % acetonitrile. Moreover, its addition produced a final yield of 43 (α-) and 63 (γ-) mg irones per kg dry orris root compared to amounts less than 29 mg irones per kg dry orris root produced by other antifungals. This could mean that ethanol and acetonitrile inactivated the enzyme, thus making imidazole look better in comparison. Imidazole addition may also adjust the pH to greater than 6, and might have created an optimum pH for the lipoxidase oxidation of orris root. Adding 100 µl of either 99 % isopropanol or of a 1% SDS solution was also evaluated (Figure 39).

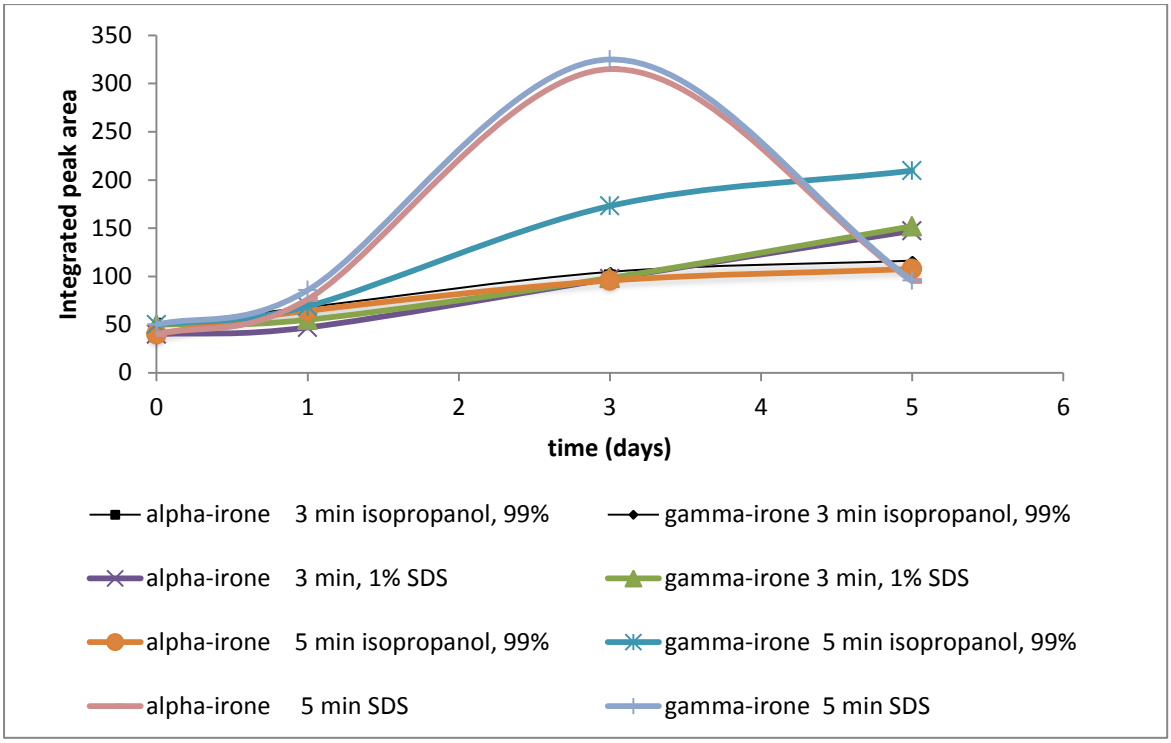


Figure 39-Testing the effect of adding isopropanol or SDS on irone production. In this experiment the root was blanched for either 3 or 5 minutes. SDS-Sodium Dodecyl Sulphate.

Addition of these antifungals did not completely stop fungal growth on the walls of the flasks. Also noted was α- and γ- irones of 31 and 34 mg/kg dry orris root at day 5 when isopropanol was incubated with orris root blanched for 3 min. When isopropanol was incubated with orris root blanched for 5 min the peak areas were around 29 and 58 mg/kg dry orris root. Beyond day 3, a sudden decline (from around 92 to less than 29 mg/kg dry orris root) in irones was noted when SDS was incubated with orris root blanched for 5 minutes. This observation contradicted the outcomes seen with SDS incubation with orris root blanched for 3 minutes, where the use of SDS resulted in a good trend of irone production. This observation could perhaps be due to degradation through microbial contamination. Hence, imidazole was evaluated further at concentrations of 0.34 mg/mL, 0.68 mg/mL and 1.36 mg/mL (Figures 40 and 41).

Although imidazole ingestion has some side effects, (Boothe, 2009-2015) the small amount of imidazole used in the current study, the fact that its concentration will be reduced in product work up, and its final use in topical application means that it is unlikely to constitute a health risk.

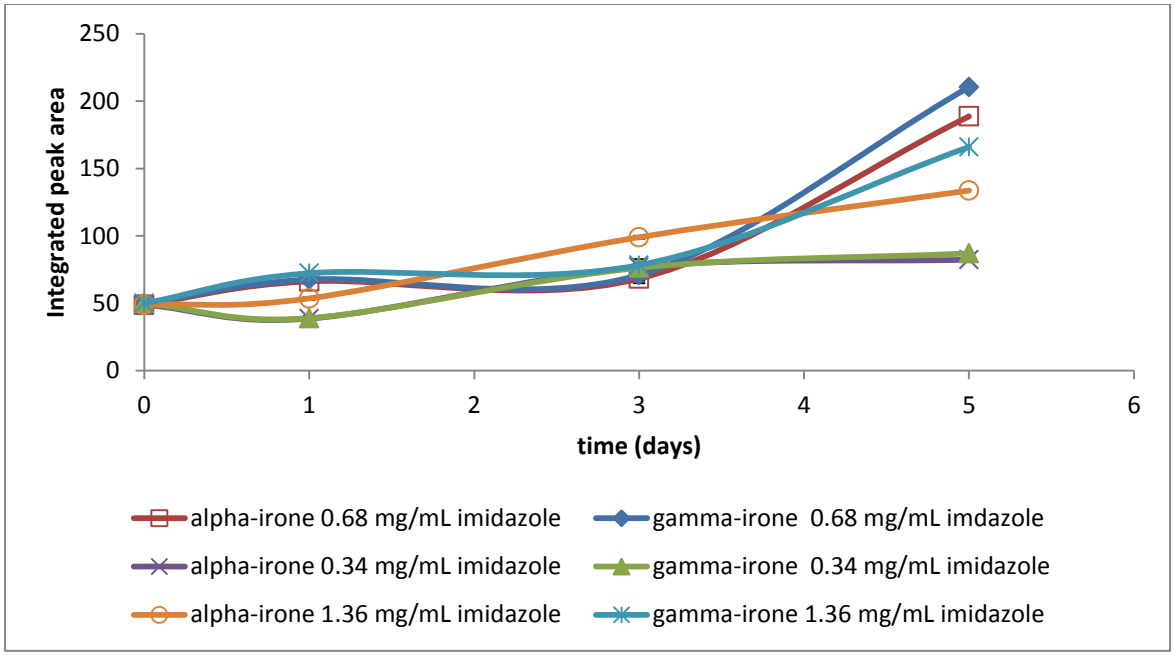


Figure 40-Comparison among the effect of incubating either 0.34, 0.68 or 1.36 mg/mL imidazole with orris root blanched for 3 minutes on the production of irones. The root was blanched for 3 min at 60 °C while the reaction occurred at 37°C.

A 0.68 mg/mL imidazole concentration resulted in 55 mg/kg dry orris root (α-irone) and 61 mg/kg dry orris root (γ-irone) at day 5 compared with lower amounts produced by 0.34 and 1.36 mg/mL imidazole concentrations.

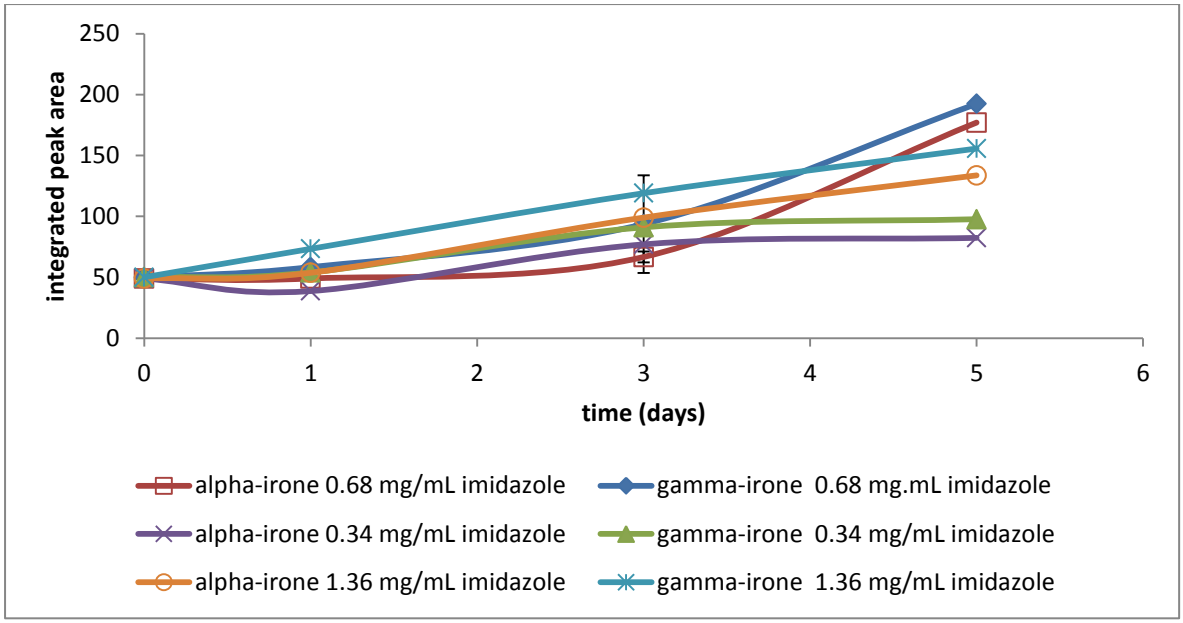


Figure 41-Comparison among the effect of incubating either 034, 0.68 or 1.36 mg/mL imidazole with orris root blanched for 5 minutes on the production of irones. The root was blanched for 5 min at 60 °C while the reaction occurred at 37°C.

A concentration of 0.68 mg/mL imidazole yielded around 57.7 and 58 mg irones/kg dry orris root. These results are in agreement with those in Figure 40 where a 0.68 mg/mL imidazole concentration

resulted in a higher irone production compared to other antifungals. In conclusion, blanching the root for 3 or 5 minutes improves the production, albeit, inclusion of imidazole had a greater effect.

4.6 Comparison of fresh root to dried root on irone yield

The optimised reactions previously conducted on dry root were tested on fresh root for comparison. The blanching time and temperature (3 min at 60°C), salts ratio (50 mg $MnCl_2$ and 25 mg $FeSO_4$), oleic acid (0.25 mL) orris root amount (5 g) and crude lipoxidase (1 g soybean flour in 25 mL borate buffer) were kept constant. The effects of using either 1 mL dioxane, 100 µL isopropanol (90 %) and 100 µL of 0.68 mg/mL imidazole buffer on the process were also investigated (Figure 42).

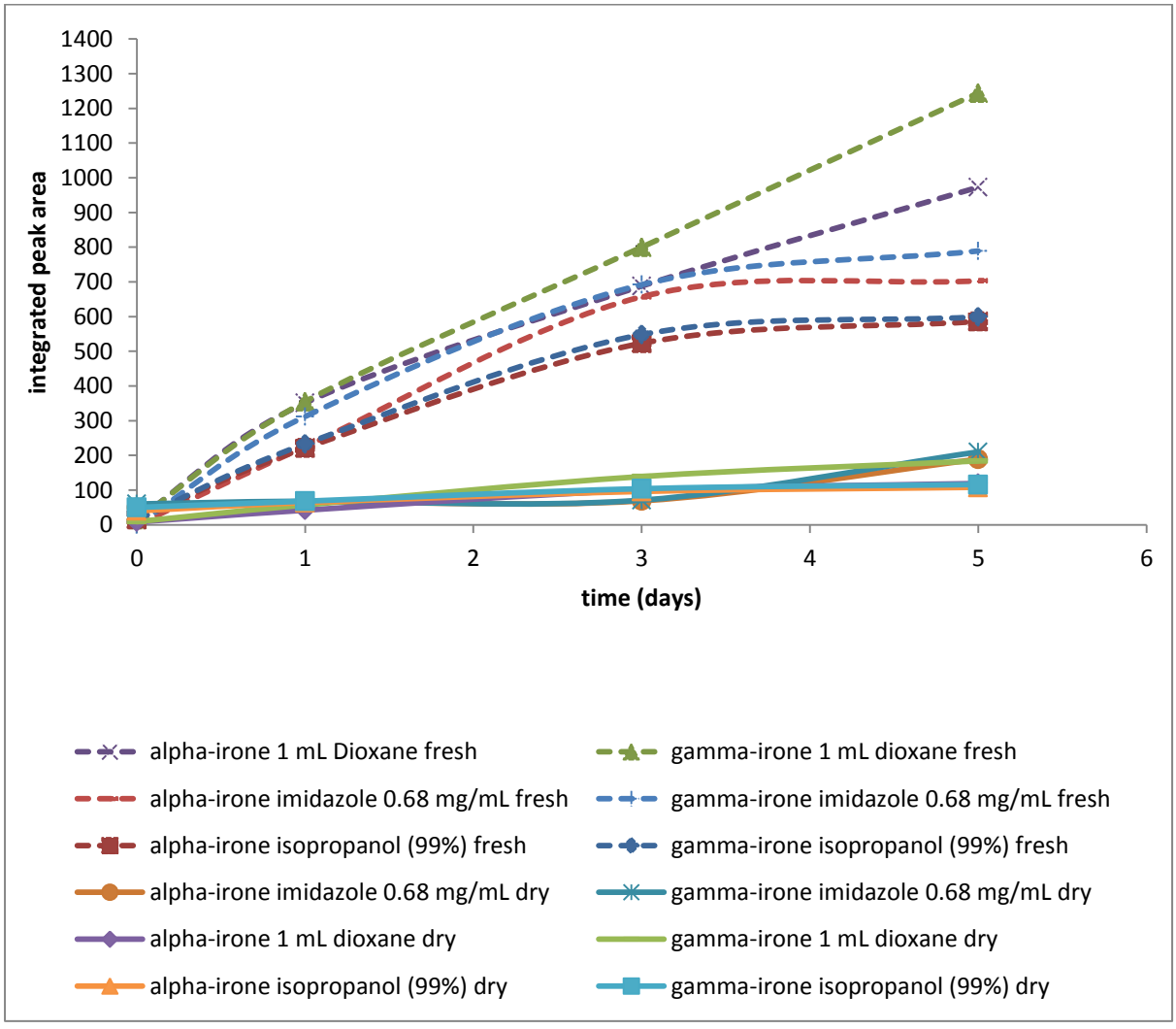


Figure 42-Comparison of fresh and dried root on irone yield. Sampling was done at 0, 1, 3, and 5 days.

Fresh orris root yielded higher irone concentrations compared with dried root for all parameters tested, with the highest amounts (282 mg/kg fresh orris root for α-irone and 361 mg/kg fresh orris root for γ-irone) seen in reactions were fresh orris root was incubated with 1 mL dioxane.

4.7 Determination of the effect of substitution of salts

The impact of replacing the salts used in the optimised reaction with other salts was evaluated to determine if the benefits of the salts are element specific or merely a function of charge. A 1:1 (25 mg: 25 mg), 2: 1 (50 mg: 25 mg) and 1:2 (25 mg: 50 mg) $CuSO_4$: $CaCl_2$ ratios were tested; also tested separately was $CuSO_4$, $CaCl_2$ and $ZnCl_2$. The optimized method with $MnCl_2$ and $FeSO_4$ was used as the positive control. The addition of other minerals did not result in any improvement in irone production (Figure 43).

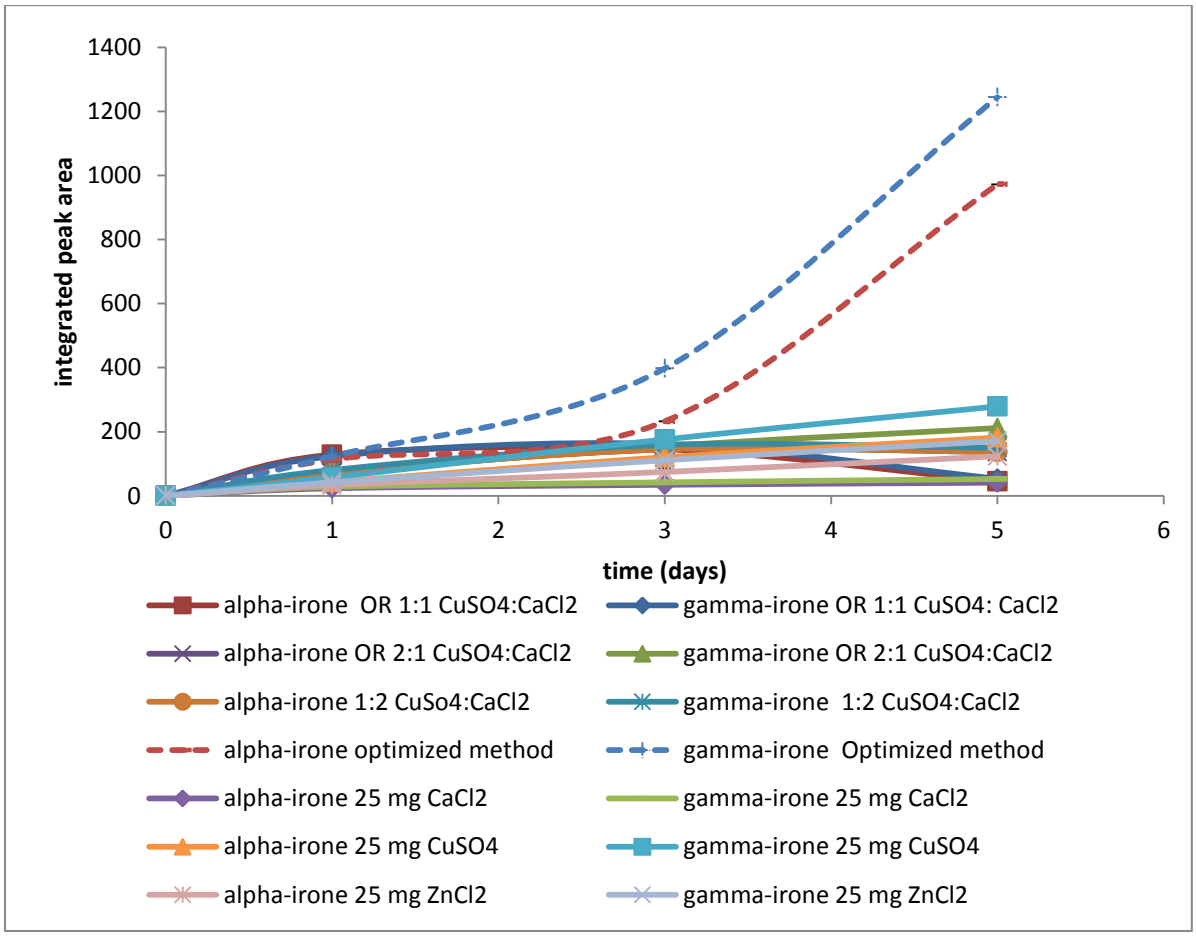


Figure 43-Determination of the influence of other minerals on incubation with fresh orris root. The optimized method with $MnCl_2$ served as the control

4.8 Comparison of the optimized method with that of benchmark processes.

The optimized biocatalytic method used here produced two fold more irones compared to the chemical oxidation method reported by Courtois *et al.* (1998) and Ehret *et al.* (2001) where fresh orris root was incubated with 0.1 - 0.5 g/L sodium nitrite at 25°C, pH 2.5 with stirring. Although the latter method was reported to have produced 1840 mg irones/kg dry rhizomes, when this method was reproduced in the present study only 131 and 153 mg/kg fresh orris root for α- and γ-irones

were generated compared to 282 and 361 mg/kg fresh orris root produced via the optimized method (Figure 44). The lower yields of the sodium nitrite reaction in this study may be due to the orris root starting material.

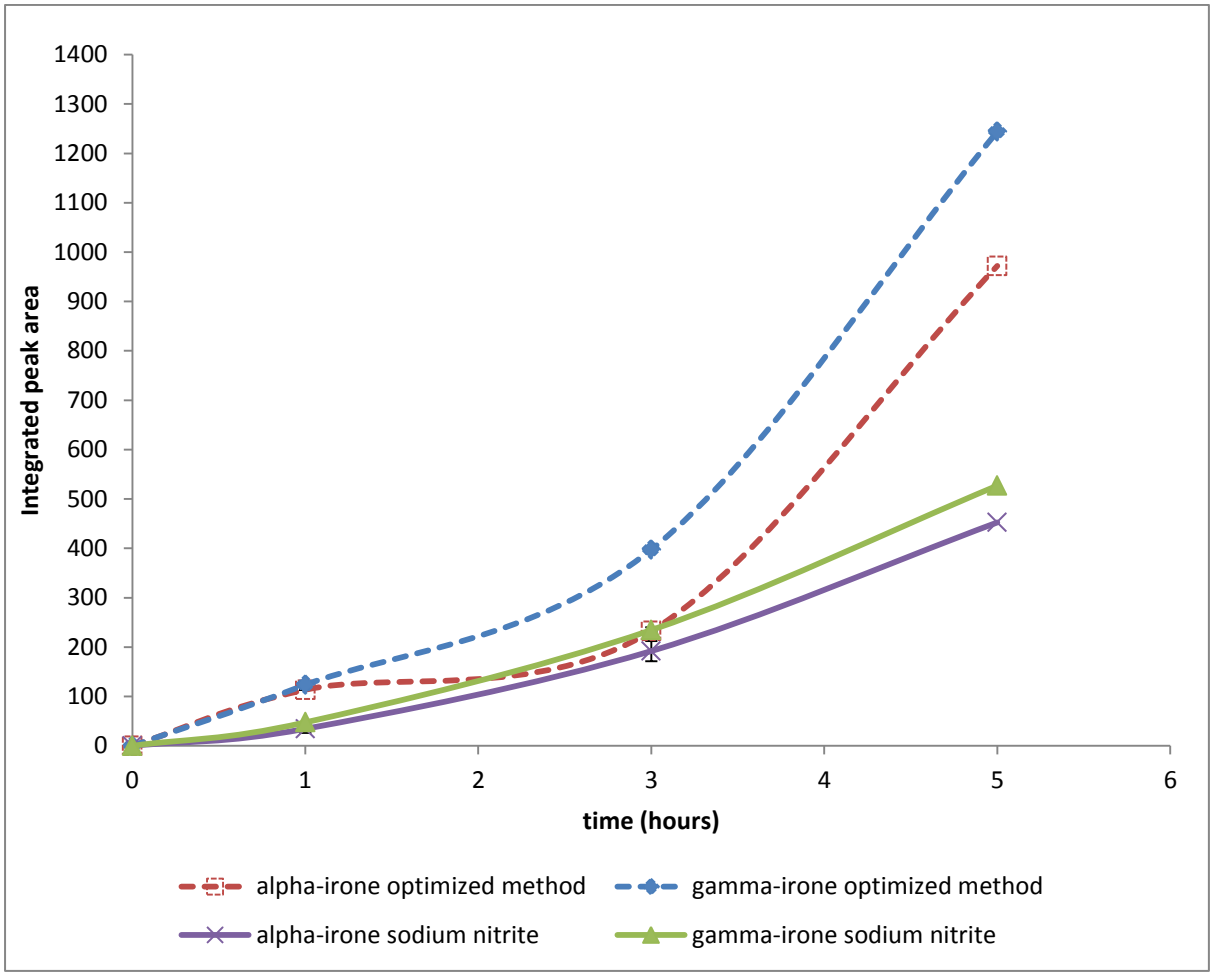


Figure 44-Comparison of the optimized biocatalytic method with that of the sodium nitrite method of Courtois *et al.*, (1998).

Scale up of the optimized method:

The effect of scaling up the reaction volume from 25 mL to 700 mL in 2 L sterile reactors on the accumulation of irones was investigated. Figure 45 illustrates the build-up in α- and γ-irones after 3 days of incubation of fresh orris root with crude soybean lipoxidase. Extracted mass chromatograms for α- and γ-irone prepared and then analysed via LC-MS. The starting material, fresh orris root, was found not contain irones upon analysis. Furthermore, the Figure elucidates the extracted mass chromatograms were compared with those of the commercial irone sample (Appendix) and that obtained via the optimized method (Figure 44).

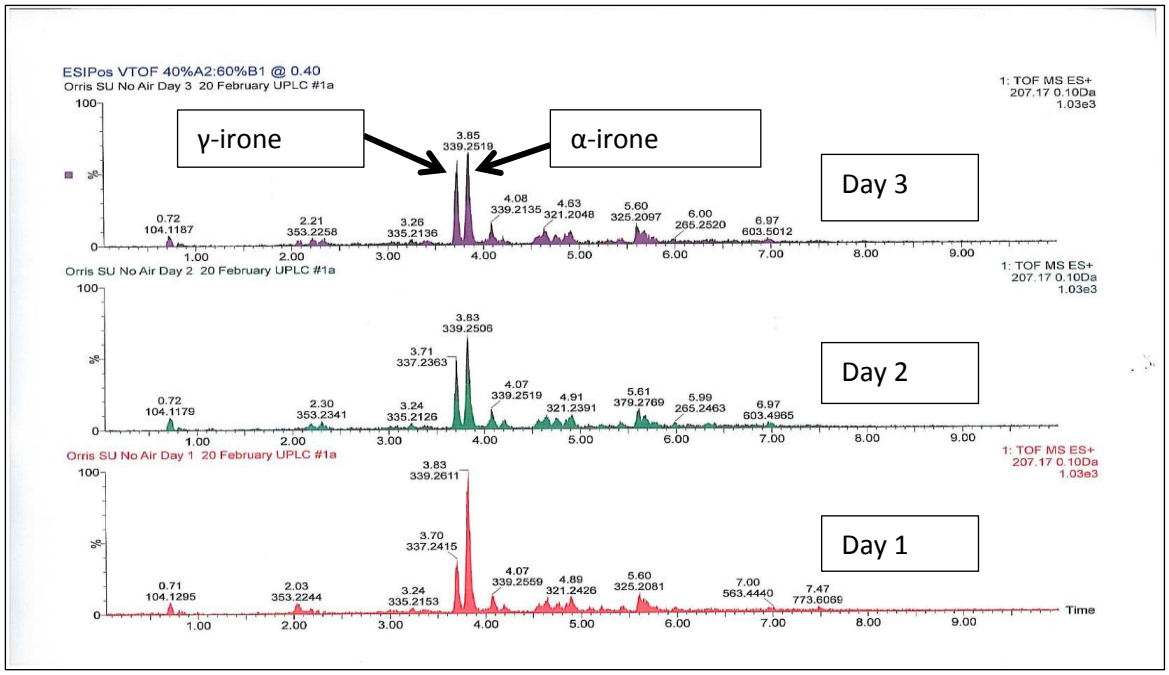


Figure 45- Extracted mass chromatograms (207 Da) for α and β irones prepared from biocatalytic reactions of fresh orris root. The reactions were carried out for 3 days with daily sampling.

Alpha-irone declined while gamma irone increased with time, the decline inα-irone was suspected to be due to lack of aeration in the 2 L reactors. Compared with the irone standard and the optimized method performed on small scale, the extracted mass chromatograms produced by this scale-up method share identical retention times and mass, albeit at low concentrations. Figure 46 represents similar results as Figure 45 but 100 % oxygen was introduced into the reactions.

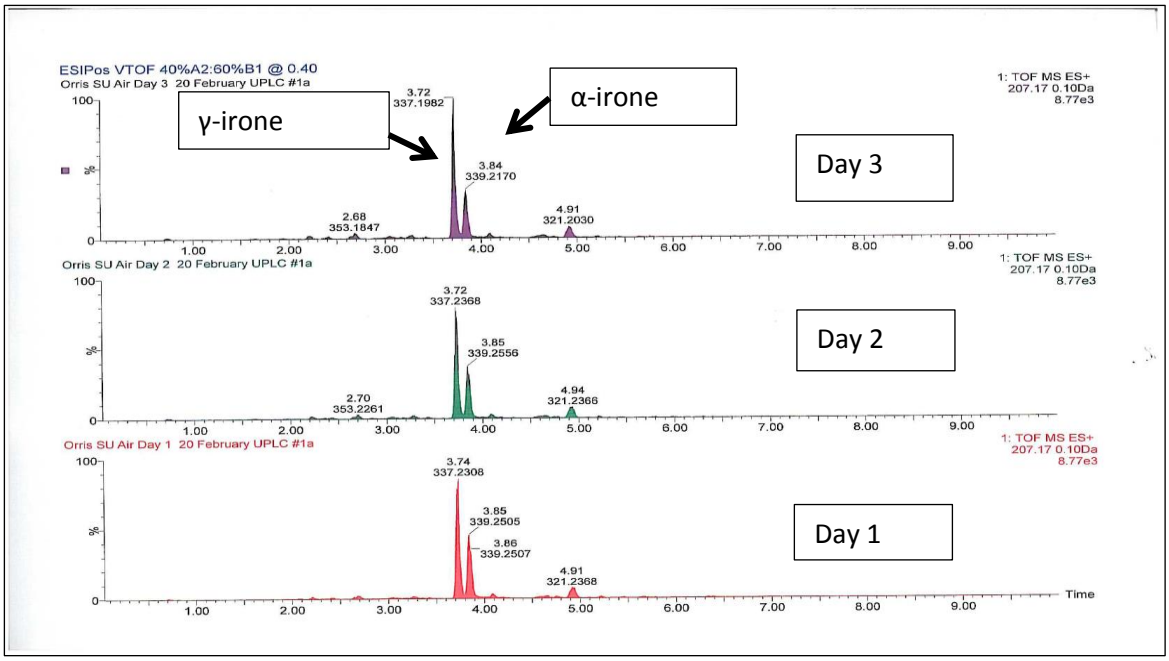


Figure 46- Extracted mass chromatograms (207 Da) for γ- and α-irone prepared from biocatalytic reactions of fresh orris root with the introduction of oxygen and analyzed by LC-MS. The reactions were carried out for 3 days with daily sampling.

Irones (α and γ) with mass 207 Daltons were detected at around 4 minutes retention time, which correspond to the mass and retention time of commercial irone sample (see Appendix). Concentrations of around 435 mg irone /kg fresh orris root were produced by the end of day 3 (Figure 46). These findings are indicative of the necessity of improved agitation and aeration of the reactions at 0.7 L bench scale. This high concentration of produced irone is similar to that seen on laboratory scale. This method was further tested at 2 L bench scale where the pH of the reaction mixture was measured and found to be 5.2; this pH was adjusted to 6 and 7 for comparison and the results are shown in Figure 47.

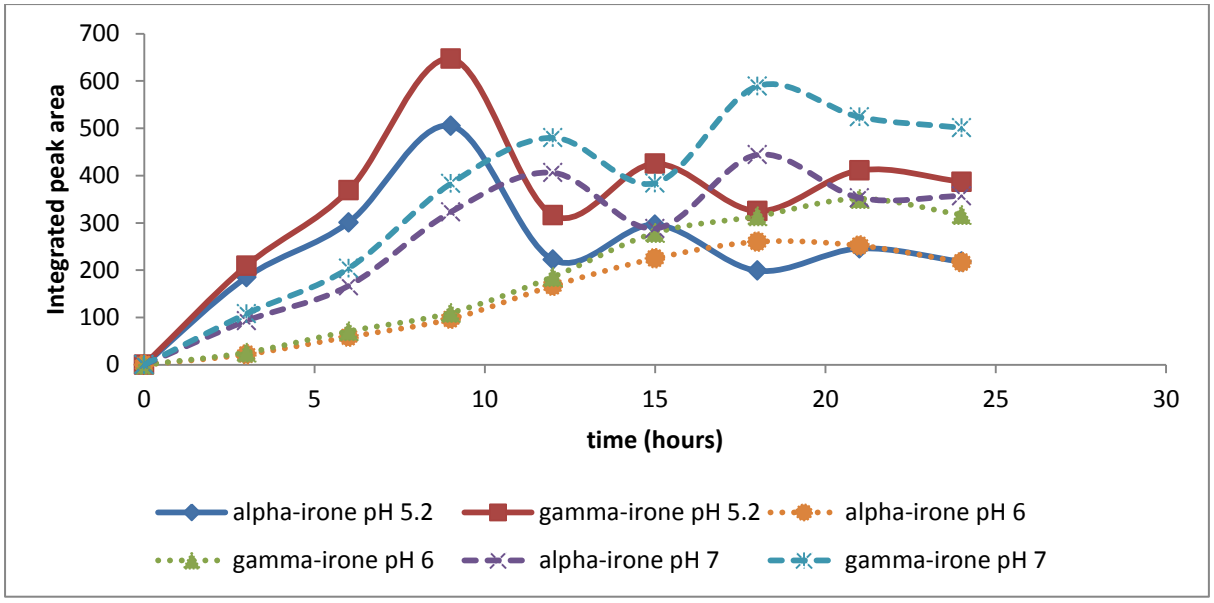


Figure 47-Effect of adjusting the pH of the reaction mixture at 2 L bench scale.

The 2 L bench scale up reaction produced α- and γ- irones (mass 207 Da) in the concentrations 63 and 112 mg/kg fresh orris root at pH 5.2 while pH 6 reactions produced 63 and 91.93 mg irones/kg fresh orris root. On the other hand, 104 and 145 mg irones/kg fresh orris root were produced by pH 7 reactions. The maturated suspension produced by this process was stored in the freezer (-20°C) until used for isolation of the final product. Thus, at 2 L bench scale the incubation period was reduced to 24 hours, this improvement in yield might be due to improved aeration and agitation.

Hence the optimal reaction conditions involved incubating 5 g orris root (peeled, cleaned, blanched for 3 min at 60°C and homogenized with a Waring blender), 1 g soybean flour in 25 mL $NaBO_3$ buffer (0.01 M, pH 9.2), 50 mg $MnCl_2$, 25 mg $FeSO_4$ ratio, 1 mL dioxane and 0.25 mL oleic acid at 37°C, 180 rpm for 5 days results in accumulation of irones as per LC-MS analysis results. This method is to the best of my knowledge, the first method to use crude soybean lipoxidase to oxidize the homogenized root for effective production of irones. This method was found to be effective at 0.7 and 2 L bench scales.

Other researchers have also explored the lipoxidase reaction. Gil *et al.,* (1992) reported the following reaction conditions as optima: 10 g of orris root extract, 90 mL borate buffer (0.01 M, pH 9.2), 10 mL dioxane, a 1 mM oleic acid and 0.17 g of 6 units per mg soybean lipoxidase, marketed by Fluka and continuous stirring in an oxygen atmosphere at 30°C for 48 h; The irone content after this maturation step is 1.8 g of irone/kg of dry weight of rhizome. However, the prior step to extract the irone precursors before subjecting them to oxidation which requires a lot of processing and energy input for the reflux distillation for 48 hours. Another drawback is the use of expensive purified commercial soybean lipoxidase instead of the extract used in the present study.

Canhoto *et al.,* (2009) reported that incubating 5 g of orris root extract with 49 mL of borate buffer (0.01 M pH 9.2), 0.125 g of Tween 80, 3 mg of linoleic acid and 1 mL of a lipoxidase (Type I-B from soybean, 131 000 units/mg solid) solution (0.44 mg/mL in buffer) at 500 rpm for 20 h at room temperature gives 46.01 mg/kg dry weight on GC-MS analysis. It should be noted that the researchers used a Likens-Nickerson distillation immediately after the 20 h incubation. These inventors further determined that the maximum incubation period is 10 days while the optimum temperature was 50°C. Also implicated in their study was that the lack of oxygen impacts irone production negatively. Similar to the study by Gil *et al.,* (1992) there is a labour-intensive step to extract the irone precursors and the use of the somewhat expensive commercial lipoxidase. These researchers further reported that when washed and blanched fresh Orris rhizome was incubated at 50°C at a relative humidity of ca. 74 % for 20 days, comminuted and extracted with diethyl ether, an irone concentration of 718 mg/kg dry weight of rhizome was produced. This was compared with an average value of 530 mg/kg dry weight for traditionally processed Orris rhizome.

This can also be compared to the chemical oxidation method of Ehret *et al.,* (2001) who reported a good conversion upon incubation of 500 g of fresh orris root with a solution of 0.001 g/mL sodium nitrite, a temperature of 30°C, a pH 2.5 and incubation period of 48 hours in a reaction vessel equipped with mechanical stirrer. This process produced up to 1.84 g/kg dry rhizomes. Hydrodistillation of the maturated mixture resulted in 1.85 g iris butter. However, sodium nitrite has been reported to be toxic to both human beings and animals (Sowers *et al.,* 2004).

4.9 Isolation of the irones from the bioconversion mixture

The maturated irone suspension produced at 2 L scale up was used in an attempt to further increase its irone content and to eliminate impurities. Petroleum ether (40°C-60°C) extraction of the suspension was carried out. This attempt resulted in elimination of most impurities without any increase in the irone content as shown in Figure 48.

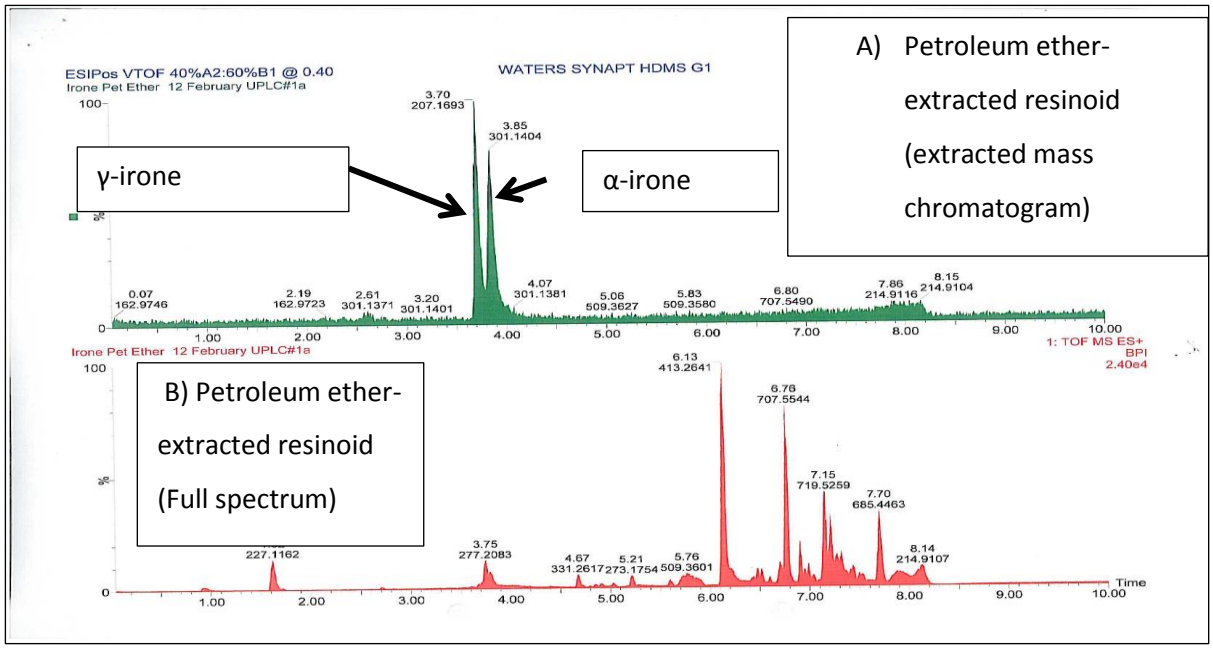


Figure 48- A full scan (EPI) representation of extracted mass chromatograms for α and β irone peaks produced by petroleum ether extraction of the maturated irone suspension. A resinoid- is a honey-like petroleum ether extract of orris root (www.thegoodscentscompany.com)

Alpha (α-) and Beta (γ-) irone (mass 207 Da, retention times 3.76 and 3.83) amounts of around 125 and 142 mg/kg fresh orris root respectively were generated from petroleum ether extraction of the irone suspension produced at 2 L reaction scale.

Also attempted was hexane (A.R. 99%) extraction of the accumulated irone suspension, the results of which are as indicated in Figure 49.

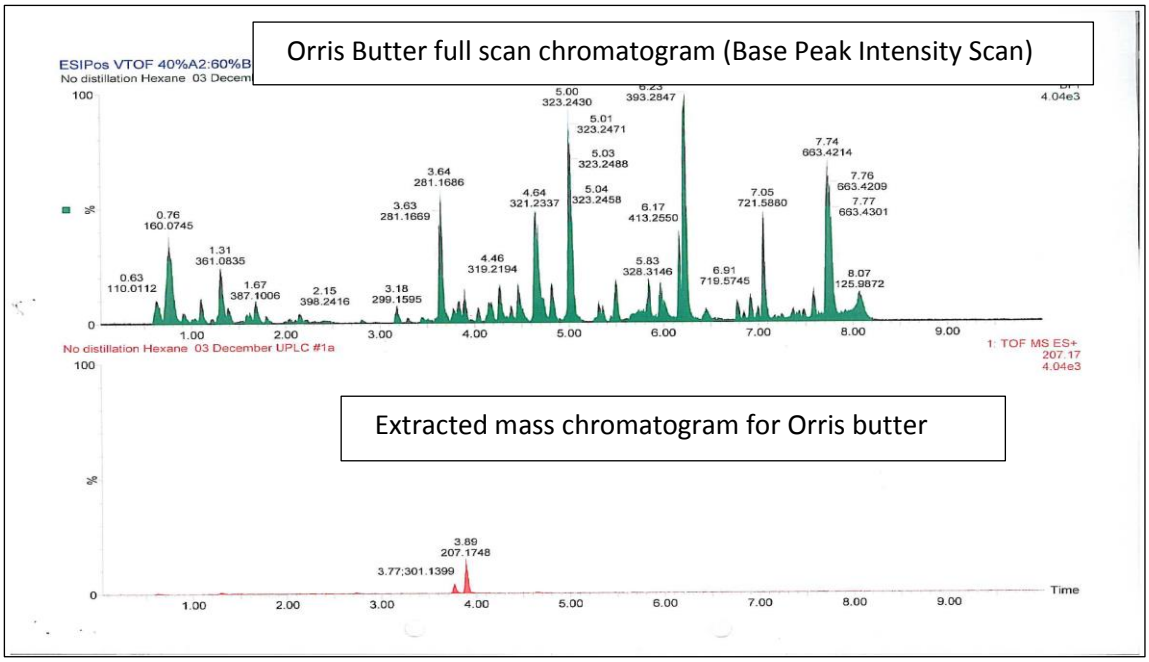


Figure 49- A full scan and an extracted mass chromatogram for resinoid produced by hexane extraction of maturated irone suspension. A resinoid- is a honey-like petroleum ether extract of orris root (www.thegoodscentscompany.com).

As shown in Figure 49, hexane-extraction of the maturated suspension produced approximately 348 mg irones/kg fresh orris root compared to the 145 mg irones/kg fresh orris root identified in the 2 L scale up irone suspension. Also noted, was a slight removal of impurities between 0-3 minutes retention times, just a minute before the irone retention time (4 minutes). Compared with petroleum ether-extraction, hexane-extraction of the suspension resulted in marginal elimination of impurities. Overall, neither hexane nor petroleum ether extraction method produced the same orris butter composition as the commercial sample (see Appendix).

Steam distillation of the maturated irone mixture followed by extraction with ethyl acetate A.R. 98% was attempted, but showed no improvement in irone content (Figure 50).

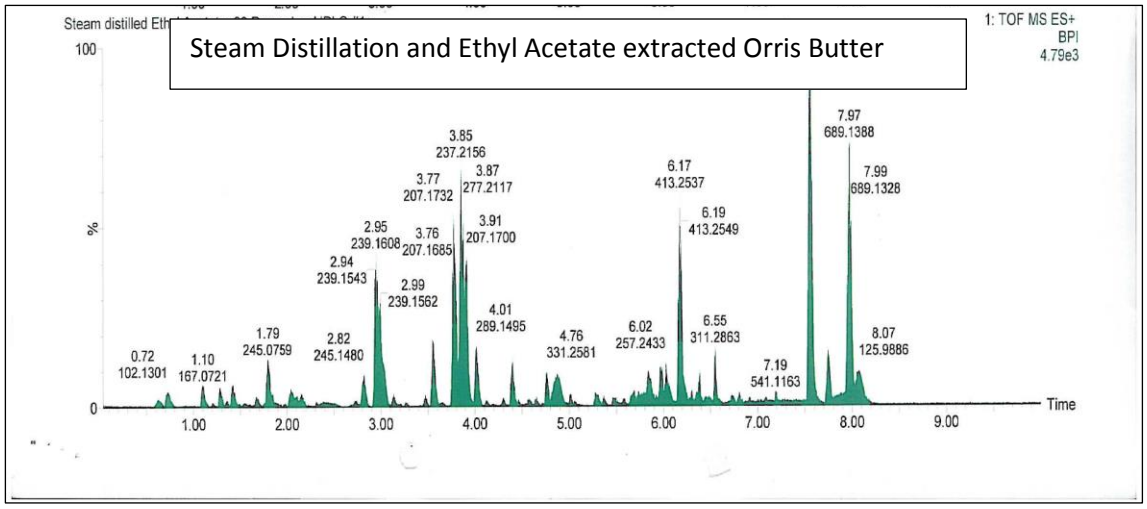


Figure 50- A full Scan (EPI) illustration of extracted mass chromatogram for γ- and α-irone generated from steam distillation (SD) followed by ethyl acetate (EA) extraction of maturated irone suspension

Figure 50 shows that the method used to attempt to concentrate the irones resulted in little elimination of impurities and no increase in irone content compared with the suspension. Compared with the commercial orris butter sample (Appendix), this method provided a butter of different composition.

Likens-Nickerson distillation was also performed as an attempt to generate orris butter and ultimately concentrate the irones in the butter while removing the impurities (Figure 51).

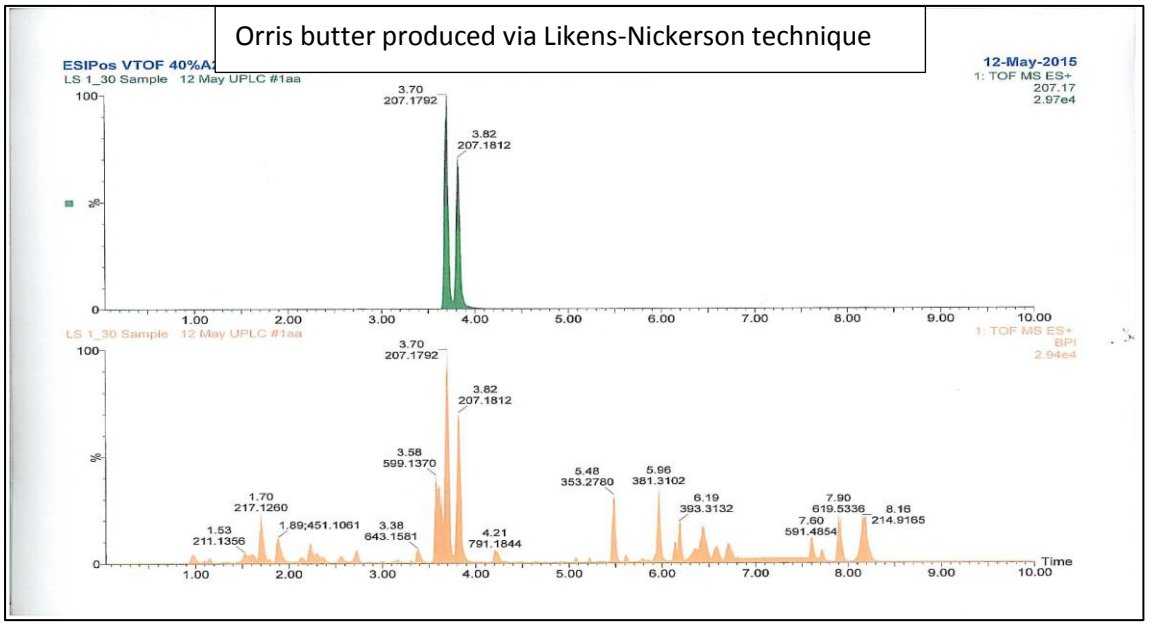


Figure 51-Illustration of extracted mass (top) and full scan (bottom) chromatograms for α and β irones generated from Likens-Nickerson distillation (SD) of maturated irone suspension produced via crude soybean lipoxidase action.

Likens-Nickerson extraction method produced 696 mg irones/kg fresh orris root from the irone suspension. Most of the impurities were eliminated from either side of the irones retention time

(around 4 minutes) giving a similar profile as commercial orris butter (see Appendix). These outcomes indicate that Likens-Nickerson distillation is the best technique for isolation of the final product from the maturated suspension.

4.10 Bioconversion of orris root to irones using purified and immobilized soybean lipoxidase.

Lipoxidase/lipoxygenase purification was conducted to enhance its catalytic activity so its maximum velocity (V_{max}) and substrate concentration at half maximum velocity (K_M), pH and temperature optima can be determined. It was believed that lipoxidase in its purified form could offer better production of irones than the crude suspension as used in earlier experiments. Immobilization of purified lipoxidase was aimed at improving lipoxidase stability and to allow for its reusability at industry if enough activity to generate irones could be preserved.

4.10.1 Purification:

An attempt to extract and purify soybean lipoxidase by an Aqueous Two-Phase system was made and the purification table is as presented in Table 5. This method was chosen on the basis of it having resulted in 125 % activity recovery with overall purification factor of 4.38 compared to crude enzyme extract as reported in a study by Lakshmi *et al.* (2009). However, the purified enzyme produced by this method was not previously tested on orris root oxidation to irones. Ii should be noted that although the activity is presented in Table 5 as µmol/min conversion to µM/min or M/min can be achieved.

Table 5- the purification table for soybean lipoxidase.

Fraction	Fraction volume (mL)	mg protein	Activity (µmol/min)	Total activity µmol/min * mL	Specific activity (µmol/min/ mg)	Fold purification	% yield
Sodium Acetate	74	0.000605	0.0074	0.55	12.31	1	100
PEG 20 000	63	0.000475	0.0073	0.53	15.30	1.24	96
PEG 6000 top	105	0.00019	0.0027	0.32	13.96	1.13	52
PEG 6000 bottom	106	6E-05	0.0022	0.23	36.23	2.94	41

Protein concentration was determined by the Bradford assay, this value was multiplied by, the volume of lipoxidase used for the assay multiplied by the dilution factor to get the mg protein; total activity (µmol/min x mL) is obtained by multiplying the activity (µmol/min) with the fraction volume; the activity in (µmol/min) is obtained by the method mentioned in the method section. Specific activity-is obtained by dividing the activity (µmol/min) with the mg of protein; purification fold- refers to the number of multiples of starting value. In this case it refers to the increase in the specific activity, i.e. the purification is obtained by dividing the specific activity at any stage with by the specific activity of the original homogenate; yield (%) - the yield is based on the recovery of the activity after each step. The activity of the original

homogenate is usually set arbitrarily set at 100 %. The yield (%) is calculated from total activity (µmol/min*mL) at each step divided by the total activity in the homogenate, multiplied by 100.

An increase in fold purification to 2.94 as depicted in Table 5 is as a result of an increase in specific activity of the enzyme suggesting a good purification, and is similar to the purification factor of 2.38 attained by Lakshmi *et al.,* (2012). The percentage yield declined slightly at the end of the first purification step (sodium acetate phase) before its slight decrease from 96 % (in the PEG 20 000 phase) to 93 % for the PEG 6000 fraction. The decrease in the yield can be as a result of a decrease in protein concentration which in itself suggests the successful removal of impurities from the crude extract.

The Michaelis–Menten kinetics of the purified soybean lipoxidase was determined (Figure 52). Different substrate concentrations were prepared and used in the assay as described in the experimental section and the activity versus substrate concentration graph was plotted to determine the K_M and V_{max}. It should be noted that although the graphs express the reaction rate as U/min and K_M as mg/mL, conversions were made to µmol/min and mol/L for comparison with literature values.

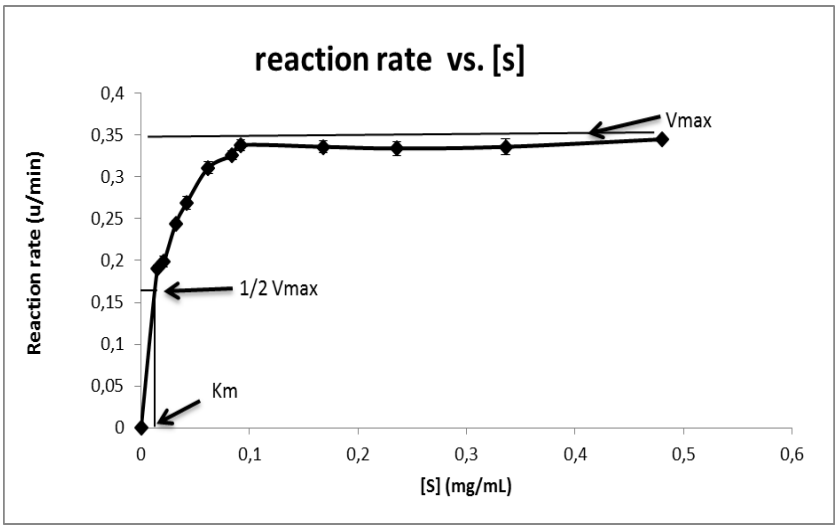


Figure 52-Michaelis-Menten plot illustrating the effect of substrate concentration on purified soybean lipoxidase activity. K_M -substrate concentration at half the enzyme's maximum reaction rate and V_{max} -the maximum reaction rate of the enzyme at substrate saturation.

As indicated in Figure 52, the purified soybean lipoxidase has an estimated maximum velocity of 0.015 µmol per min, and a ½ V_{max} of 0.0075 µmol per minute), at which the K_M can be extrapolated.

Lineweaver-Burk (top left), Hanes-Woolf (top right) and Eadie-Hofstee (bottom left) plots were constructed in order to determine the exact V_{max} and K_M (Figure 53). The r^2 values of above 0.95 indicate a good approximation of the real data points by the regression lines.

For Lineweaver-Burk plot, the y-axis represents the inverse of the reaction rate (1/v) for soybean lipoxidase expressed in min/U, the x-axis represents the inverse of the substrate concentration 1/[S] expressed in mL/mg. The slope is expressed as K_M/V_{max} while the y-intercept is expressed as $1/V_{max,}$ the x-intercept is expressed as $-1/K_M$, V_{max} is calculated as 1/y-intercept while K_M is calculated as Vmax x (K_M/V_{max}). For Hanes-Woolf plot, the y-axis represents substrate concentration per lipoxidase rate expressed in mg/mL x U while the x-axis represents the substrate concentration in mg/mL. The slope is expressed as $1/V_{max}$, while the y-intercept is expressed as K_M/V_{max}, the x-intercept is expressed as $–K_M$. An excellent estimate of the real data points by the regression line is supported by the r^2 value of 0.999. For Eadie-Hofstee plot, the y-axis represents the reaction rate, v (U/min) while the x-axis represents the reaction rate, v (U/min) over substrate concentration, [S] in mg/mL. The slope of the Eadie-Hofstee plot is expressed as $-K_M$, while the x-intercept is expressed as V_{max}/K_M, the y-intercept is expressed as v. The data for V_{max} and K_M determined by each method are as shown in Table 6.

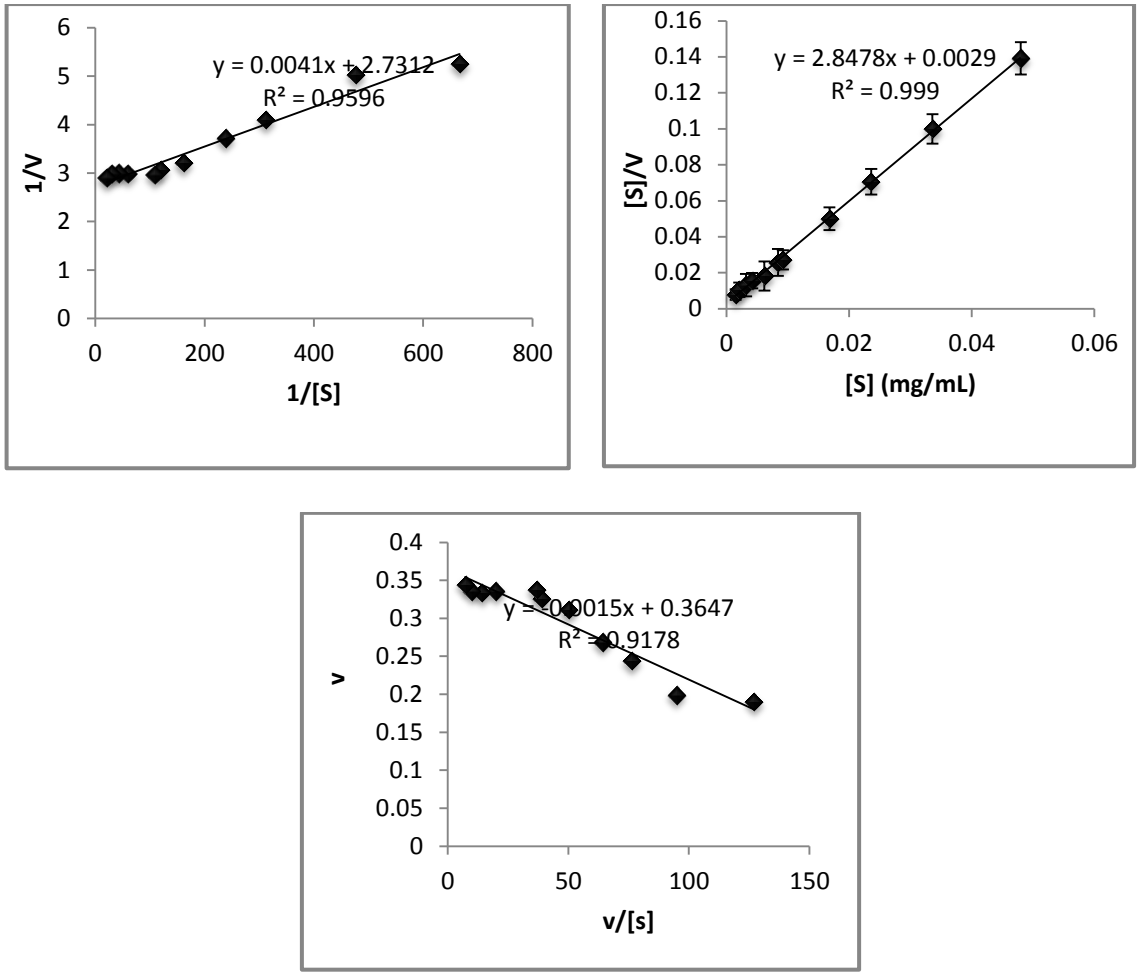


Figure 53- The effect of linoleic concentration on purified soybean lipoxidase activity by Lineweaver-Burk (top left), Hanes-Woolf (top right) and Eadie-Hofstee (bottom) plots.

Table 6- Comparison of lipoxidase kinetic parameters determined form multiple plots.

Soybean lox (0.12 mg/mL)	Lineweaver-Burk plot	Hanes-Wolf plot	Eadie-Hofstee plot	Mean	SD
V_{max} (µmol/min)	0.0159	0.0152	0.0159	0.0157	0.0004
K_M (mo/L)	5.34 E-06	3.92 E-06	5.34 E-06	4.87E-06	8.2E-07

As indicated in Table 6, no noteworthy variance exists when K_M and V_{max} are determined by either method. We obtained Km and Vmax values of 4.87E-06 mol/L and 15.7 µM/min. A study on overproduction, purification, and characterization of extracellular lipoxygenase of *Pseudomonas*

aeruginosa in *Escherichia coli* reported the K_M and V_{max} of 4.89 X 10^{-5} mol/L and 0.226 µM/min (Lu *et al.,* 2013). The reason for the discrepancy between the values for K_M and V_{max} in the present study and that of Lu *et al.,* (2013) can partially be linked to the use of different levels of purity and also different enzymes which might have different kinetic properties. The current study reproduced (Lakshmi *et al.,* 2012) and these researchers did not determine K_M and V_{max}, making it difficult to do a comparison.

Determining the effect of enzyme concentration on the activity of purified soybean lipoxidase is outlined in Figure 54.

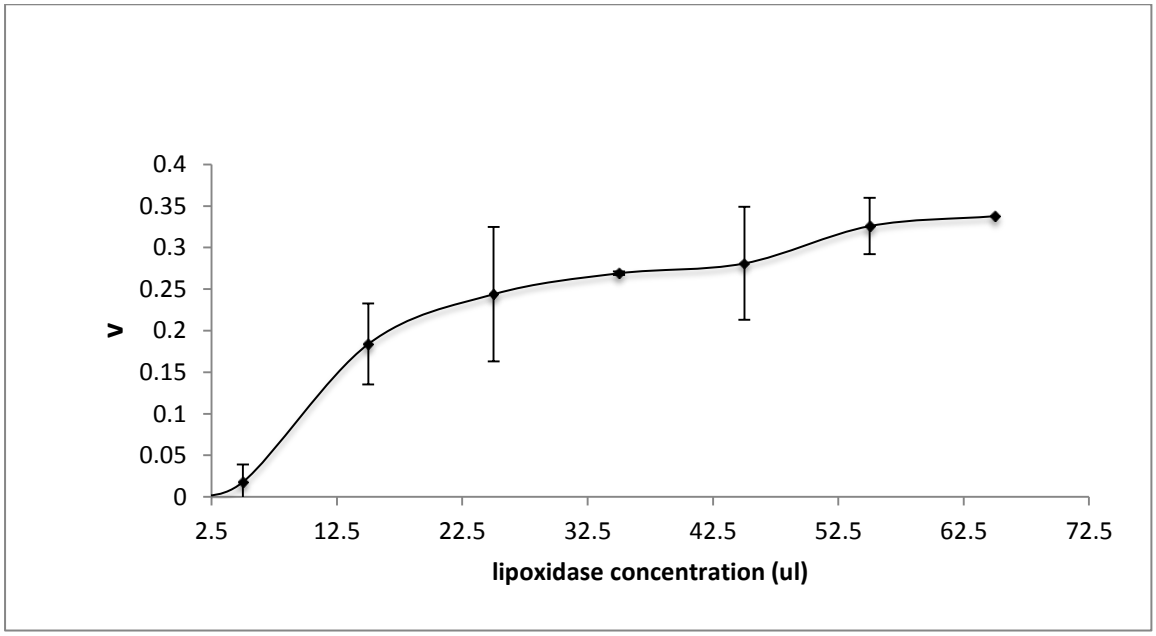


Figure 54-Effect of varying soybean lipoxidase concentration on the reaction rate. The y-axis represents the reaction rates (V) in U/min while the x-axis represents increasing amounts of soybean lipoxidase [E] in µl. The enzyme concentrations were prepared from a stock of 0.12 mg/mL. The error bars represents mean ±SD of three replicate samples (3n).

It was determined from Figure 54 that at a certain concentration of the enzyme (25 µl) soybean lipoxidase reaction rate stopped increasing exponentially such that further increase in enzyme concentration makes no significant difference to the rate.

Figure 55 outlines the influence of either pH or temperature on purified Soybean lipoxidase activity.

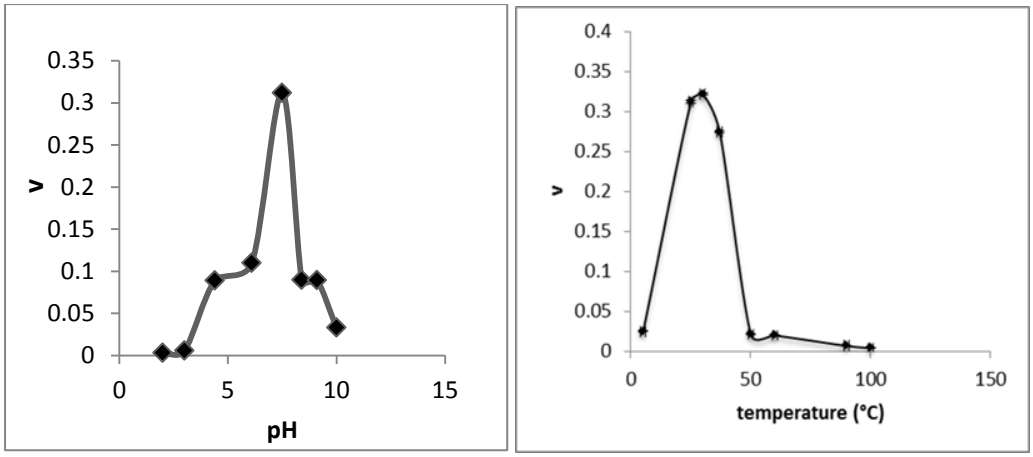


Figure 55- Illustrations of the effect of pH (left) and temperature (right) on purified soybean lipoxidase activity. The graphs above represents reaction rate (V) in U/min of soybean lipoxidase versus either pH or temperature.

It was determined that pH 7.5 is the pH optima for purified soybean lipoxidase. The activity declined markedly at pH values below or above 7.5. These findings are in agreement with the findings of (Surrey, 1964) and Lu *et al.,* (2012). A temperature of 30°C was identified to be the optimum for LOX catalytic activity; a study by Lu *et al.,* (2012) reported ambient temperature as the temperature optima for lipoxygenase of *Pseudomonas aeruginosa* in *Escherichia coli*.

Enzyme from the purification steps was tested on the bioconversion of iridals in fresh orris root to α- and γ-irone (Figure 56) to ensure that we still had the appropriate activity.

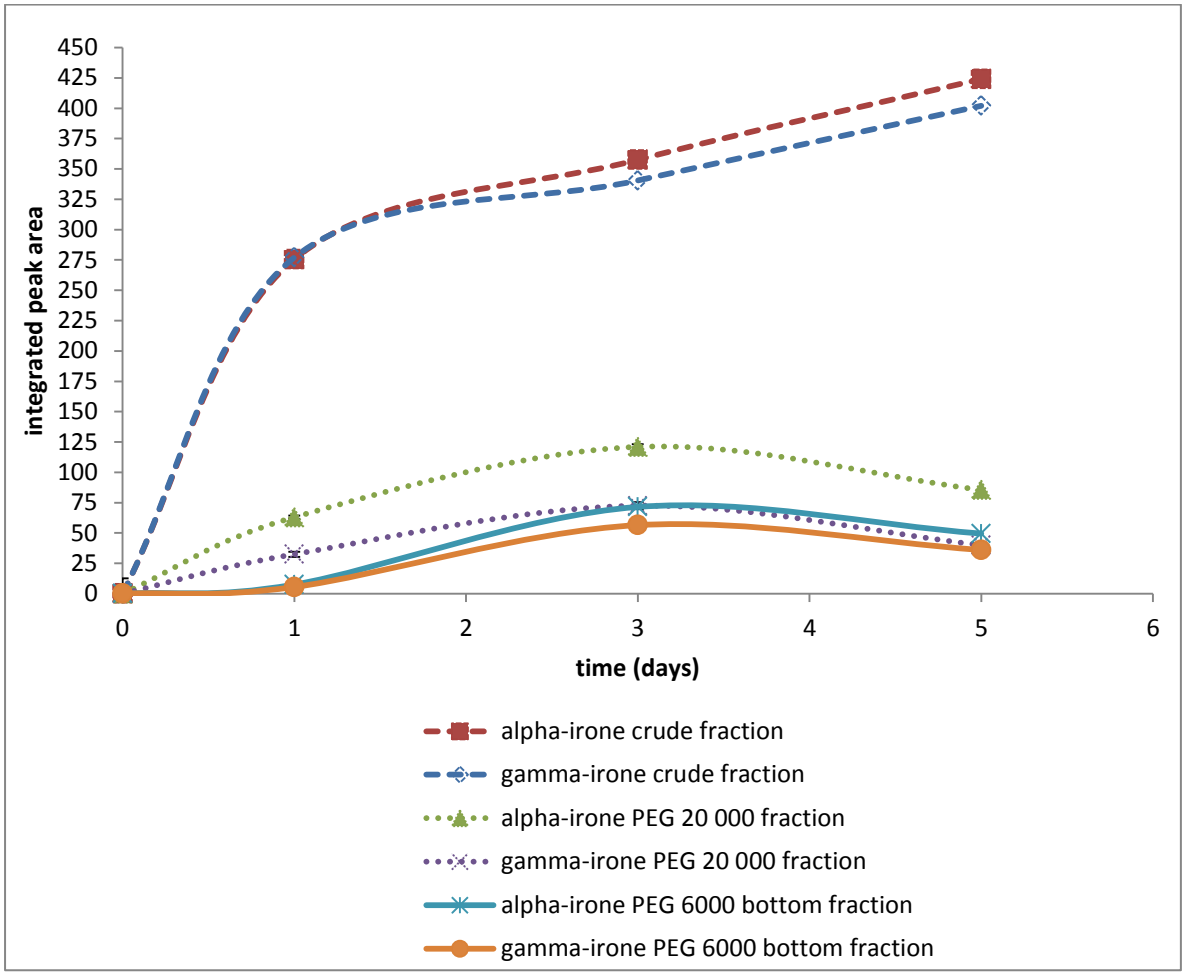


Figure 56-The influence of purified lipoxidase fractions (PEG 20 000 and PEG 6000) on the process.

Purified Soybean lipoxidase was able to oxidize iridals in fresh orris root to release quantifiable amount of irones. However, very low concentrations (around 14 mg irones/kg fresh orris root) were produced by the last purification fraction compared with 25 and 116 mg irones per kg fresh orris root produced by PEG 20 000 and crude fractions at day 5. This could mean that the enzyme is less stable in its purified form, and has denatured over time. Gil *et al.,* (1992) and Canhoto *et al.,* (2001) utilized a commercially prepared and stabilised purified soybean lipoxidase, and 0.17 g of 6 units/mg soybean provided by Fluka was used in former whilst Type I-B lipoxidase from soybean, 131 000

units/mg solid supplied by Sigma was utilized in the latter study for oxidation of orris root, and they both led to production of a quantifiable amount of irones.

4.10.2 Immobilization:

The activities (U/min) of soybean lipoxidase immobilized on either alginate gel (BSLOX) or dendrispheres (DSLOX) are as presented in Figure 57. Substrate was prepared by dissolving 2.1 mg linoleic acid sodium salt in 25 mL of 0.15 M sodium phosphate buffer pH 7.5. Soybean lipoxidase was prepared by dissolving immobilized enzyme in appropriate volume of 50 mM phosphate buffer pH 7 for DSLOX; beads (BSLOX) produced via immobilization were used as they were.

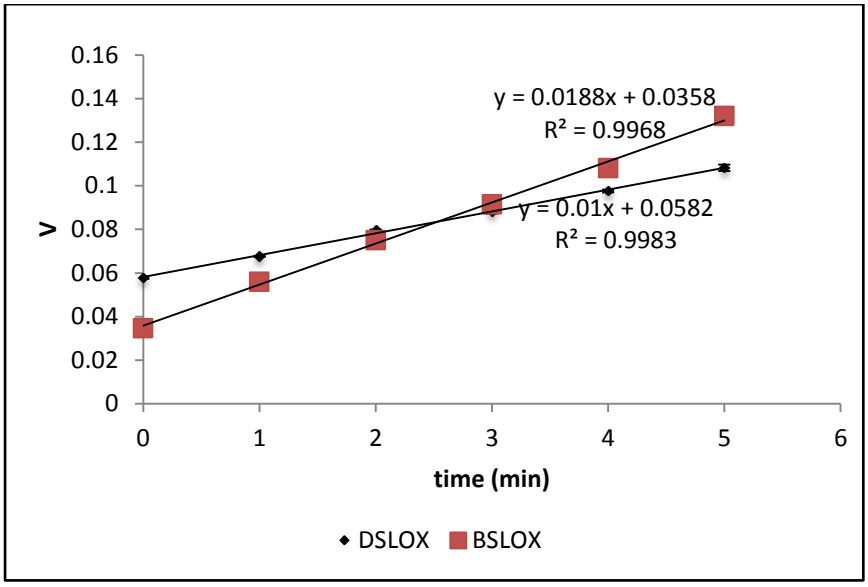


Figure 57-represents absorbance over time graph for soybean lipoxidase enzyme immobilized on either dendrispheres (DSLOX) or alginate gel (SLOX).

A linear increase in activity (U/min) with time was attained for both immobilized enzymes, suggesting the ability of the immobilized enzymes to act on linoleic acid and generate hydroperoxides (Figure 55).

Michaelis–Menten plot: determination of the effect of varying linoleic acid concentration on the activity of soybean lipoxidase immobilized on either dendrispheres or alginate gel was centre to the current test (Figure 58). It should be noted that although the substrate concentration and reaction rate (V) are reported in the following graphs as g/100 mL and U/min, conversions to mg/mL, g/L then to mol/L and conversions from U/min to µmole/min and sometimes to µM/min were made before a comparison with K_M and V_{max} values from literature was made.

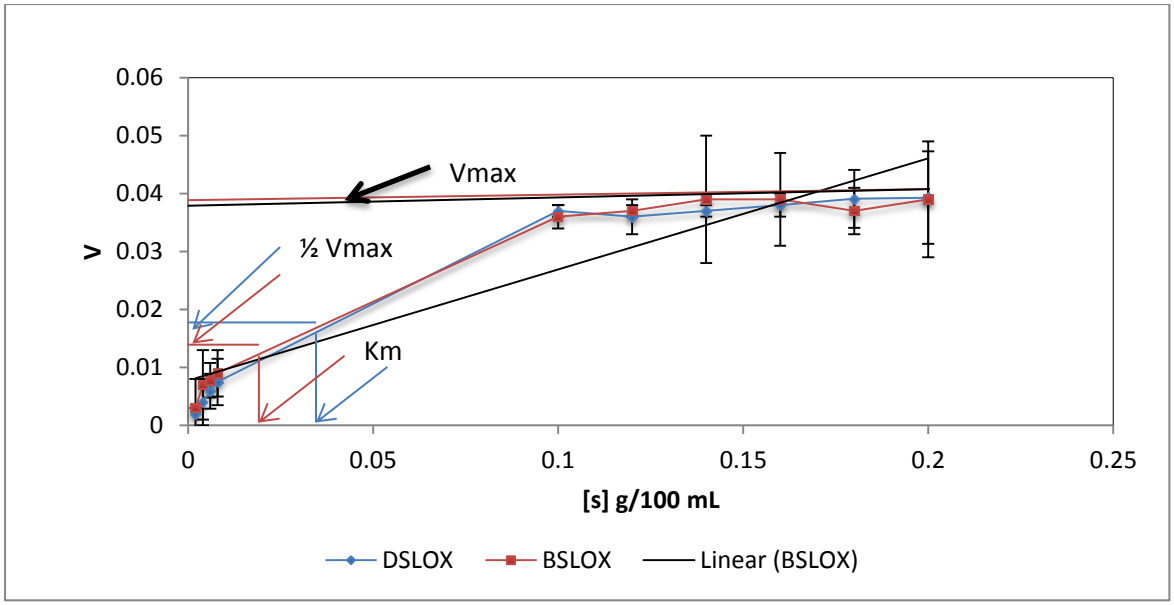


Figure 58-The effect of linoleic acid concentration on the activity of soybean lipoxidase immobilized on either dendrispheres (DSLOX) or alginate gel (BSLOX). The y-axis represent reaction rate in U/min; the x-axis represents substrate con centration in g/100 mL. The error bars represents mean ±SD of three replicate samples (3n).

Both plots (DSLOX and BSLOX) indicated that soybean lipoxidase becomes saturated at certain concentration 1 mg/mL of linoleic acid sodium salt, beyond this concentration no sufficient change in enzyme activity occurs when the enzyme is fed with more substrate. The estimated V_{max} as highlighted in Figure 64 was determined to be 1.69 µM per minute for DSLOX and 1.61 µM for BSLOX. These estimated V_{max} values were above 100 % lower than that of the unbound enzyme. The two values were halved to determine ½ V_{max} from which the estimate K_M was extrapolated as shown in the graph.

Figure 59 gives the Lineweaver-Burk (top left), Hanes-Woolf (top right) and Eadie-Hofstee (bottom) plots used to determine K_M and V_{max} for dendrispheres and alginate gel immobilized lipoxidase (DSLOX and BSLOX). The K_M and V_{max} data is given in Table 7.

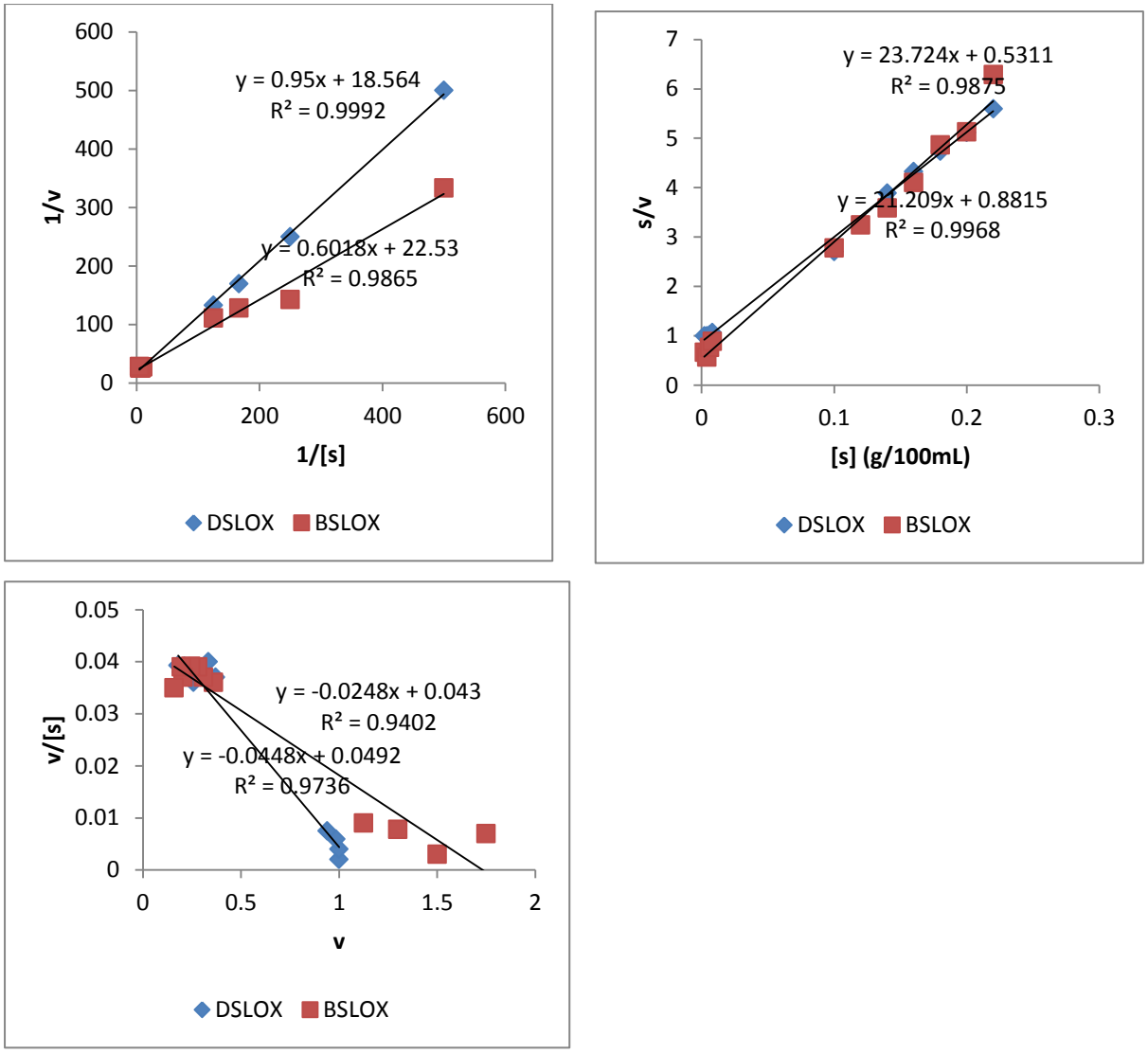


Figure 59-The effect of linoleic acid concentration on the activity of Soybean lipoxidase immobilized on dendrispheres (DSLOX) or alginate gel (BSLOX) by Lineweaver-Burk (top left), Hanes-Woolf (top right) and Eadie-Hofstee (bottom).

Table 7- Comparison of immobilized enzyme kinetic date derived from various plots.

	Lineweaver-Burk plot	Hanes-Woolf plot	Eadie-Hofstee plot	Mean	±SD
Dendrispheres- immobilized LOX					
K_M (mol/L)	1.71E-3	1.46E-3	1.60E-3	1.59E-3	1.30E-4
Sodium alginate-entrapped LOX					
K_M (mol/L)	9.62E-4	7.84E-4	8.79E-4	8.75E-4	8.91E-5
Dendrispheres-immobilized Lox					
V_{max} (µmol/min)	0.0023	0.002	0.0021	0.0021	5.77E-5
Sodium alginate-entrapped lox					
V_{max} (µmol/min)	0.0019	0.0018	0.0019	0.0019	5.77E-5

The following series of tests were undertaken with a goal to determine pH (top curve) and temperature (bottom curve) optima for LOX immobilized by two different methods as already

indicated above. As stated in Figure 60, DSLOX signifies soybean lipoxidase immobilized on dendrispheres whereas BSLOX represents immobilization of soybean lipoxidase on alginate gel.

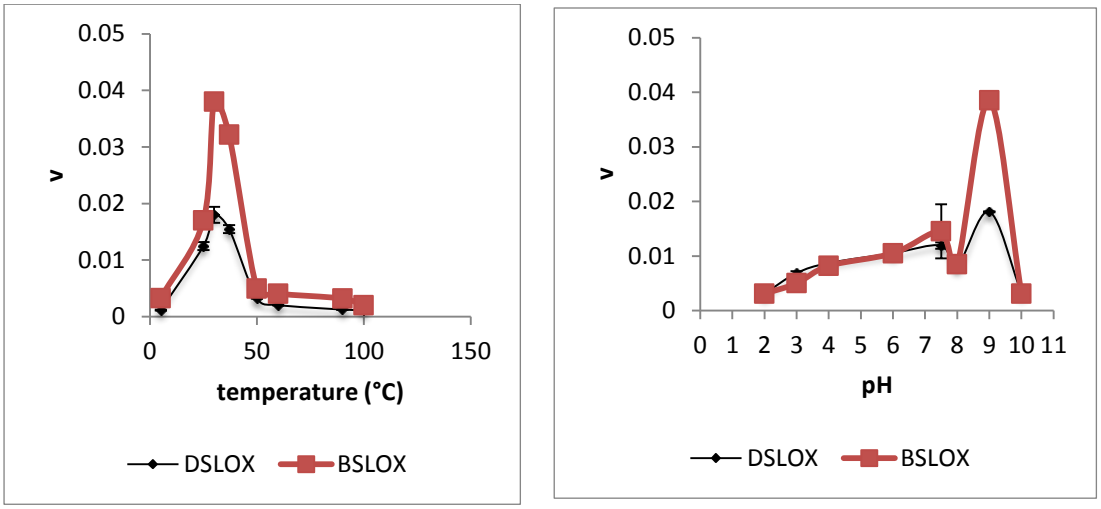


Figure 60- The effect of either temperature (left) or pH (right) on the activity of dendrispheres- or sodium alginate-entrapped soybean lipoxidase.

The graph above represents the reaction rate (v) in U/min of soybean lipoxidase versus pH or temperature. All reactions were prepared in triplicate and means ±SD were used to plot the graph.

Different pH values were tested for identification of pH optima, and pH 9 was identified as the optimum for lipoxidase immobilized on alginate gel or dendrispheres as outlined in Figure 68. However, a perceptibly higher activity (36 % more) was noted with lipoxidase entrapped on alginate gel. This could suggest that either immobilization procedure is effective; however, alginate gel-entrapment at pH 9 is preferred. These outcomes are in accord with those in a study by Hsu *et al.,* (1997) on immobilization of lipoxygenase in alginate-silicate sol-gel matrix, albeit that the two studies employed somewhat different methodologies and assays. In comparison, the assay utilized in the present study is more efficient in that it is not time consuming. Piazza *et al.,* (1993) also identified pH 9 as the optimum for fatty acid hydroperoxide in the presence of organic solvent using lipoxygenase immobilized using carbonyldiimidazole-activated matrix, termed Reacti-Gel.

The present study found that the optimum temperature for soybean lipoxidase immobilized on either dendrispheres or sodium alginate gel is 30°C. Although an outstandingly higher (35.7 % higher) activity was seen with alginate gel entrapped- soybean lipoxidase, it is apparent that immobilization on either support is achievable. Dendrispheres and alginate immobilized enzymes had 89 % and 78.9 % lower activity compared with the unbound enzyme. The optimal temperature for hydroperoxide formation in a study by Parra-Diaz *et al.,* (1993) was reported to be 15°C. In that

study, a commercial preparation of soybean lipoxygenase (EC 1.13.11.12) was covalently immobilized on a commercial carbonyl-imidazole activated support.

It will be interesting for researchers to explore other efficient ways (reported in literature) to attempt purifying soybean lipoxygenase without tempering with its ability to efficiently convert iridals in orris root into irones. It will also be interesting for scientists to attempt immobilizing the crude enzyme (after dialysis) to allow for its reusability at industry if the producer prefers the extraction of the iridals over the use of homogenised orris root. The immobilized enzyme was not tested on the process because we could not make enough of it due to shortage of materials. However, it is without any reasonable doubt that the crude soybean lipoxidase used in the present invention offered an efficient way to convert iridals in fresh orris root to irones instead of the conventional maturation process of 2 to 3 years.

CHAPTER 5 CONCLUSION

In summation, the findings of the present study suggest that incubation of fresh orris root in the presence of dioxane, oleic acid, magnesium, iron atmospheric oxygen and crude soybean lipoxidase as a catalyst at 37°C leads to a substantial accumulation of irones final yield of 696 mg irones/ kg dry orris root compared with a mere 530 mg irones/kg orris root seen for traditionally processed orris root. Although Gil *et al.* (1992) had a higher yield of 1.8 g of irone/kg of dry orris root after 48 h at 30°C; the drawback is the labour intensive prior step to extract the irone precursors before subjecting them to oxidation and the use of expensive purified commercial soybean lipoxidase instead of the extract used in the present study. Canhoto *et al.,* (2009) yielded a mere 46.01 mg irones/kg dry orris root after 20 h at 25° C despite having subjected the mixture to Likens-Nickerson distillation. These researchers also yielded a mere 718 mg irone/kg dry orris root when washed and blanched fresh orris rhizome was incubated at higher temperature of 50°C for 20 days, comminuted and extracted with diethyl ether. Ehret *et al.* (2001) generated an excellent yield of 1.85 g irones/kg dry orris root through chemical oxidation method at 25°C for 48 hours and further subjecting the product to hydrodistillation. However, sodium nitrite (catalyst) has been reported to be toxic to both human beings and animals (Sowers *et al.,* 2004).

Oleic acid serves a substrate for lipoxidase. Lipoxidases act on oleic acid to generate a hydroperoxide responsible for oxidation of irone precursors in the orris root. Dioxane is a co-solvent which improves the dispersibility of the hydrophobic molecules in the aqueous medium without denaturing the lipoxidase. The metal salts (specifically iron and magnesium) are responsible for enhancing the enzyme activity, and iron is found in the active site of the enzyme. Hence our original hypothesis that crude soybean lipoxidase requires mineral salts for efficient bioconversion of orris root to irones has been demonstrated. It can be deduced from these results that the higher reaction temperature of 37°C is preferable. This is a considerable improvement of the 3 year maturation method. Soybean lipoxidase purified in the present study yielded quantifiable amount of irones when tested on the process. However, higher irone-generating activity was seen with the crude fraction, indicating instability and denaturation, or possibly reduction of the active site iron, with time in the purified fraction with time in the purified fraction. Bioconversion was not performed with the immobilized enzyme due to the limited availability of immobilized material.

Blanching the fresh root before the bioconversion had a definite positive effect on the yield of the irones. Blanching was aimed at inactivating the orris root enzymes (e.g. tyrosinase) to prevent colour formation by tannins (which could get into the final product and also have a negative effect on extraction). During scale up it was established that an adequate supply of oxygen during the

reaction is crucial, and enhanced agitation and addition of air resulted in a much improved bioconversion time.

The present study also found that subjecting the maturated mixture to solvent extraction with 40°C-60°C petroleum ether resulted in elimination of most of the impurities, but a drawback was its inability to concentrate the irones from the maturated mixture any further.

However, this research discovered that subjecting the maturated irone mixture to Likens-Nickerson distillation for circa 3 hours leads to the correct profile for commercial orris butter (see appendix) and to an increase in irone concentration. LC-MS proved to be a robust and accurate analytical method for detecting and quantifying irones in complex mixtures. The present study also reports a sampling procedure where equal proportions of acetone and DMSO are used to extract the irones from the maturated mixture prior LC-MS analysis. The desired profile consist of irone (α- and γ-) peaks, myristic acid peak and other small peaks (see Appendix).

This optimised method may be sufficient for commercial production of orris butter. The current study eliminated the step required for extraction of irone precursors prior to enzymatic oxidation as reported in Gil *et al.,* (1992). The current work is also the first reported where minerals were added and made a substantial difference in the production of the irones. Another economic benefit of the current process is the use of a cheap, commercially available soybean flour to produce a crude soybean lipoxidase enzyme for the bioconversion.

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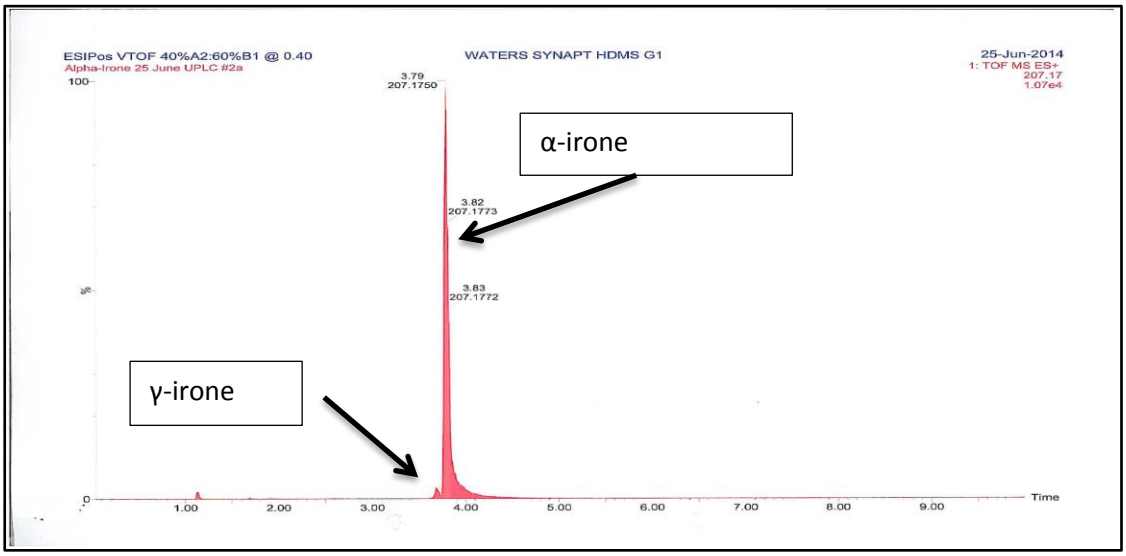
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Appendix

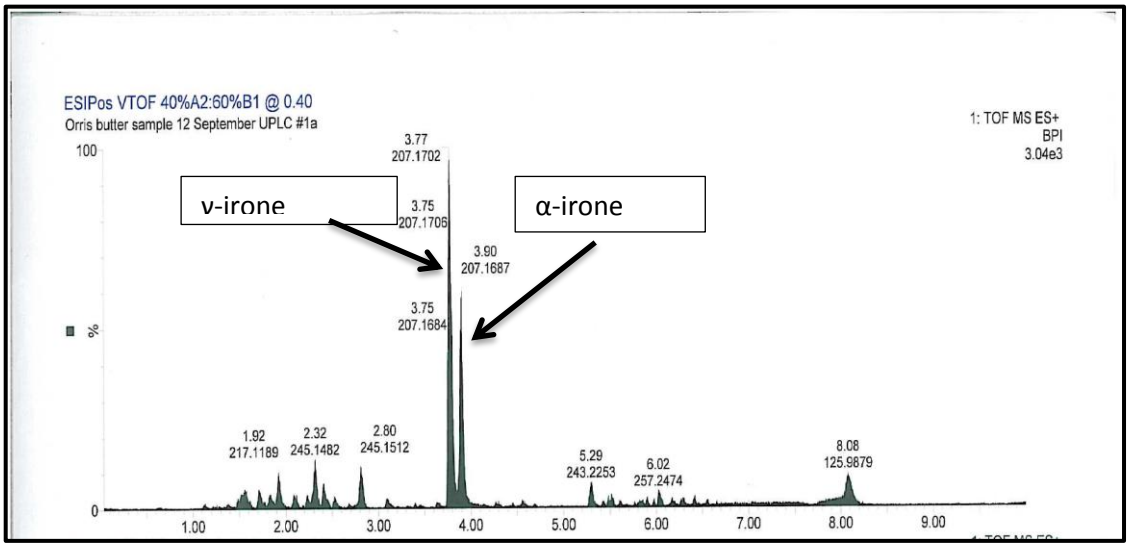
1. Commercial α- irone sample:

The following figure represents the LC-MS chromatogram for the commercial α irone sample



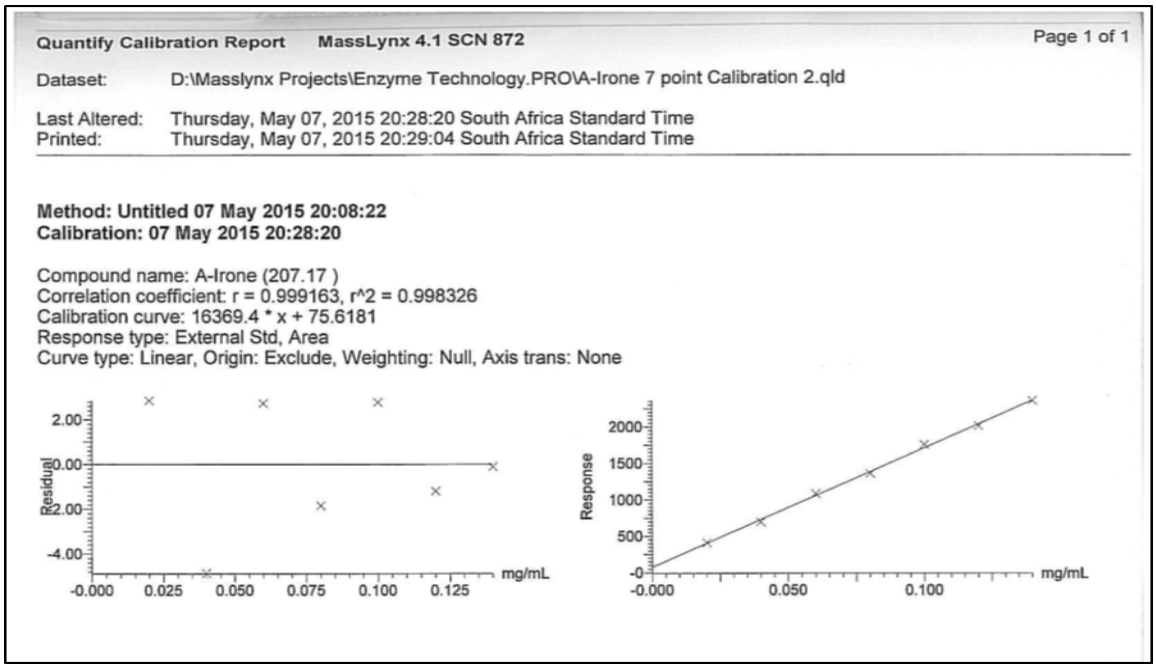
The numbers 3.79 and 3.82 appearing at the top of γ- and α-irones in Figure 28 represents the retention times. It was determined by LC-MS that the irone standard shows two irone peaks, the taller peak being α- and the small peak at the base of the α- irone peak is suspected to be γ-irone. Our suspicion was based on the fact that literature reported α- and γ- irones as the major products of orris root iridals oxidation. The proportions of these irone isomers depends upon the Iris species used to obtain the root. When *Iris pallida* is used more γ-irones compared to α-irones are produced and when *Iris florentina* is used the opposite occurs as it is the case in the Figure.

2. Commercial orris butter sample profile

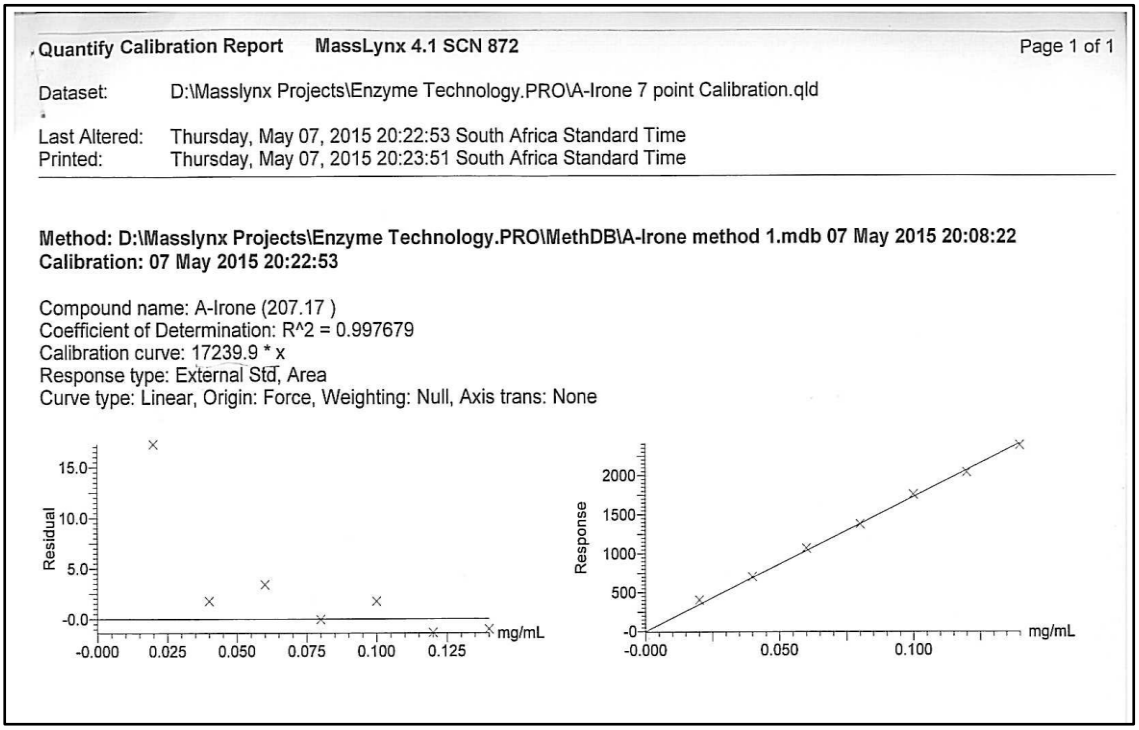


The butter consists of irones (α- and γ-), myristic acid and marginal amounts of impurities. More γ-irones compared to α- irones were generated. As indicated in the literature, one would assume that the orris root used in here was from *Iris pallida*.

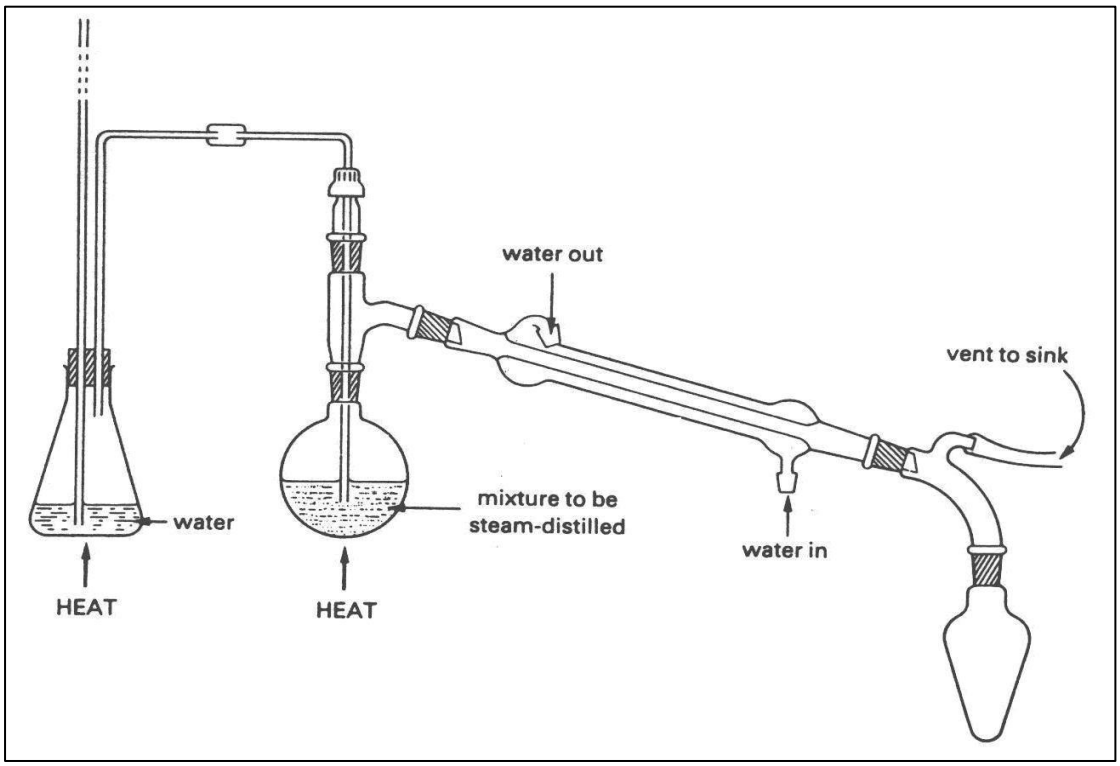
3. Commercial α-irone calibration curve



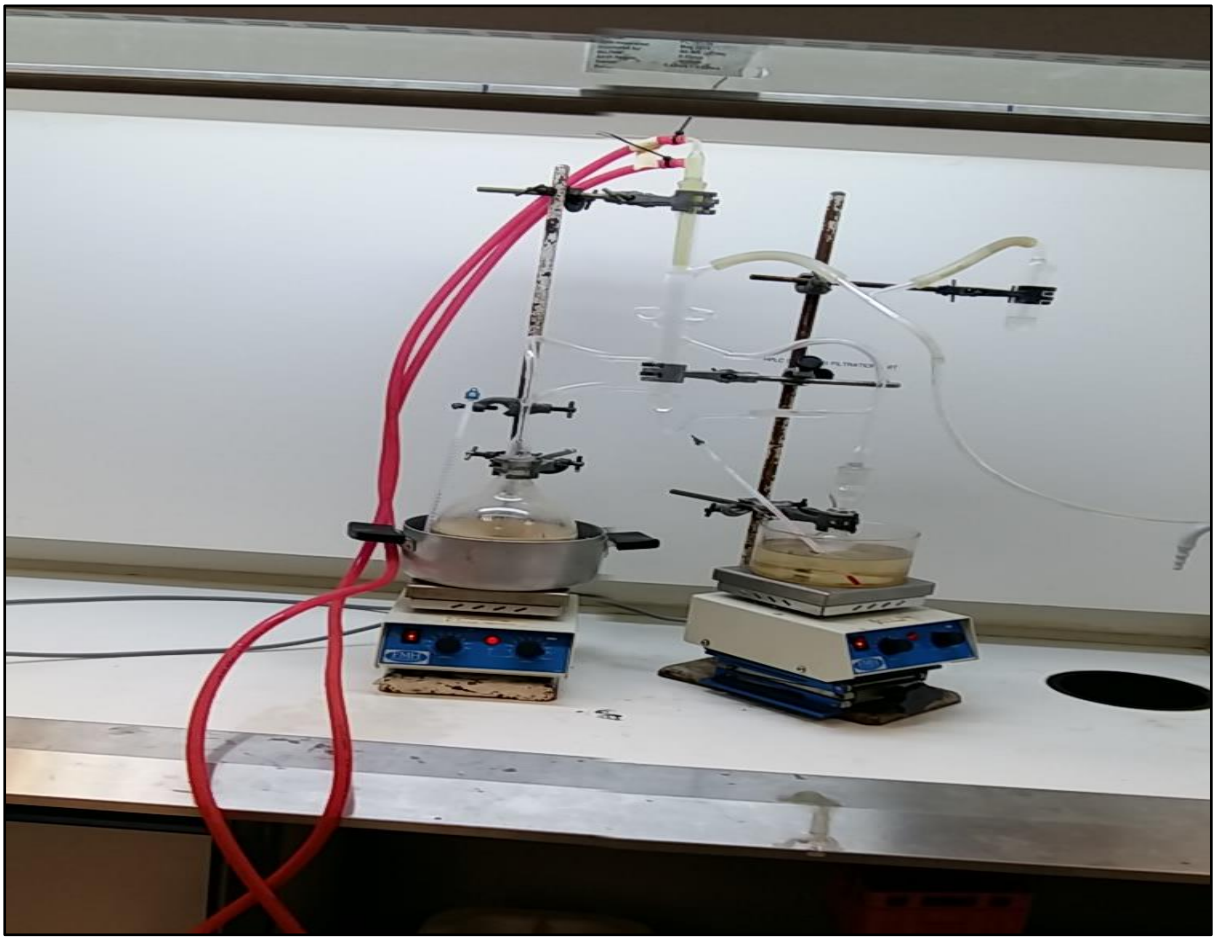
4. Commercial α- irone calibration curve adjusted so that the regression line starts from the origin.



5. Steam distillation apparatus

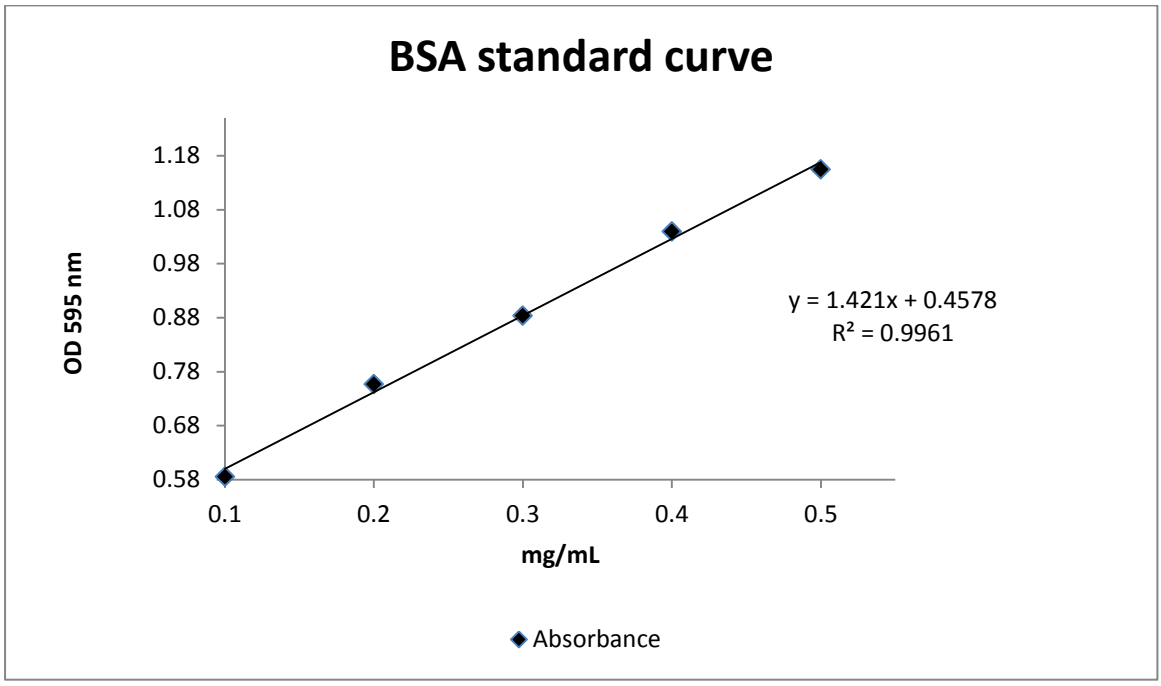


6. Likens-Nickerson distillation apparatus photo



7. BSA standard curve

The following figure shows BSA standard curve used to extrapolate protein concentrations in the enzyme purification and immobilization sections.



8. GC analysis:

Gas chromatography-flame ionization technique was reported to have intrinsic advantage of being attractive for the characterization and quantification of terpene mixtures (Zubyk and Conner, 1960). The column used was a 30 metre × 0.32 mm i.d. with a 5 mm film thickness of DB wax. The GC programme that he used was as follows:

Injection volume: 1 mL; initial temperature: 100°C; initial time: 1 minute; rate: 5°C/min; final temperature: 200°C, du: 10 minutes.

Analysis was also attempted using the non-chiral gas chromatography method with the same conditions as above. However, the column utilized was Rtx-Sil MS W/Integra Guard (Restek), 30 m × 0.25 mm id × 0.25 µm DF, s/n 68900 and the final time was 20 minutes. This method was able to elute the commercial α-irone standard and the compounds of interest at the retention times of around 11.706 for α- and 12.232 for the compound suspected to be γ-irone. This method was

discontinued due to lack of availability of analytical standards that made the results difficult to interpret, so a LC-MS method was adopted.

9. Table 1-Constituents found in the total extract of *Iris pallida* and the ionization modes utilised for their MS characterization.

		Ionization modes				
		PCl		**NCl**		
Component	MW (Da)	EI	NH3	CH4	*i*-CH10	NH3
(1) lriflophenone	246	X	X	X	X	X
(2) lridine	522	X	X	X	X	X
(3) lrigenine	360	X	X	X	X	X
(4) lrisflorentine	386	X	X	X	X	X
(5) lristectogenine B	330	X	X	X	X	X
(6) lsoiridogermanal	474	X	X	X	X	X
(7) Unidentified iridal	474		X	X	X	X
(8) Spiroiridal-1	486	X	X	X	X	X
(9) Spiroiridal-2	528	X	X	X	X	X
(10) Spiroiridal-3	470		X	X	X	X
(11) lridal	472	X	X	X	X	X
(12) lripallidal	486	X	X	X	X	X
(13) lriflorental	486	X	X	X	X	X
(14) lsoiridogermanal myristate	684	X	X	X	X	X
(15) lripallidal rnyristate	696	X	X	X	X	X
(16) lriflorental rnyristate	696	X	X	X	X	X
(17) Spiroiridal-2 myristate	738		X	X	X	X
(18) Unidentified iridal ester	710			X	X	X
(19) Unidentified iridal ester	730		X	X	X	X

Figure 1 displays the structure of the compounds identified in the *Iris pallida* extracts investigated by Bicchi *et al.* (1993).