Development of an optimized process for commercial production of (-) ambrafuran

Dissertation submitted for the qualification

Master of Science in Molecular and Cell biology (full time)

University of Witwatersrand

By

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Date: **01 February 2016**

ABSTRACT

Ambergris is a large lump excreted by whales, when exposed to sunlight, air and sea water, it oxidatively decomposed through inorganic reactions to form different compounds including (-) ambrafuran. (-) Ambrafuran is a highly fragrant compound and it is considered to be a stronger perfume ingredient than other constituents in ambergris. It is used as a fixative agent to stabilize perfumes by reducing the rate of evaporation of volatile substances. A two-step process for production of (-) ambrafuran starting from sclareol was developed at CSIR for an industrial partner, Teubes cc. The current commercial production of (-) ambrafuran entails a chemical process consisting of at least 8 steps and require very harsh chemicals and elevated temperatures. In the current study, relevant technologies for the optimization of a process for commercial production of (-) ambrafuran were investigated. The project objective has been to optimize fermentation conditions on laboratory scale for the conversion of sclareol to an intermediate diol using the microorganism *Hyphozyma roseoniger* and to subsequently test different zeolites for conversion of diol to (-) ambrafuran. Production of ambradiol was achieved in potato dextrose broth media in 13 days compared to a patented method which took 16 days. The method was also scaled-up in a 2 L fermentation bioreactor and the yield of 93% was achieved after 24 hours of reaction. Following the initial use of the zeolite CBV320, two new zeolites (CFG-1 and ZD0614) were identified which have the potential to convert ambradiol to (-) ambrafuran without undergoing an activation process. Zeolite CFG-1 have been recognized to be highly effective for converting the intermediate ambradiol to the resulting (-) ambrafuran. One of the greatest outcomes of this research project is that the amount of zeolite required per substrate has been reduced from between 1:6 and 1:9 to 1:2. The substrate concentration has been increased from 5 mg/mL to 100 mg/mL which also resulted in the reduction of the volume of solvent required for the cyclodehydration step. The study allowed for scale-up and following further optimisation on larger scale should result in a process on commercial scale.

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.

Signature 01/02/2016

ACKNOWLEDGEMENTS

I would like to thank the Council for Scientific and industrial Research (CSIR) especially to the Biosciences Unit, under the Protein Technologies research group for having granted me the opportunity to further my studies. Thank you to the funders, National Research Foundation (NRF) THRIP. Thank you to Teubes cc. who acted as both the industrial partner and the funder. Thank you to my supervisor, Dr Karl Rumbold from Wits University School of Molecular and Cell Biology and co- supervisor, Dr. Lucia Steenkamp from CSIR (Principal researcher) for their support and guidance and David Morrison with assistance in editing the thesis. I would also like to thank Mr Kgama Mathiba and Prof Paul Steenkamp for valuable guidance with the analytical elements of the project. I would also like to thank my family and friends for their full support and gratuity during this course of my study.

Table of Contents

Abbreviations

AlO_4	Aluminium tetraoxide
CrO_3	Chromium trioxide
DCM	Dichloromethane
dH_2O	Distilled water
DMSO	Dimethylsulphoxide
FID	Flame ionization detector
GLC	Gas liquid chromatography
HOMO	Highest occupied molecular orbital
IBDA	Iodobenzenediacetate
LC-MS	Liquid chromatography and Mass spectroscopy
$LiAlH_4$	Lithium aluminium hydroxide
LUMO	Lowest unoccupied molecular orbital
NMR	Nuclear magnetic resonance spectroscopy
PDB	Potato dextrose broth
p-TsOH	p-Toluenesulphonic acid
RuO_4	Ruthenium odxide
SAR	Structural activity relationship
SiO_4	Silicon tetraoxide
tBuOK	tert-Butoxide
THF	Tetrahydrofuran
TLC	Thin layer chromatography
YNB	Yeast nitrogen base without amino acids
YPD	Yeast extracts peptone and dextrose

LIST OF TABLES

LIST OF FIGURES

DEDICATION

I dedicate this dissertation to my late Grandfather, Mbhazini James Mongwe; my lovely wife Anastesia Mayimele; my son Nhlaluko Mongwe; all my siblings, and my parents Chucha Silence Mongwe and Cathrine Mpenyani.

COPYRIGHT PAGE

DECLARATION OF ADHERENCE TO ETHICAL CONSIDERATIONS

I have adhered to the ethics of Wits University, Faculty of Science, School of Molecular and Cell Biology, and the CSIR Biosciences in Bio-manufacturing industry and development centre research group.

SIGNATURE PAGE

Student signature: _____Date:

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Co-supervisor's signature: _____Date:

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Supervisor's signature:

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Examiner's signature: _____Date:

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CHAPTER 1

1. INTRODUCTION

1.1 Ambrox

Choudhary and Musharraf (2005) described (-) - ambrox (a trade name of Firmenich S.A) as a highly fragrant constituent of ambergris, which is a metabolic product of *Physeter macrocephalus L,* commonly known as the sperm whale, digestion of squid beaks. Ambergris drifts in the sea for extended periods of time and during this time; it is being oxidatively decomposed by the action of sea water, air and sunlight, resulting into several inorganic reactions. The combination of these inorganic reactions result in the ambergris producing many odorous compounds such as ambrinol, α-ambrinol, γ-homocyclogeranyl chloride, ambreine, ambrox and dihydro-γ-ionone (Tanimoto and Oritani, 1997; Barrero *et al*., 1988).

(-) Ambrox has a strong amber-like odorous fragrance compared to its chiral compound (+) ambrox and has been rated as good as "Civet" and "Musk" (Martres *et al*., 1993). (-) Ambrox is also considered to be a stronger perfume ingredient/additive than (+) ambrox (Choudhary and Musharraf, 2005). Different techniques for (-) ambrox and its racemate production had been established starting with natural occurring "sesqui or di-terpenes" compounds such as sclareol (Bolster *et al*., 2001), a compound extracted from the plant *Salvia sclarea* or clary sage as described by (Carey, 2013b).

The reasonably high price of (-) ambrox which is estimated to be $1000 or more per kilogram have promoted ongoing research for new chemical synthetic routes of producing this compound and its derivatives. The synthesis normally starts from low-cost natural available labdanes as substrates (Bolster *et al*., 2001). Many industries are currently using sclareol as the starting material for (-) ambrox production (Fráter *et al*., 1998), because of its stereochemical features that are more related to that of ambrox (Jean *et al*., 2001).

1.2 Problem statement

Consumers have developed a "chemophobia attitude" over chemical or synthetic compounds that are associated with food and home-care products (Krings and Berger, 1998; Vandamme, 2003). Normally, biotechnological processes are less harmful to the environment and often

produce the desired enantiomeric compounds (Krings and Berger, 1998). Currently (-) ambrafuran, used in the fragrance industry can be obtained naturally from ambra excreted by whales but the whales are a threatened species, or synthetically by at least an eight step chemical process using harsh chemicals. A client, Teubes cc requested the development of a "green process" to produce (-) ambrafuran on commercial scale so that the company can market the product as "natural".

Farbood and Willis, (1989) described the biological conversion of sclareol to ambradiol using the microorganism *Hyphozyma roseoniger*. Steenkamp and Taka (2010) patented a method for the conversion of the intermediate diol to the ambrafuran (ambrox) product using zeolites. The drawbacks were that the zeolites have to be activated at high temperature and the high volume of organic solvent was not feasible on large scale and have to be optimised.

1.3 Hypothesis

Conditions can be optimised for the conversion of sclareol to the intermediate diol and then to the final (-) ambrafuran so that the process can be considered "green" and the conditions can be scaled up for the development of a commercial process. The main contributors to a process feasible on large scale will be increasing the substrate concentration and reducing the volume of the organic solvent and finding the best zeolite for the process.

1.4 OBJECTIVES OF THE STUDY

1.4.1 Research objectives

The overarching objective is the development of a "green" process at bench scale for the production of (-) ambrafuran. The purpose of this study is to optimise the conditions for the process of (-) ambrafuran production starting from sclareol. The project will involve the optimisation of the fermentation conditions to obtain an intermediate diol from the sclareol as well as optimisation of conditions around the conversion of the diol to the final (-) ambrafuran. This second step will involve testing different zeolites and solvents in order to use less solvent for the final step and to be able to scale up the complete process to bench scale. The specific objectives of the research were:

- Development of analytical methods to separate and detect the sclareol, intermediate diol (ambradiol) and final product (ambrafuran) as well as quantification of the different compounds.

- To obtain and test different zeolites for their ability to convert intermediate diol to (-) ambrafuran.

- Test different solvents which are considered to be acceptable for use in production of "natural products" for optimum conversion to final product.

- To reduce the quantity of zeolite and solvent required for conversion of the diol to the ambrafuran.

- Optimise fermentation conditions at laboratory scale for convert sclareol to the intermediate diol using *Hyphozyma roseoniger*.

- Development of a downstream process for the extraction and purification of the final (-) ambrafuran

- Scale up of the complete technology to 2 L bench scale.

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CHAPTER 2

2. LITERATURE REVIEW

2.1 Biocatalysis

Biocatalysis is specified as the use of enzymes and whole-cells for the conversion of chemical and biological materials. Both whole-cells and enzymes are widely being used as biocatalysts, yet whole-cell biocatalysts are considered to be advantageous over the isolated enzymes due to their ability to use cheap and abundant raw materials and can certainly be used without undergoing enzyme purification or addition of coenzymes (Goretti *et al*., 2013). Enzymes and whole-cells biocatalysts are normally suitable to produce optical pure stereoisomer compounds due to their complex chiral composition (Panke *et al*., 2004). It has been found that most reactions catalysed by these biological systems show great selectivity in terms of chemo-, regio-, and stereo-selectivity and are environmentally friendly based on their activity for taking place under milder conditions than chemical synthetic routes which uses toxic chemicals and operate under harsh conditions (Goretti *et al*., 2013).

Biocatalytic methods fulfil the objectives and ethics of "green chemistry" which is becoming increasingly important in industrial processing (Kazlauskas and Kim, 2011). The use of biocatalytic methods in the production industries are highly recommended and are called "green processes" because they are environmentally more acceptable as they are sustainability, use milder conditions and due to the recyclability of the biocatalyst (Kazlauskas and Kim, 2011). In most cases, biocatalytic tools are considered to be enantioselective compared to chemical tools (Kazlauskas and Kim, 2011).

2.2 Natural valuable fragrances

Fragrance production started thousands years ago through extraction from natural resources (Serra *et al*., 2005). Historically, ambergris, jasmine and iris essential oil are being considered as the most valuable flavours and fragrance compounds (Brenna *et al*., 2002). These natural products are reported to be costly due to their low natural concentrations and considered to produce better results contrary to similar synthetic materials (Brenna *et al*., 2002). Biocatalytic routes for ambergris and jasmine fragrances are largely being investigated to completely replace or to avoid the use of chemical synthetic routes to minimise the use of hazardous substances (Serra *et al*., 2005).

2.3 Flavouring Compounds of Ambergris

For many years, ambergris has been regarded as one of the most highly rated perfumery materials due to its extraordinary use as a fixator. The term ambergris came from the French "Ambre gris" which means grey amber dissociating itself from brown amber which is a fossilized resin. Ambergris is a substance excreted by the sperm whale (*Physeter macrocephalus L*.) as a metabolic product which possibly formed as the result of its diet or injuries to the gut (Ohloff, 1990). It is reported that ambergris is composed of 40-46% cholestanol type steroids and about 25-45% ambrein, which is a triterpene (áde Groot, 1991). Its possible formation remains unknown though some suggestion states that it may have been formed as a result of an irritation reaction occurring in the intestine of the whale (áde Groot, 1991). When ambergris is released into the sea as a lump, is subsequently exposed to sunlight, air and sea water and it can be reduced into several compounds such as ambrinol, α-ambrinol, γ-homocyclogeranyl chloride, ambreine, ambrox and dihydro-γ-ionone (Barrero *et al*., 1988). In 1954, the biggest lump of ambergris weighing around 400 kg was recovered from a whale killed by the Whaling Vessel Southern Harvest" and it is still regarded as the largest piece recovered (Bolster, 2002).

Several chemical pathways for the degradation of ambergris have been developed in which some of the products are odourless while many have odours reminiscent of seaweed, wood, and moss with peculiar, sweetness which is a characteristic of ambergris, but with a dry undertone with unequalled perseverance (Hanson, 2001). One of these desired fragrances is (-) ambrafuran, which is considered to be the most important compound amongst the ambergris compounds because it possesses the animalic note characteristic of ambergris (Zinkel *et al*., 1971)

Due to the decline in the number of whales, partly due to their hunting for the collection of ambergris, the hunting of the baleen whale (*Physeter macrocephalus L*.) is currently banned and alternative ways to find or produce the odorous products consisting of the ambergris fragrances are currently underway (Topçu *et al*., 1999). Currently, the ambrafuran materials used in perfumes are entirely from synthetic or semi-synthetic sources. Different types of plants such as podocarparcea (*Pandanus family*), *Salvia sclarea* (clary sage), oak moss and several fungi consist of compounds that can be converted to ambergris-like-odorants (Fráter, 2000). Sclareol from clary sage is currently the most widely used starting material for

industrial production of ambrafuran. Several laboratory routes are presently being designed to produce ambrafuran and its racemate using sclareol as starting material (Bolster, 2002).

2.4 Structural activity relationships of ambergris-odour fragrances

It has been hypothesized that the structural elements of the labdane skeleton is highly associated with that of the ambergris scent, with ambrafuran being considered to be the vital compound amongst other ambergris odorants. The availability of a decalin skeleton with either one alcohol, ether or ester function and alkyl substituents at specific position is considered as the basic structural component of this group and all ambergris odorants contain these general features (Cirera, 1993). This does however not mean that compounds with similar features will contain amber-like-type odour and therefore configuration and conformational features are of great importance for odour profile and strength.

Studies on structural activity relationships (SAR) revealed the "triaxial rule" of odor sensation conducted by Ohloff in 1971 as shown in the Figure 1 below (Kovatcheva *et al*., 2004). Based on the outcome of the rule, it is reported that all ambergris odorants encompass a *trans*-decalin system with axial substituents in which either of these R1, R2 and R3 axial groups containing an oxygen atom is a geometric prerequisites for an amber-type-odour (Cirera, 1993).

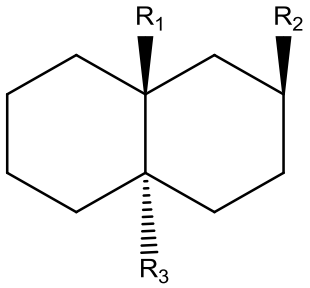


Figure 1: A diagram representing the "Triaxial rule" by Ohloff (1971)

However, Vlad *et al*., (1983) reported that the cyclohexyltetrahydrofuran, which is an ambergris compound, does not contain a decalin ring system as indicated in the Figure 2 below.

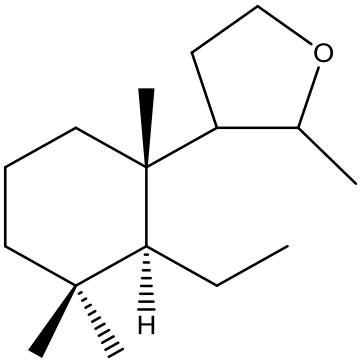


Figure 2: A structure of the cyclohexyltetrahydrofuran (Cheng *et al*., 2009)

This compound led Vlad and co-workers to pursue another way of studying the ambergris structure odor relationship. In their findings, it is reported that all ambergris odorant possesses the so called "ambergris triangle" shown in Figure 3 below (Bersuker *et al*., 1985).

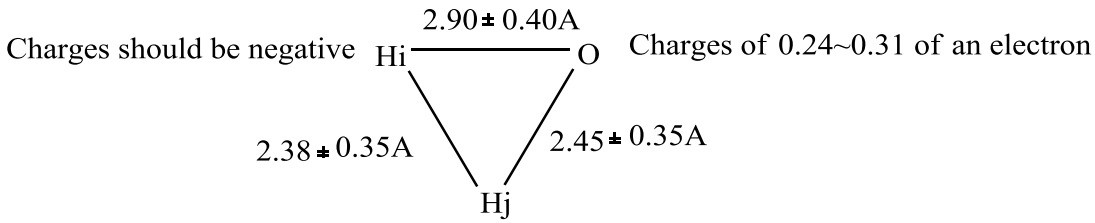


Figure 3: A diagram representing the "ambergris triangle" by Vlad and co-workers (Bersuker *et al*., 1985)

It is reported that both hydrogen atoms and an oxygen atom play a critical role to the lowest unoccupied molecular orbitals (LUMO) of the ambergris chemicals and it is postulated that electron transfer occurs between the active molecules and the odorant receptor site within the LUMO (Bersuker *et al*., 1985). In 2009, a "new ambergris triangle" was reported by Cheng and colleagues in a follow-up studies of the ambergris structure (Cheng *et al*., 2009).

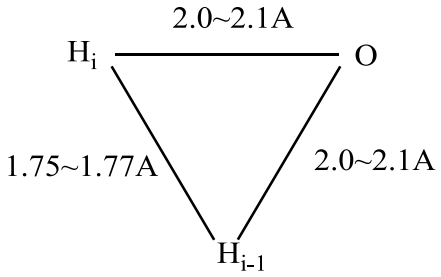


Figure 4: A diagram representing the "new ambergris triangle" by Cheng and colleagues (Cheng *et al*., 2009)

The ambergris compounds such as *trans*- and *cis*-isomers of 1-[2,2,6-trimethylcyclohexanyl]hexan-3-ol, trans- and *cis*-isomers of 4-isopropenyl-1,3,3,5,5-pentamethylcyclohexanol are being used in the study that led to the formulation of the "new ambergris triangle" shown in Figure 4 (Cheng *et al*., 2009). It is found that the two hydrogen atoms and an oxygen atom were supposed to contribute to the highest occupied molecular orbitals (HOMO) rather than the LOMO as revealed by Vlad and co-workers (Cheng *et al*., 2009). Bersuker *et al*., (1985) proved that ambergris triangles determine the structural arrangements and the intensity of the ambergris odorants. Cheng and colleagues also proved that the lowest energy isomers cannot contain the strongest odor in relation to the other isomers (Cheng *et al*., 2009).

2.4 *Hyphozyma roseoniger*

It is a biocatalyst for conversion of sclareol to ambradiol. It is known as a hyphomycetes fungus with a yeast form and demonstrates pink colonies when grown in yeast mold agar, malt extract agar, and it is light orange-pink when grown in corn meal agar (Farbood and Willis, 1989). It is sometimes referred to as a filamentous yeast or a yeast-like fungus (De Hoog and Smith, 1986). It is reported to have been extracted from soil sample found in central New Jersey in United States of America and the organism was characterized as *Hyphozyma roseoniger* based on its distinctive physiochemical as well as morphological features by Centraalbureau voor Schimmelculture and the American Type Culture Collection with CBS 214.83 and ATCC 20624 identities (Farbood and Willis, 1989). It forms a true hyphae when grown on malt extract agar, potato and rice slides after 2-3 weeks (Farbood and Willis, 1989) and has the ability to grow in different nutritious media (De Hoog and Smith, 1986). The microorganism grows from 12-30˚C (Farbood and Willis, 1989).

2.5 Zeolites

Zeolites are mostly used as catalysts in cyclodehydration reactions. In 2010, Steenkamp and Taka reported the use of calcium type zeolite (CBV320A) for conversion of ambradiol to ambrafuran. They are referred to as the solid crystalline structures built with silicon, aluminium and oxygen that form substructures containing cavities and channels inside where cations, water and small molecules can be accommodated as depicted in Figure 5 (Baerlocher *et al*., 2007). They are also well known as the aluminosilicate components of microporous solids called molecular sieves (Wali *et al*., 1996). It is reported that zeolites can be naturally formed from volcanic rocks and ash layers reacted with alkaline ground water as compared to industrial zeolites which are artificially created (Rollmann *et al*., 2007). In this study only a brief general description of zeolites and their structural channelling dimensions are given.

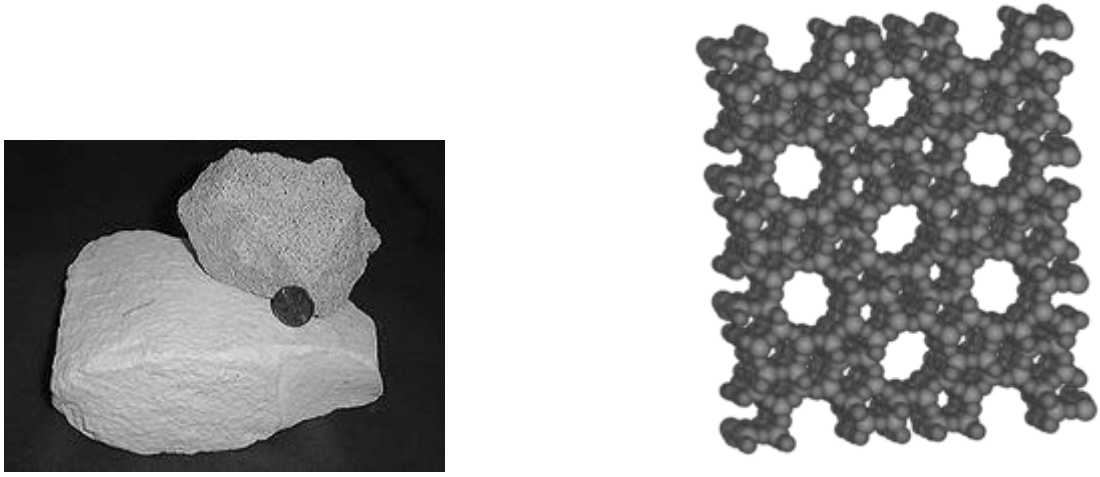


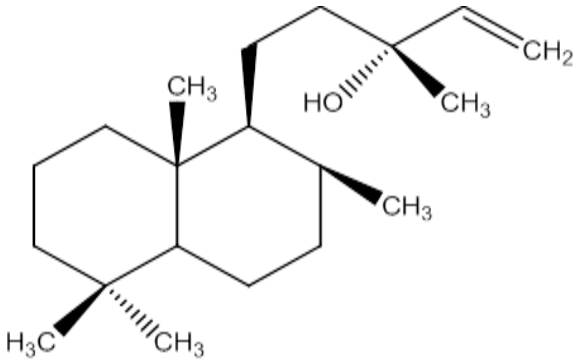
Figure 5: Configuration of the zeolites with their microspore structure and dimensions (http://en.wikipedia.org/wiki/Zeolite accessed on 17 May 2015)

Zeolites can lodge various cations including Na^{+}, K^{+}, Mg^{2+}, Ca^{2+} and many more in their microporous compartment. Their distinctive porous structure and a modifiable acidity play a crucial role in their efficiency in different complex reactions (Vjunov *et al*., 2014)**.** Zeolites acts as catalysts for various chemical reactions which take place within the internal cavities and it is also claimed to have dominated some processes in the field of petroleum refining as well as petrochemistry around early 1962 (Scherzer and Gruia, 1996). Industrial zeolites are usually manufactured by means of heating alumina solutions as well as silica with sodium hydroxide (Scherzer and Gruia, 1996). They are applicable as catalysts as well as in different

industries such as petrochemical, nuclear industry, biogas industry and other commercial industries (http://en.wikipedia.org/wiki/Zeolite accessed on 17 May 2015)

2.6 Sclareol

Caniard *et al*., (2012) described sclareol as a diterpene Natural product of high value for the fragrance industry. It is a commercially available labdane extracted from the flowers and leaves of *Salvia sclarea* (clary sage) which belongs to the family of *Laminaceae* (Schalk *et al*., 2012). Sclareol is highly rated as a potential starting material for semi-synthesis of various commercial compounds especially for ambrafuran production due to its labdane carbon skeleton and its hydroxyl group which resembles that of (-) ambrafuran (Caniard *et al*., 2012). It is primarily collected in essential oil-producing trichomes coated in flower calices of clary sage (*Salvia sclarea*), even though it has been reported that sclareol can be isolated from several plants such as *Cistus creticus (Cistaceae), Nicotiana glutinosa (Solanaceae),* and *Cleome spinosa (Brassicaceae,* but clary sage remains the most predominant source (Caniard *et al*., 2012).



Sclareol

Figure 6: A typical structure of Sclareol (Schalk, 2013)

Kuźma *et al*., (2006) described clary sage as a biennial herb native species of Southern Europe, Mediterranean basin and Iran, "therefore" it is mainly cultivated for its essential oil in European countries like France, Hungary and Bulgaria as well as North America.

There are two common ways of extracting sclareol from clary sage - one being where sclareol is extracted by means of a two-step industrial process involving hydro-distillation of the

aerial parts of the plant, followed by solvent extraction of the plant material left, and the other technique is using bioengineered enzyme terpene synthases "which however has been" reported to produce limited product (Laville *et al*., 2012). Since there's a high demand for sclareol for the replacement of ambergris from sperm whale for the production of ambrafuran, many industries are currently using apolar solvent extraction technique for isolation of sclareol from clary sage, as indicated in Figure 7 below (Laville *et al*., 2013).

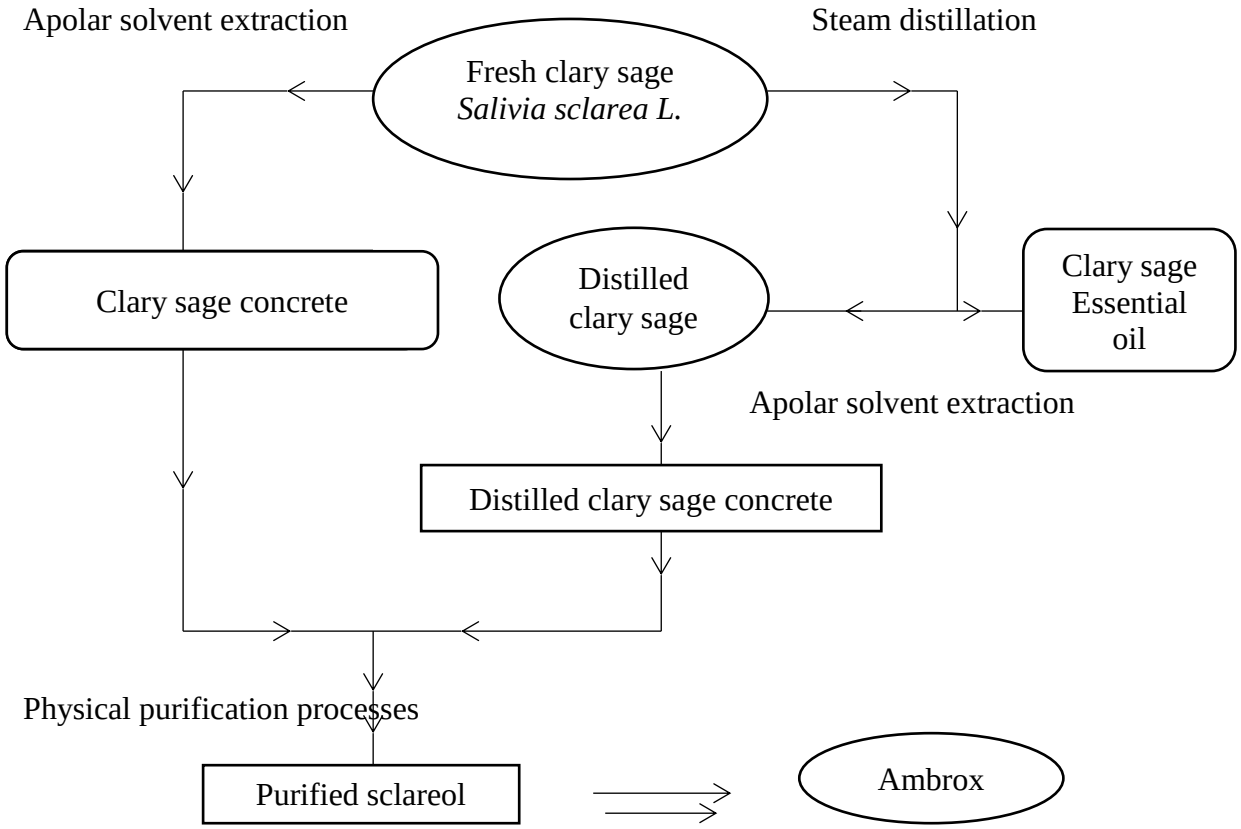


Figure 7: An illustration of the sclareol extraction process from Clary sage (*Salvia sclarea L.*) (Laville *et al*., 2013)

This illustration scheme depicts how sclareol was extracted from clary sage by apolar solvents on an industrial scale, generating extracts known as concretes which undergo physical purification processes to produce pure sclareol. Through a series of different mechanisms, sclareol is successfully being used as the starting material for the production of ambrafuran (Frija *et al*., 2011).

2.7 Chemical syntheses of ambradiol (Tetranorlabdane diol) from sclareol

Some years ago, ambradiol was reported to be produced through chemical syntheses starting from sclareol in 7 steps as indicated in the diagram below (Figure 8) (Moulines *et al*., 2001). Sclareol is reported to be oxidatively degraded into sclareolide or/and ambradiol which serves as an important precursors for ambrafuran production. In a reaction that involved sclareol with 0.023 mol/mol ruthenium chloride hydrate and sodium periodate carried out in a tedious process (Carlsen *et al*., 1981), a purity of 88.5% of sclareolide with acetoxy-acid was produced. The main drawback in this process is the constant recovery of ruthenium salts (Moulines *et al*., 2001). The solution for this obstacle was discovered by Moulines and co-workers through epoxidation, Payne rearrangement and acid cyclization of sclareol to yield 8,13-epoxylabdane-14,15-diol in solid form (Moulines *et al*., 2001). The 8,13-epoxylabdane-14,15-diol was dissolved in methanol in a solution containing 53.9 mol/mol sodium periodate and the reaction was carried out for 16 hours at 25°C in the dark to produce 8,13-epoxy-15-norlabdane-14-al, which underwent oxidation using Jones reagent and resulted in the sclareolide without traces of acetoxy-acid. Sclareolide was then reacted with lithium aluminiumhydroxide ($LiAlH_4$) for 3 hours at room temperature and reported to yield ambradiol. Ambradiol is regarded as the most suitable intermediate compound for ambrafuran production (Moulines *et al*., 2001).

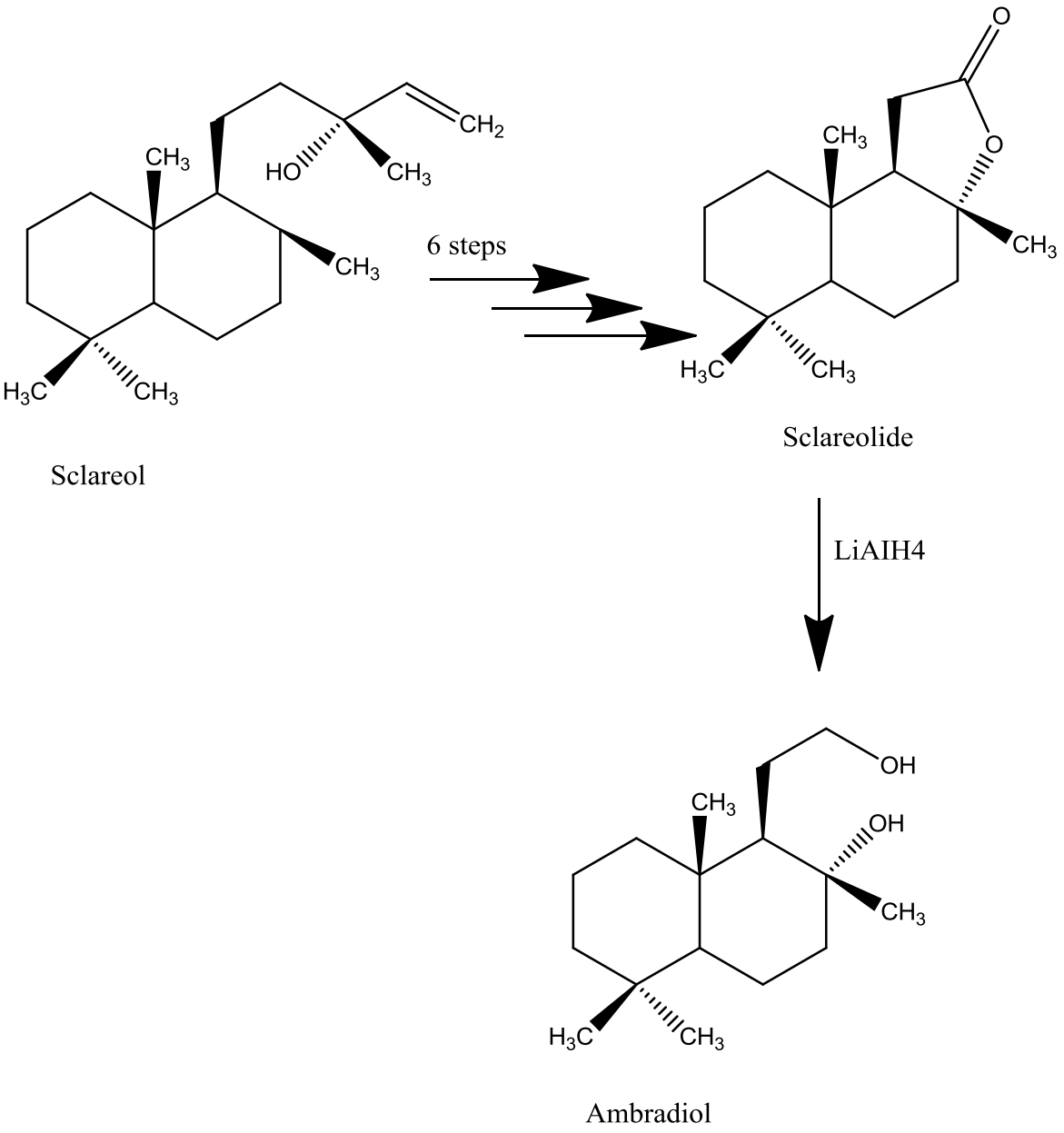


Figure 8: Schematic representation of chemical syntheses of ambradiol from sclareol (reproduced from Steenkamp and Taka, 2010)

It is reported that production of ambrafuran through chemical syntheses generally results in racemic mixtures rather than isolated enantiomers. Therefore, alternative processes have been adopted where biological conversion of sclareol using microorganisms to yield either sclareolide or ambradiol were investigated and patented by Farbood *et al*., (1990).

2.8 Biotransformation of Sclareol to ambradiol (Tetranorlabdane diol)

Biotransformation of terpenes is being widely considered in a number of industries, especially in pharmaceutical and cosmetic industries because it is reported to yield enantiomeric fragrance compounds under favourable conditions and such products are being classified as "natural" (De Carvalho and da Fonseca, 2006).

Biotransformation of sclareol to ambradiol using the microorganism *Hyphozyma roseoniger* with the identifications of either CBS 214.83 or ATCC 20624 was reported by Farbood and Willis, (1989). The organism is described to have distinct yeast and filamentous forms showing the same biological features and ability to perform transformations. In 1990, another microorganism known as *Bensingtonia ciliata* (ATCC 20919) was also discovered to convert sclareol to ambradiol in a similar way as that of *Hyphozyma roseoniger* (Downey *et al*., 1990). It is reported that these microorganisms are capable of converting the sclareol to ambradiol under aerobic conditions between the temperature of 20-25°C in aqueous nutrient medium within a period of 7-15 days (Carey, 2013a). By using *Bensingtonia ciliata* ATCC 20919 under aerobic conditions in aqueous nutrient medium at a temperature of 25°C, 1 g sclareol is reported to have resulted in 0.35 g ambradiol. *Hyphozyma roseoniger* CBS 214.83 or ATCC 20624 transformed sclareol into ambradiol in aqueous nutrient medium in 10 days, after 3 days of cultivation at 25°C.

Fermentation process for production of ambradiol using *Hyphozyma roseoniger* in an aqueous solution comprising nutrient media of 0.1 g NH_4NO_3, 0.1 g KH_2PO_4, 0.05 g $MgSO_4.7H_2O$, trace minerals, and vitamin B complex in 100 mL scale was described by Farbood and Willis, (1989). The microorganism was reported to have been pre-inoculated in the same media for 3 days and induced with 10 mg sclareol mixed with (5 mL) 50% glucose and (0.1 mL) Tween-80 and incubated for further 3-4 days at 25˚C on a rotatory shaker (200 rpm). Sclareol (8 g) was dissolved in Tween-80 (8 mL) and transferred into the media for another 4 days to transform sclareol to ambradiol. The contents were isolated with ethyl acetate (200 mL) and desiccated in sodium sulphate anhydrous. A crude extract of 4 g ambradiol was reported to have been obtained after solvent evaporation and a purity yield of 100% analysed by gas liquid chromatography (GLC) after recrystallization by hexane with chloroform, and the resulting amount of ambradiol was 2.4 g (Farbood and Willis, 1989).

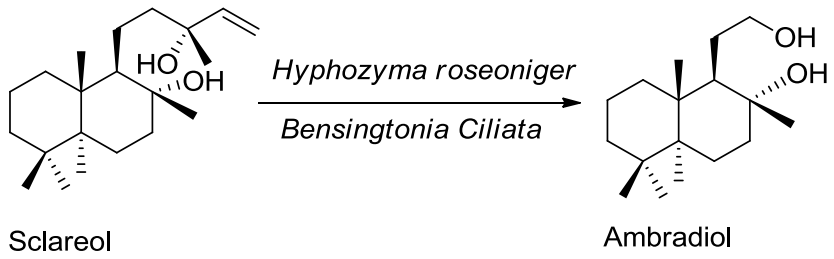


Figure 9: Typical schematic representation of biological conversion of sclareol to ambradiol using microorganisms *Hyphozyma roseoniger* and *Bensingtonia ciliata* (Downey *et al*., 1990; Farbood and Willis, 1989)

Conversion of sclareol to ambradiol in aqueous nutrient media consisting of 0.1 g NH_4NO_3, 0.1 g KH_2PO_4, 0.05 g $MgSO_4.7H_2O$, with yeast extract (0.1 g) replacing trace minerals and vitamins from fermentation method mentioned above was also established by (Farbood and Willis, 1989). In this patent, sclareol was utilized at different levels (2 g, 3 g and 5 g in 100 mL media) and reported to have been recrystallized in hexane, pulverized, sieved through 50-mesh sieve then mixed with equivalent amount of Tween-80 before being added into the induction inoculum for biotransformation. The reaction was allowed to occur for a further 4 days with the 2 g sclareol yielding 81% isolated ambradiol while 3 g and 5 g sclareol yielded 74% and 71% isolated ambradiol respectively. In 2010, Steenkamp and Taka also reported the use of *Hyphozyma roseoniger* from ATCC to convert sclareol into ambradiol in aqueous medium containing yeast nitrogen base without amino acids at 20°C for 10 days as shown in Figure 9. It is estimated to have yielded ≥98% purity of ambradiol without any by-products and the structure was confirmed using LC-MS (Steenkamp and Taka, 2010).

Farbood and Willis, (1989) further described the fermentation process for production of ambradiol using isolated cells (*Hyphozyma roseoniger*) from culture broth and washed in an aqueous solution of 30 µM phosphate buffer pH 7.2 after 3 days of pre-inoculum. The harvested and washed cells were then disseminated into the same buffer and incubated for 4 days on a rotatory shaker (200 rpm) at 25˚C. Sclareol (0.5 g) was dissolved in Tween-80 (5 mL) and regularly dispensed into the buffer for another 3 days to convert sclareol to ambradiol. Thin layer chromatography (TLC) was used to identify the progress for conversion of sclareol to ambradiol and reported that 98% was attained in an isolated yield with a GLC purity of 99% (Farbood and Willis, 1989).

2.9 The syntheses of Ambrafuran

The variety of processes for the syntheses of (-) ambrafuran is widely reported and is mainly divided into four major routes which are: (a) Synthesis based on the cyclization of linear or monocyclic precursors, (b) Synthesis starting from cyclic monoterpenoids or drimenol, (c) Synthesis starting from labdanes, and (d) Synthesis starting from other natural products. Amongst all these routes, syntheses of (-) ambrafuran from labdanes has been regarded as the most suitable starting material because they are easily obtained from nature (Bolster *et al*., 2001). (-) Ambrafuran is being referred to as a "tetranor labdane" which is a class of diterpene by Bolster and co-workers, which makes it very efficient for the labdanes diterpenoids to be considered as the most preferred starting material for its production. In this study, focus will be more on syntheses of ambrox starting from labdane diterpenes, especially sclareol.

2.9.1 Syntheses of ambrafuran starting from the Labdanes

Labdanes are considered to produce massive groups of diterpenes which are often being used as the starting material for the production of different natural products including (-) ambrafuran (Swift, 2002). It has been reported that 4 carbon atoms have to be omitted from the labdanes through oxidative procedures, for (-) ambrafuran to be synthesized in the end (Bolster *et al*., 2001). There are many labdanes used for the production of (-) ambrafuran but amongst them only a few tha are reported to have been successful in the production of ambrafuran are selected for this discussion.

2.9.1.1 (-) Ambrafuran production starting from labdanolic acids

Labdanolic acid is regarded as one of the labdanes that have the possibility to act as a precursor in the synthesis of (-) ambrafuran and it is available in nature. It is reported to have been easily extracted from *Cistus ladaniferus L.* well known as "Rock-rose". through steam distillation or by treatment of plant material with hot water (aqueous base) and soaking with n-Hexane, then evaporation (Bolster *et al*., 2001). The oxidative degradation of labdanolic acid is described to be very difficult due to its carboxyl group being the only functional group to be accessible in its side chain (Allemann and Jenny, 2001).

Bolster and co-workers have investigated the synthesis of (-) ambrafuran from labdanolic acid in 7 steps as indicated in Figure 10 below. Labdanolic acid was degraded in a solution

containing N,N-dimethylaniline with acetyl chloride overnight to form a pure acetate compound which underwent a process called iododecarboxylation which is regarded as the key step. The iododecarboxylation process was carried out using iodobenzenediacetate (IBDA) with iodine (I_2) under a 100 W tungsten lamp within 45 mins to produce the iodide compound reported to be unstable due to contamination by iodobenzene (Bolster *et al*., 2001).

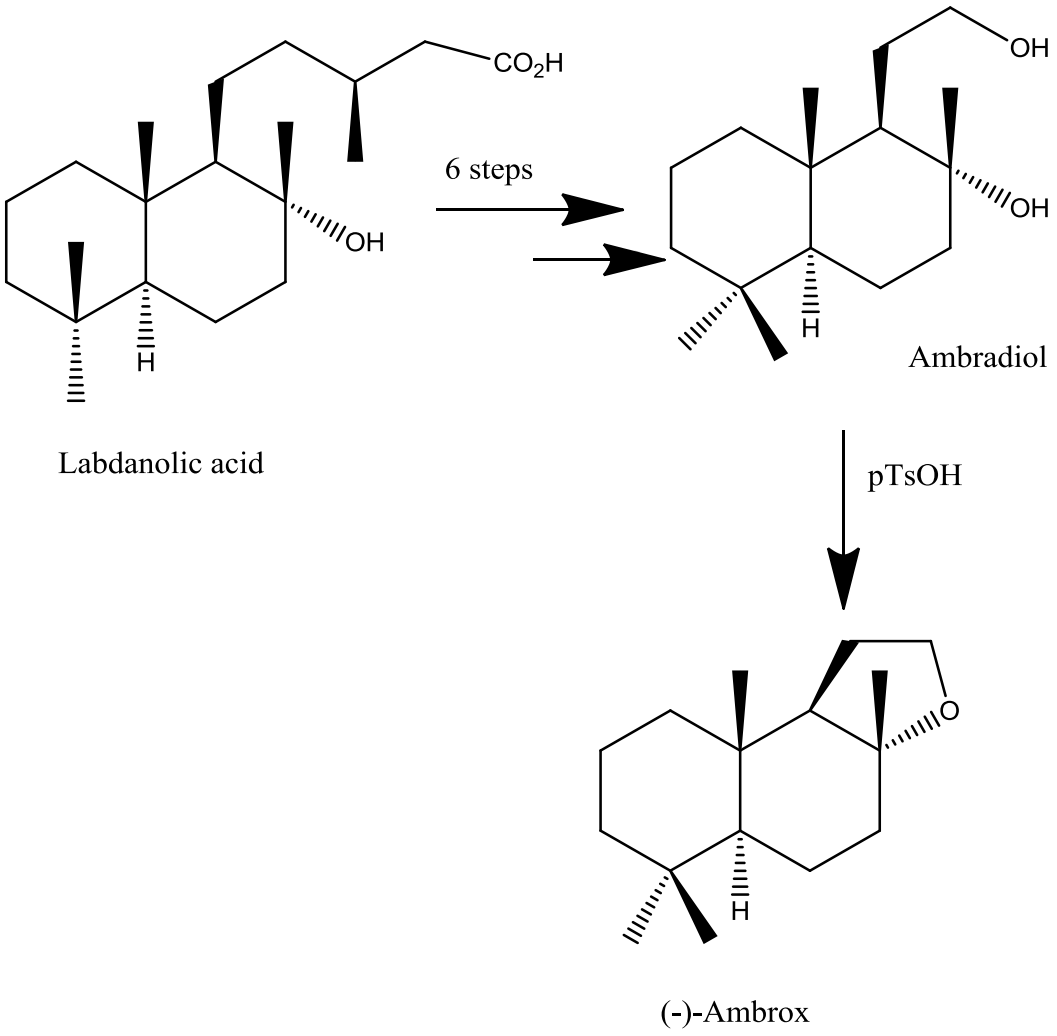


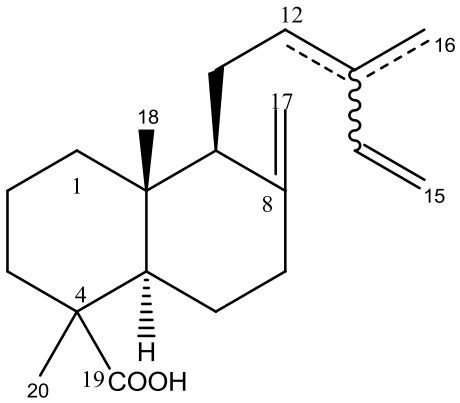
Figure 10: Schematic representation of synthesis of (-) ambrafuran from labdanolic acid (Allemann and Jenny, 2001)

The iodide compound was treated with tert-Butoxide (tBuOK) in tertrahydrofuran (THF) at 25°C and resulted in methyl ketone which subsequently cyclized to yield sclareol oxide. The sclareol oxide was then reported to have undergone ozonolysis to remove the enol ether which led to the formation of an aldehyde and acetate group compound which was later reduced in a solution mixture of methanol and dichloromethane at -78°C to yield ambradiol

with 60% purity. Ambradiol was treated with *p*-toluenesulfonic acid in nitromethane at room temperature for a period of 3 hours and resulted into 87% purity of (-) ambrafuran (Bolster *et al*., 2001). Starting with 10 g of crude labdanolic acid, a yield of only 0.08 g (-) ambrafuran was achieved Bolster *et al*., (2001).

2.9.1.2 Ambrafuran production starting from communic acids

Communic acids are labdane diterpenes mostly obtained in different species of the *Cupresaceae* family (Allemann and Jenny, 2001). The *trans* and *cis*-communate are communic acids reported to be the most commonly used compounds for the synthesis of different kinds of perfumery fixatives including ambrafuran. These communic acids are isolated from the *Juniperus sabina L* of the genus *Juniperus* via hexane extraction and later crystallized for preparation of perfume fixatives (Barrero *et al*., 1993). As indicated in Figure 11, communic acids contain a carboxylic group at C19, an exocyclic methylene at C8- C17 with a dienic side chain system which is considered to be instrumental for production of perfume fixatives (Barrero *et al*., 2012).



Trans and *Cis*-Communic acids

Figure 11: The structure of communic acids (Barrero *et al*., 1993)

It was reported that ambrafuran was produced in two different approaches, either from methyl-trans-communate and methyl-cis-communate or their mixture as shown in Figure 12 below (Barrero *et al*., 1993). The first approach described the transformation of the communic acids (trans/cis-communate) to an aldehyde compound (compound A) in Figure 12 by either ozonolysis at -78ºC using trioxygen (O_3) in dichloromethane (CH_2Cl_2) or by

hydrogenation at 0ºC with diimide which resulted in degradation of C12-C13 by Osmium tetroxide (OsO_4)/Sodium periodate ($NaIO_4$) for a period of 2.5 days.

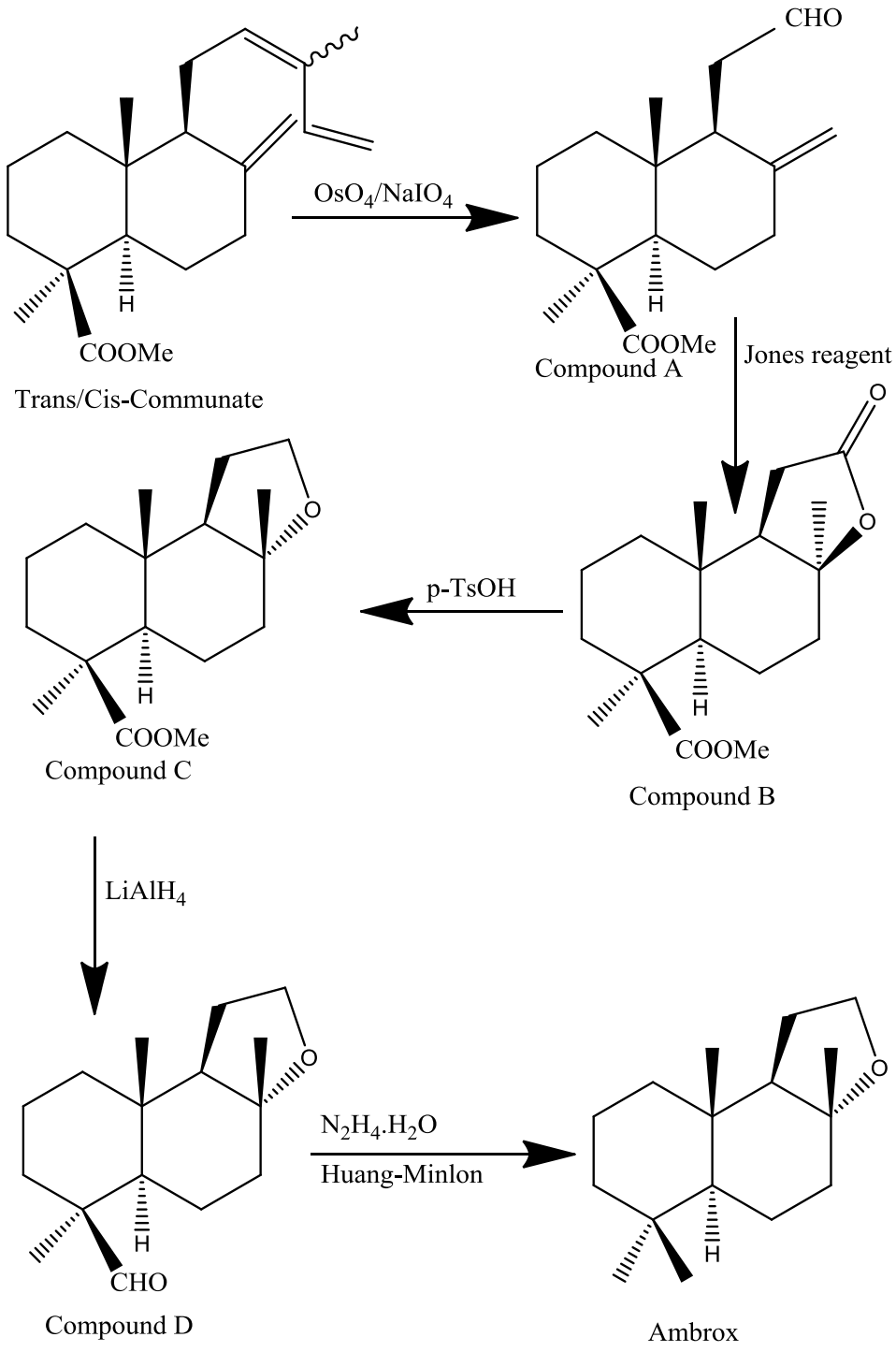


Figure 12: A schematic representation for ambrafuran production from communic acids through a chemical route (Barrero *et al*., 1993)

The aldehyde compound was later being oxidised by acetone/Jones reagent at 0ºC and cyclized with *p*-toluenesulphonic acid (p-TsOH) in toluene by refluxing for an hour which resulted into the formation of γ-lactone compound (Compound B) in Figure 12. This underwent a reduction by lithium aluminium hydride ($LiAlH_4$) in tertrahydrofuran (THF) and proceeded for an hour at 25ºC to produce a tetrahydrofurane derivative (Compound C) in Figure 12. It is reported that ambrafuran was produced in 3 steps from the tetrahydrofurane derivative in which another aldehyde compound formed (Compound D) as shown in Figure 12 was treated with tetrazene-water ($N_4H_4.H_2O$) in potassium hydroxide (KOH) or Huang-Minlon conditions and refluxed for an hour (Barrero *et al*., 1993).

The second approach involved the direct conversion of the trans/cis-communate into a diol compound by ozonolysis with O_3 in CH_2Cl_2 at -78ºC and cyclized with p-TsOH in CH_3NO_2 to yield an alcohol compound which served as an intermediate of an aldehyde compound (Compound D) in Figure 12 (Barrero *et al*., 2012). It was reported that in this processes, a total yield of 19% ambrafuran was obtained. This process involved the use of hazardous chemicals and takes almost a week to reach to the final product with low yield.

2.9.1.3 Ambrafuran production starting from sclareol

The production of (-) ambrafuran from sclareol is highly investigated in many pharmaceutical industries including cosmetic industries around the world through hemisynthesis (Fráter *et al*., 1998). In most cases, sclareol is reported to have undergone three phases for ambrafuran production which are (a) Oxidative degradation of the side chain of sclareol, (b) The reduction process to yield ambradiol, and lastly (c) The cyclodehydration of ambradiol to form (-) ambrafuran (Allemann and Jenny, 2001). 13,14,15,16-Tetranorlabdane-diol which is well known as ambradiol is reported to be the easiest compound to be cyclized and an important intermediate for ambrafuran production in the final step (Moulines *et al*., 2001).

In 1987, Decorzant and co-workers invented a two-step process for the transformation of sclareol to (-) ambrafuran shown in the Figure 13 below. The side chain degradation of sclareol was reported to have being carried out using chromium trioxide (CrO_3) that led to the formation of sclareolide. The two-step transformation started from the reduction of sclareolide by ($LiAlH_4$) to form ambradiol which was later cyclized using β-naphthtalenesulfonic acid and resulted into ambrafuran formation with a purity of 50% (Decorzant *et al*., 1987).

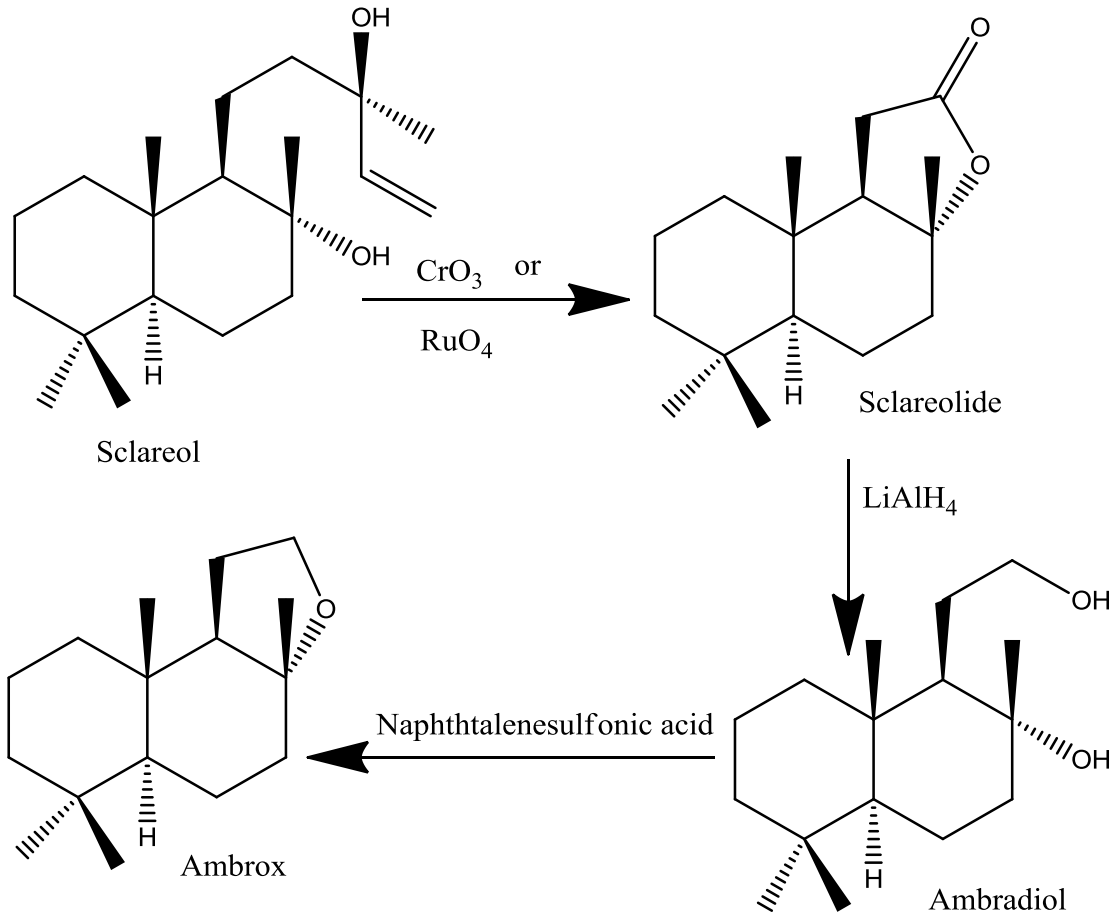


Figure 13: A schematic representation of ambrafuran production starting from sclareol using chemical pathways (Decorzant *et al.*, 1987)

Ruthenium oxide (RuO_4) is reported to have substituted chromium trioxide (CrO_3) in the degradation step of the side chain of sclareol and resulted in an increase in purity of (-) ambrafuran by 30% (Martres *et al.*, 1993). Many different chemicals have been used for oxidation of sclareol, such as chromic acid which is reported to be problematic for use in industrial processes. Aluminium lithiumhydride has been proven to be unsuitable for use in industry because it is highly flammable (Kawanobe and Kogami, 1985). In many cases, chemical production of ambrafuran leads to a racemic form rather than the (-) enantiomer (Carey, 2013a). Therefore, the use of these hazardous chemicals in industry is not feasible due to lack of enantiospecificity and green processes are being proposed which are considered to be environmentally friendly (Steenkamp and Taka, 2010).

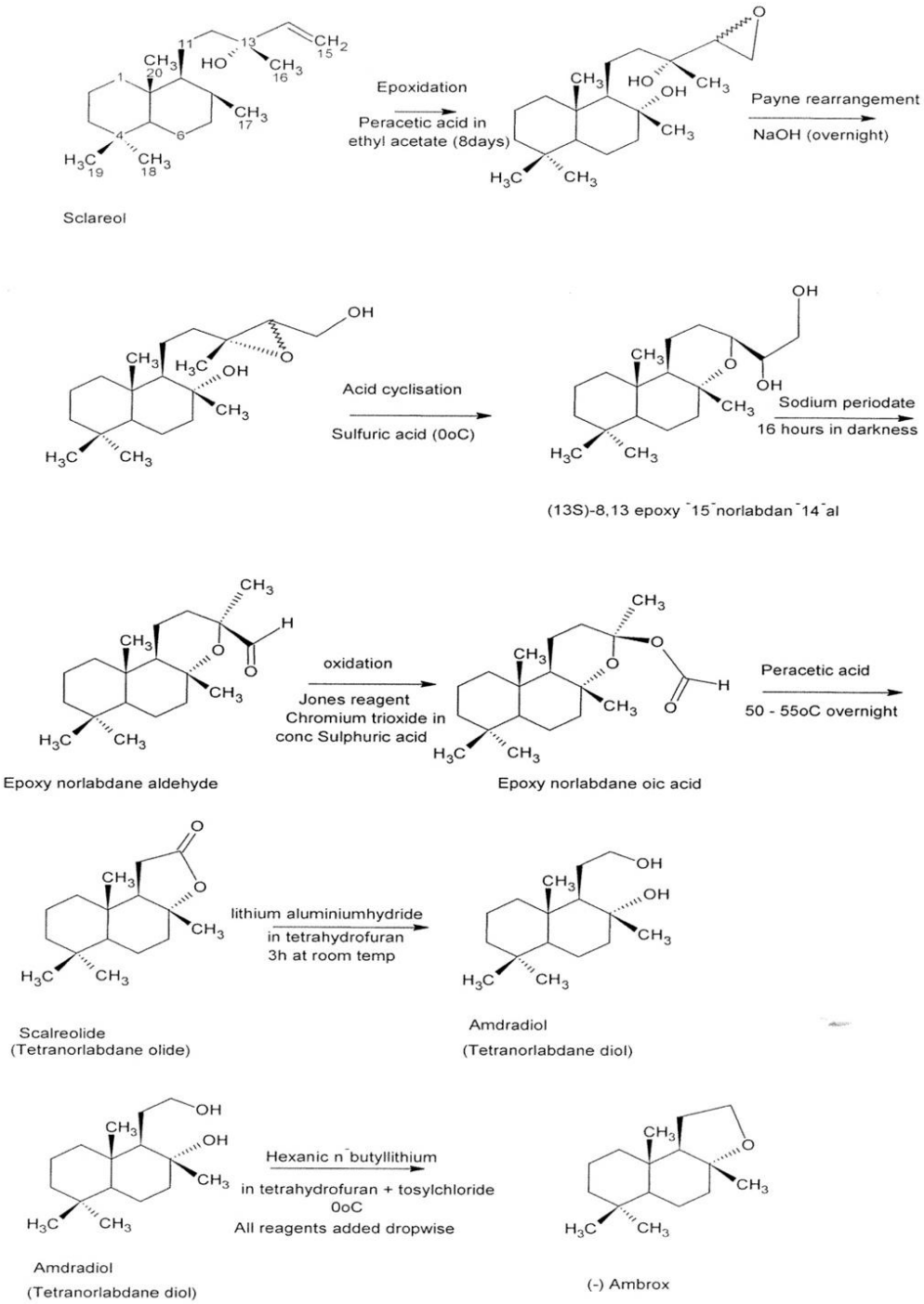


Figure 14: Shows the chemical pathway process for commercial production of ambrafuran (Steenkamp and Taka, 2010)

Eight steps are required for production of (-) ambrafuran in industry starting from sclareol as shown in Figure 14 above. In this process, sclareol was oxidized for 8 days via paracetic acid in ethyl acetate in the first step and the process is known as epoxidation. The process is

explained above in chemical syntheses of ambradiol section 2.7. The final step defines how ambradiol was converted to (-) ambrafuran. Ambradiol (10.1 g) dissolved in 100 mL dry tetrahydrofuran and the solution was stirred at 0˚C under nitrogen. A drop wise solution of 1.6 M hexane *n*-butyllithium (26 mL) was added into a stirring solution (ambradiol & tetrahydrofuran) for 15 mins. Tosylchloride (7.6 g) was added and dissolved into the solution and cooled at 0˚C for further 15 mins. Another 1.6 M hexanic *n*-butyllithium (26 mL) solution was added in a drop wise manner while stirring for another 15 mins. A white precipitate was reported to have formed during the addition of 1.6 M hexanic *n*-butyllithium for the third time and the reaction was left to stir for 2 hours at room temperature. It was further reported that the residual material after evaporation of tetrahydrofuran was partitioned between 100 mL water and ethyl acetate. Brine was used to wash the ethyl acetate and dried on sodium sulfate anhydrous. The yield of (-) ambrafuran was reported to be 96% and 9 g white crystalline found after evaporation of ethyl acetate. The (-) ambrafuran elements were confirmed by NMR spectroscopy (Moulines *et al*., 2001). This commercial process takes long to reach to the final product and uses harsh chemicals along the way.

The use of microorganisms such as *Hyphozyma roseoniger* and *Bensigtonia ciliata* for preparation of ambradiol from sclareol was reported in 1989 and it is considered to be a green process (Farbood and Willis, 1989) due to the use of a natural microorganism as explained in section 2.8. Ambradiol is a good precursor for production of ambrafuran. (Steenkamp and Taka, 2010) further invented a two-step process for producing ambrafuran starting from sclareol as indicated in Figure 15 below.

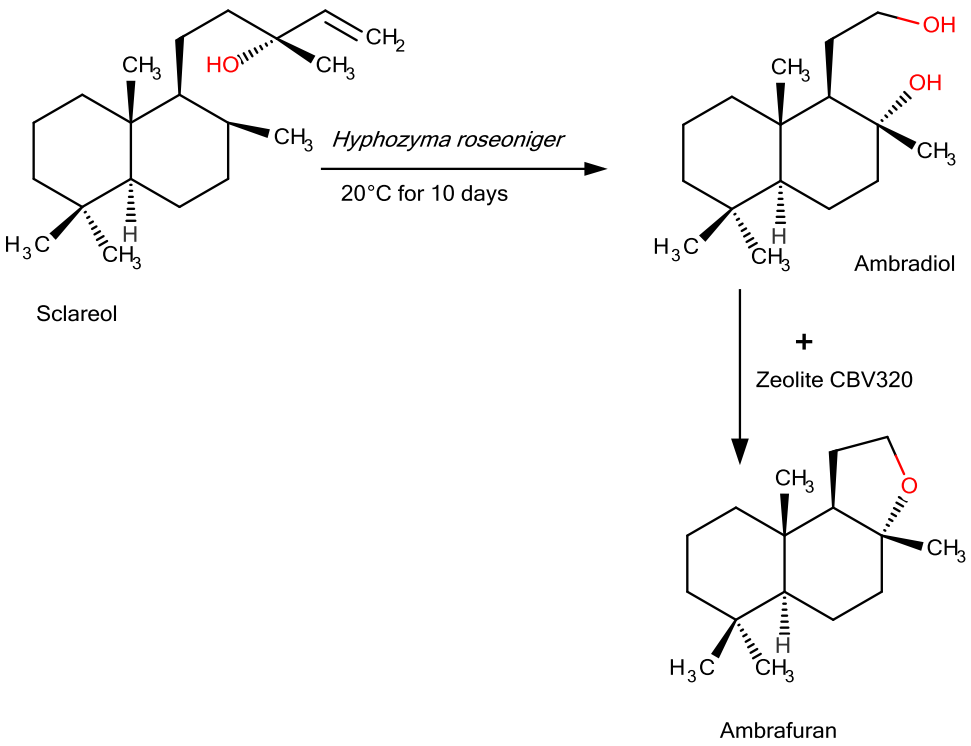


Figure 15: A schematic representation of ambrafuran production from sclareol using zeolites (Steenkamp and Taka, 2010)

The microorganism *Hyphozyma roseoniger* was used in the first step to transform sclareol into ambradiol which was later transformed to (-) ambrafuran using the zeolite CBV320 in hexane or toluene through a cyclization procedure as indicated in Figure 15. The zeolite CBV320 was heated in a conventional microwave at 500 W for activation. The reaction was carried out in an orbital shaker incubator at 25˚C for 6-24 hours with a ratio of 1:6 (5 mg diol:30 mg zeolite CBV320) to 1:9 (5 mg diol:45 mg zeolite CBV320) ambradiol per zeolite in 1-20 mL of the solvent (Hexane) and the yield of 96-98% (-) ambrafuran was reported to have been achieved after the removal of zeolites via filtration and removing the solvent under reduced pressure (Steenkamp and Taka, 2010). Although it is considered as a green process, the problem with this method was that it involved the use of high quantities of zeolites and the large volume of the solvent. The activation of the zeolite which takes place in a conventional microwave is also a problem because at larger scale it will require a special microwave unit. Our study will focus on reducing the amount of zeolites by testing its recyclability and efficiency. Solvent reduction will also be considered.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

Orbital shaker incubator was from New Brunswick Scientific (USA). Allegra™ X-22R Centrifuge with a SX4250 rotor and an AvantiTM J-25I centrifuge with a JA-14 rotor were from Beckman Coulter™ Inc. (California, United States), Heraeus Pico 17 Centrifuge was from Thermo-Scientific. DU® 800 Spectrophotometer was from Beckman Coulter™ Inc. Weighing balance BP 2100S from Sartorius and WAS220/X from RADWAG®. Gas chromatography 6890N Network system equipped with 7683B Series Injector was from Agilent Technologies. Gas chromatography 5890 Series II GC System was from Hewlett Packard HP. 2 mL Crimp Neck Vials, 32x12 mm-clear glass and Syringe Filters-Clarinert™ were purchased from Stargate Scientific. Restek Rtx-Sil MS W/Integra Guard 30 m x 0.25 mm id x 0.25 µm df s/n 689000 GC column was purchased from Restek and was used for analysis in this study. Olympus BX40 Microscope coupled with CC12 Soft Imaging system, Japan.

3.1.2 Reagents

Hexane A.R, Heptane A.R, Ethyl Acetate A.R, and Toluene A.R were purchased from RADCHEM Laboratory suppliers. Diethyl Ether, Propan-2-ol HPLC, Decane, Dimethylsulphoxide, and Methanol were purchased from Lab Scan Analytical Science. Dichloromethane, Propane-1,2-diol, Chloroform, Tween 80, Ammonium nitrate, Magnesium Sulphate Anhydrous, Magnesium Sulphate Heptahydrate, D(+) Glucose Anhydrous, tert-Butanol, and Yeast Extract Powder were from Merck Chemicals. 1,2-Butanediol was purchased from SA fine chemicals (SAFC). Tetrahydrofuran (THF) was purchased from Fluka Chemika. Kaiser Alumina A-2 was from Harshaw Chemie BV. Potato Dextrose Broth (PDB) was purchased from Scharlan Microbiology. Difco™ Yeast Nitrogen Base without Amino Acids was supplied by Labretoria. Sclareol (Natural) was from Ventos SA (ERNESTO). *Hyphozyma roseoniger* was purchased from CBS. Zeolites CBV320, CFG-1, ZD0614, CBV500-X16, CBV8062, CBV100 and Alumina were all kind gifts from Zeolyst

International. Water generated from a Systec VX-95 and Millipore Elix UV 5 was from Merk.

3.2 Methods

The following methods are the basis to that of the standard developed methods from CSIR Biosciences. The method developed at CSIR formed the basis for all the methods below. All experiments were done in triplicate except where it is stated otherwise

3.2.1 Analytical methods

Gas chromatography-Flame Ionization Detection (FID) technique was used for analysing samples in the majority of this work. In this study we chose Gas Chromatography (GC) analysis due to its intrinsic advantages of being attractive for the characterization and quantitative of terpenes mixtures as investigated by (Zubyk and Conner, 1960).

3.2.1.1 Quantitative method for analysis of sclareol, intermediate ambradiol and ambrafuran

The following method is a non-chiral gas chromatography method which was used in this study to detect and separate sclareol, intermediate ambradiol and the final product (-) ambrafuran:

Temperature gradient: 180˚C-300˚C

Injection temperature: 270˚C

Detection temperature (FID): 310˚C

Rate: 15˚C/min

Head pressure: 15 psi

Split flow: 82.2 mL

Septum purge: 2.63 mL/min

Injection volume: 1 µL

3.2.1.2 LC-MS method used to confirm the identity of the compounds

The following Liquid Chromatography-Mass Spectroscopy (LC-MS) method was used for confirmation of the characteristics of ambradiol and (-) ambrafuran in this study. A Waters Ultra Performance Liquid Chromatogrpahy UPLC coupled in tandem to a Waters photodiode array (PDA) detector and a SYNAPT G1 HDMS high definition mass spectrometer was used to separate the compounds and generate accurate mass data.

Chromatographic separation of the reaction mixture was done utilising a Waters BEH C8 column (150 mm x 2.1 mm, 1.8 µm) thermostatted at 60℃. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (natural pH of 2.3) and methanol (Eluent B) containing 10 mM formic acid. The initial conditions were 30% A at a flow rate of 0.4 mL/min for 2 mins, followed by a gradient (Curve 8) to 5% A at 6 mins. The conditions were kept constant for 1 min and then changed to the initial conditions. The runtime was 10 mins and the injection volume was 1 -3 µL depending on the concentration of the compounds of interest. The PDA detector was scanned between 200 nm and 500 nm (1.2 nm resolution) and collecting 20 spectra per second.

The SYNAPT G1 mass spectrometer was used in V-optics and operated in electrospray mode to detect the compounds of interest. Leucine enkephalin (50 pg/mL) was used as reference calibrant to obtain typical mass accuracies between 1 and 3 mDalton. The mass spectrometer was operated in positive ionisation mode with a capillary voltage of 3.5 kV, the sampling cone at 20 V and the extraction cone at 4 V. The scan time was 0.2 s covering the 100 to 800 Dalton mass range. The source temperature was 120℃ and the desolvation temperature was set at 450℃. Nitrogen gas was used as the nebulisation gas at a flow rate of 550 L/h. Mass accuracy was obtained using leucine encephalin as lockmass calibrant (556.2771 Da) at a flow rate of 0.1 mL/min to obtain a typical mass accuracy of 3 – 5 mDa. The software used to control the hyphenated system and do all data manipulation was MassLynx 4.1 (SCN 704).

3.3 Optimization processes for the production of intermediate ambradiol from sclareol by *H. roseoniger*

3.3.1 Cell banking

Cultivation of the biocatalytic microorganism, *H. roseoniger*, was conducted in 2010 at CSIR and the cells were placed into 2 mL cryovials as the master cell bank. The master cell banks were frozen at -80˚C for future use. The working cell banks were made into 1 mL cryovials from the master cell bank and were frozen at -80˚C. All experiments for the conversion of sclareol to ambradiol in this study were carried out using the working cell bank generated from the master cell bank as explained below.

Potato dextrose broth (PDB) (0.53 g) was weighed into 250 mL Erlenmeyer flask and 25 mL dH_2O was added. The media was autoclaved at 121˚C for 15 mins and cooled. *H. roseoniger* (1 mL) from a cryovial from the Master cell bank was added and placed into an orbital shaker incubator at 25˚C for 3 days and agitation at 180 rpm. Yeast extract (0.2 g) and 0.4 g peptone were weighed into 250 mL Erlenmeyer flask and 19 mL dH_2O was added. The media was autoclaved at 121˚C for 15 mins and cooled. Glucose (1 mL) was added into the media to make up 20 mL. *H. roseoniger* (5 mL) from PDB was inoculated into the media and placed in an orbital shaker incubator at 25˚ for 3 days with agitation at 180 rpm. The cells were transferred into a sterile 50 mL Falcon tube and centrifuged at 4500 rpm for 20 mins. The supernatant was discarded and the cells were re-suspended in a minimal volume (20 mL) of 100 mM potassium phosphate buffer pH 6.5. The cell suspensions were mixed with an equal volume (20 mL) of 50% glycerol (v/v) and 1 mL of the mixture was each transferred into the cryovials and placed in a freezer at -80˚C as a working cell bank.

3.3.2 Determining the growth of *H. roseoniger*

The growth of *H. roseoniger* was determined for 72 hours in this experiment. The pre-inoculums were from a working cell bank 1 mL cryovial and inoculated into 100 mL PDB media. The experiment was conducted in triplicate as follows:

PDB (2.65 g) was weighed into 1 L Erlenmeyer flask and 100 mL dH_2O was added. The media was autoclaved at 121˚C for 15 mins and allowed to cool and incubated into an orbital shaker for 72 hours at 25°C and agitation at 180 rpm. Samples (1 mL) were collected twice a day into an Eppendorf tube at 0, 8, 32, 48, 56 and 72 hours. The optical density was measured by UV/Visible spectroscopy in 1 mL cuvettes at 600 nm.

3.3.3 Examination for conversion of sclareol to ambradiol using *H. roseoniger* in pre-inoculums of 24, 48 and 72 hours at 25˚C

The conversion of sclareol to ambradiol using *H. roseoniger* pre-inoculums of 24, 48 and 72 hours at 25˚C was measured. The PDB media from Pronadisa was used according to the manufacturer's instructions (26.5 g potato dextrose in 1 L dH_2O). *H. roseoniger* pre-inoculums were from 1 mL cryovials and were inoculated into 100 mL sterile (2.65 g) PDB media and incubated at 25˚C for 24, 48 and 72 hours respectively. The cells were induced with a substrate (sclareol) for 3 days in all reactions. The following method details the biotransformation of sclareol to ambradiol in all the pre-inoculums:

PDB (2.65 g) was weighed into 1 L Erlenmeyer flask and 100 mL dH_2O was added. The media was sterilized by autoclave at 121˚C for 15 mins and allowed to cool. Sclareol (20 mg) was added and incubated into an orbital shaker incubator at 25˚C and agitation at 180 rpm. Sclareol (1 g) and 1 mL Tween 80 were added into each media for 24, 48 and 72 hours respectively and incubated in an orbital shaker incubator at 25˚C and agitation at 180 rpm for 7 days. Sample (1 mL) was taken into Eppendorf tubes in day 7 and mixed with 1 mL ethyl acetate. The mixture was centrifuged at 13000 rpm for 2 mins. The upper layers were collected into vials and analysed by GC.

3.3.4 An investigation for ambradiol production using *H. roseoniger* from 24, 48 and 72 hours pre-inoculums at 25˚C

The same PDB media used in 3.3.3 was also used in this experiment. *H. roseoniger* from 1 mL cryovials was pre-inoculated for 24, 48 and 72 hours in PDB media and 5 mL *H. roseoniger* from each pre-inoculum was transferred into 100 mL fresh sterile media. The cells were induced with a substrate (sclareol) for 3 days. The following process entails a full description of how this experiment was performed:

PDB (2.65 g) was weighed into 1 L Erlenmeyer flask and 100 mL dH_2O was added. The media was sterilized by autoclave at 121˚C for 15 mins and allowed to cool. *H. roseoniger* (5 mL) from 24, 48 and 72 hours pre-inoculums was inoculated into the media. Sclareol (20 mg) was added and incubated in an orbital shaker incubator at 25˚C and agitation at 180 rpm for 3 days. Sclareol (1 g) and 1 mL Tween 80 were added and incubated in an orbital shaker incubator at 25˚C and agitation at 180 rpm for 7 days. Sample (1 mL) was taken into

Eppendoff tubes in day 7 and mixed with 1 mL ethyl acetate. The mixture was centrifuged at 13000 rpm for 2 mins. The upper layer was collected into the vial and analysed by GC.

3.3.5 Testing different media for conversion of sclareol to ambradiol

Different media were tested for conversion of sclareol to form intermediate ambradiol using *H. roseoniger* at 20˚C for 10 days. Experiments were done in triplicate. The *H. roseoniger* (1 mL) was inoculated into 20 mL potato dextrose broth media. The cultures were grown for 3 days at 25˚C and agitation at 180 rpm. The microorganism from this culture was then transferred into each media to be tested for conversion of sclareol. The following media were tested for production of ambradiol: (i) 0.1% NH_4NO_3 (w/v), 0.1% KH_2PO_4 (w/v), 0.05% $MgSO_4.7H_2O$ (w/v) and Yeast extract; (ii) 0.1% NH_4NO_3 (w/v), 0.1% KH_2PO_4 (w/v), 0.05% $MgSO_4.7H_2O$ (w/v), 0.5% (D+) Glucose (w/v) and Yeast extract; and (iii) Difco™ Yeast nitrogen base without amino acids with 0.1% NH_4NO_3 (w/v). Media were prepared as follows:

i. Media number 1

NH_4NO_3 0.1 g, 0.1 g KH_2PO_4, 0.05 g $MgSO_4.7H_2O$ and 0.2 g Yeast extract were weighed into 1 L Erlenmeyer flasks and 95 mL dH_2O was added into each. The media was autoclaved at 121˚C for 20 mins.

ii. Media number 2

NH_4NO_3 0.1 g, 0.1 g KH_2PO_4, 0.05 g $MgSO_4.7H_2O$, and 0.2 g Yeast extract were weighed into 1L Erlenmeyer flasks and 95 mL dH_2O was added into each. The media was then autoclaved at 121˚C for 20 mins. A mass of 0.5 g dextrose in 100 mL dH_2O was autoclaved separately and added into the media.

iii. Media number 3

NH_4NO_3 (0.1 g) was weighed into 1 L Erlenmeyer flasks and 100 mL dH_2O was added to each. The flasks were autoclaved for 15 mins at 121˚C and allowed to cool. Difco™ Nitrogen base without amino acids (5 mL) was filter sterilised into each flask.

3.3.5.1 Procedure for sclareol conversion

H. roseoniger (5 mL) grown as described above, was inoculated into each media in duplicate, 1 g sclareol and 1 mL Tween 80 were added into each. The flasks were incubated for 10 days at 20˚C and agitation at 180 rpm. Sampling was conducted on days 1, 3, 7 and 10 by taking 500 µL of each sample and mixing with 500 µL ethyl acetate into Eppendorf tubes. The mixtures were centrifuged at 13000 rpm for 2 mins and the supernatants were collected into vials and analysed by Gas chromatography.

3.3.6 Recrystallization of ambradiol

An experiment for ambradiol production was done in NH_4NO_3 0.1 g, 0.1 g KH_2PO_4, 0.05 g $MgSO_4.7H_2O$, 0.5 g Dextrose and 0.2 g Yeast extract (media 2) as explained in the previous experiment in 3.3.5. Ethyl acetate was added into the media (100 mL media per 100 mL ethyl acetate) and dried in $MgSO_4$ anhydrous. The solvent was evaporated using a "rota-vapour" instrument and the mass of crude extracts was determined. Crude extracts (5 mg) was weighed into the vial and 1 mL ethyl acetate was added. The sample was analysed by GC. This experiment was carried out in triplicate. The crude extracts were combined and recrystallized as explained in the method below:

Crude extracts (2.28 g) were dissolved in 20 mL ethyl acetate in 250 mL Erlenmeyer flask and boiled for 3-5 mins and allowed to warm. Hexane 500 µL was gently pipetted into the solution and allowed to cool at room temperature. The solution was placed in a cold room for overnight to allow the formation of ambradiol crystals. The solvent was decanted into another 250 mL Erlenmeyer flask and placed in a cold room at 4˚C for further crystallization for overnight. The crystals was washed with 2 mL ethyl acetate and suspended into a glass plate and placed in a fume hood for overnight. The final amount of crystals was determined. The crystals (5 mg) were weighed into vial and 1 mL ethyl acetate was added. The sample was analysed by GC.

3.3.7 Determining cell biomass of *H. roseoniger* in different media and different conditions for biotransformation of sclareol to ambradiol

After testing different media for the production of ambradiol the cell biomass of the microorganism from different media and harvesting at various conditions were determined. The growth of *H. roseoniger* was carried out for 3 days at different temperatures using the

different media. In this experiment, *H. roseoniger* cells were harvested from the following media: (a) Potato dextrose broth (PDB), (b) Difco™ yeast nitrogen base without amino acids, (c) Difco™ yeast nitrogen base without amino acids with 1% glucose (w/v), and (d) a media composed of 0.2% yeast extract (w/v), 1% glucose (w/v), 0.2% NH_4NO_3 (w/v), 0.1% KH_2PO_4 (w/v) and 0.1% $MgSO_4.7H_2O$ (w/v). 40g (D+)-Glucose was weighed into 100 mL Schott bottle and 80 mL dH_2O was added to make up a 50% glucose (w/v) solution. The glucose solution was sterilised for 20 mins at 121°C in an autoclave machine. Glucose was taken from the 50% glucose solution for media that required (1%) glucose and added aseptically. The *H. roseoniger* from the cryovials was initially grown in potato dextrose broth (PDB) for 3 days at 25˚C and agitation at 180 rpm. Then each media (100 mL) was inoculated with 5 mL *H. roseoniger* grown in the potato dextrose broth. Substrate 20 mg (sclareol) was used to induce the cells before harvesting. The original patent by Farbood and Willis, (1989) stated that harvested cells may be used for conversion and this was one of the parameters that needed to be tested. The following temperatures were used for the growth of *H. roseoniger* on each of the media above; 20˚C, 25˚C, 28˚C & 30˚C respectively. The conversion of sclareol to ambradiol was conducted in Difco™ yeast nitrogen base without amino acids or 1 mM sodium phosphate buffer pH 7.2 at 25˚C for 7 days. Both sodium phosphate buffer and Difco™ yeast nitrogen base were prepared as 1 L each. Difco™ yeast nitrogen base without amino acids was prepared according to the manufacturer's instructions and entailed using 6.7 g of the Yeast nitrogen base without amino acid in 98 mL distilled water and 2 mL of 50% glucose (w/v). The glucose was sterilised separately. This experiment was also performed with and without cell induction. Reactions were performed in 1 mM sodium phosphate buffer or in Difco™ yeast nitrogen base without amino acids as follows:

H. roseoniger cells from the media mentioned above at different temperatures were divided into 2 sets of 10 mL fractions in sterile Falcon tubes. The cells were centrifuged at 4500 rpm for 20 min. The supernatants were discarded and the cells were re-suspended into either 50 mL Difco™ yeast nitrogen base without amino acids or the other set in 50 mL sodium phosphate buffer pH 7.2. Sclareol (1 g) and 1 mL Tween 80 were added into each in a 250 mL Erlenmeyer flask. The flasks were incubated in an orbital shaker at 25˚C and agitation at 180 rpm for 7 days. Samples (1 mL) from each flask were taken into Eppendorf tubes on day 1, 3, 5 and 7 respectively and mixed with 1 mL ethyl acetate. The mixtures were centrifuged at 13000 rpm for 2 mins. The supernatants were placed into vials and analysed by GC.

3.3.8 Measuring the production of ambradiol in Difco™ yeast nitrogen base without amino acids & potato dextrose broth at 20˚C and 25˚C using *H. roseoniger*

In this experiment, induction and non-induction for conversion of sclareol to ambradiol in both potato dextrose broth and Difco™ yeast nitrogen base without amino acids media were conducted at 20˚C and 25˚C for 7-10 days. As in the previous experiments above (section 3.3.7), microorganism (1 mL) from cryovials was inoculated into 35 mL potato dextrose broth (PDB). The cultures were grown for 3 days at 25°C and agitation at 180 rpm. This pre-inoculum is used as the source of the microorganism to inoculate different media for determining the conversion of sclareol to the intermediate diol. The following experiments were conducted in duplicate following the production of the pre-inoculum:

i. Non-induced reaction for ambradiol production in Difco™ yeast nitrogen base without amino acids (YNBaa) at 20 and 25°C

YNBaa (1.44 g) was weighed into 50 mL glass beaker and 40 mL sterile dH_2O was added. YNBaa (5 mL) was then filter sterilized (0.45 µL filter paper) into sterilized 95 mL dH_2O in 1 L Erlenmeyer flask. *H. roseoniger* (5 mL) was inoculated into the flasks. Sclareol (1 g) and 1 mL Tween 80 were added and incubated in an orbital shaker at 20˚C and agitation at 180 rpm for 10 days. Samples (1 mL) were taken into Eppendorf tubes from day 1-10 and mixed with 1 mL ethyl acetate. The mixture was centrifuged at 13000 rpm for 2 mins. The upper layers were placed into vials and analysed by GC.

ii. Induced reaction for ambradiol production in Difco™ yeast nitrogen base without amino acids (YNBaa) at 20 and 25˚C

YNBaa (1.44 g) was weighed into 50 mL glass beaker and 40 mL sterile dH_2O was added. YNBaa (5 mL) was then filter sterilized (0.45 µL filter paper) into sterilized 95 mL dH_2O in 1 L Erlenmeyer flask. *H. roseoniger* (5 mL) was inoculated into the flasks. Sclareol (20 mg) was added to each flask and incubated in an orbital shaker at 20˚C and agitation at 180 rpm for 3 days to determine the effect of induction with a low concentration of the substrate. Following the initial 3 day induction, sclareol (1 g) and 1 mL Tween 80 were added and incubated in an orbital shaker at 20˚C and agitation at 180 rpm for 7 days. Samples (1 mL) were taken from day 1-7 and mixed with 1 mL ethyl acetate. The mixture was centrifuged at 13000 rpm for 2 mins. The upper layers were collected into vials and analysed by GC.

iii. Non-induced reaction for ambradiol production in potato dextrose broth (PDB) media at 20 and 25˚C

PDB (2.4 g) was weighed into 1L Erlenmeyer flask and 100 mL dH_2O was added and sterilized by autoclave at 121˚C for 15 mins and allowed to cool. *H. roseoniger* (5 mL) was inoculated into the media. Sclareol (1 g) and 1 mL Tween 80 were added and incubated in an orbital shaker at 20˚C and agitation at 180 rpm for 10 days. Samples (1 mL) were taken from day 1-10 and mixed with 1 mL ethyl acetate. The mixture was centrifuged at 13000 rpm for 2 mins. The upper layers were collected into vials and analysed by GC.

iv. Induced reaction for ambradiol production in potato dextrose broth (PDB) media at 20 and 25˚C

PDB (2.4 g) was weighed into 1 L Erlenmeyer flask and 100 mL dH_2O was added and sterilized by autoclave at 121˚C for 15 mins and allowed to cool. *H. roseoniger* (5 mL) was inoculated into the media. Sclareol (20 mg) was added and incubated in an orbital shaker at 20˚C and agitation at 180 rpm for 3 days. Sclareol (1 g) and 1 mL Tween 80 were added and incubated in an orbital shaker at 20˚C and agitation at 180 rpm for 7 days. Samples (1 mL) were taken into Eppendorf tubes from day 1-7 and mixed with 1 mL ethyl acetate. The mixture was centrifuged at 13000 rpm for 2 mins. The upper layers were collected into vials and analysed by GC.

3.3.8 Testing conversion of ambradiol on a larger scale in potato dextrose broth (PDB) at 25˚C using *H. roseoniger* in an orbital shaker incubator

In this experiment, a reaction for ambradiol production was executed in a 1 L Fernbach flask at a reaction scale of 700 mL. The *H. roseoniger* from 1 mL cryovials was inoculated into a 35 mL sterile (16.8 g) potato dextrose broth for pre-inoculum and incubated at 25˚C for 3 days. The cells were then induced with 140 mg sclareol for 3 days after pre-inoculum in 700 mL potato dextrose broth. The reaction was done in triplicate as follows:

PDB (16.8 g) was weighed into 1 L Fernbach flask and 700 mL dH_2O was added. The media was sterilized by autoclave at 121˚C for 15 min and allowed to cool. *H. roseoniger* (35 mL) was inoculated into the media. Sclareol (140 mg) was added and incubated in an orbital shaker at 20˚C and agitation at 180 rpm for 3 days. Sclareol (7 g) and 7 mL Tween 80 were added and incubated in an orbital shaker at 25˚C and agitation at 180 rpm for 7 days. Samples

(1 mL) were taken into Eppendorf tubes in day 7 and mixed with 1 mL ethyl acetate. The mixture was centrifuged at 13000 rpm for 2 mins. The uppers layer were collected into vials and analysed by GC.

3.3.10 Test for ambradiol production in 2 L fermentation bioreactor using *H. roseoniger*

Conversion of sclareol to ambradiol was examined in fermentation reaction in this test in a 2 L reaction vessel. In this trial, a reaction volume of 1.2 L was used and the *H. roseoniger* pre-inoculum was prepared from 2 x 1 mL cryovials into 60 mL sterile 1.59 g PDB media and grown for 3 days at 25˚C. The pre-inoculum was then transferred into the fermenter reactor containing sterile potato dextrose broth media 31.8 g in 1.2L dH_2O and induced with 240 mg sclareol for 3 days. Following induction, the sclareol was mixed with Tween 80 and transferred into the fermenter for biotransformation to take place. Sclareol (12 g) and 12 mL Tween 80 were mixed and transferred into the fermenter. The reaction took place at 25˚C, agitation at 300 rpm and oxygen level of 0.2% (aeration). Sample (1 mL) was taken into Eppendorf tubes at 24, 31 and 48 hours and mixed with 1 mL ethyl acetate. The mixture was centrifuged at 13000 rpm for 2 minutes. The upper layer was collected into the vial and analysed by GC.

3.4 Optimisation of processes for production of (-) Ambrafuran

The process developed at CSIR for production of ambrafuran by zeolite CBV320 form the basis for the following optimized processes. The CSIR process used a ratio of 1:9 and 1:6 (intermediate ambradiol per zeolite CBV320) as well as high volumes (5-10 mg substrate/1–20 mL) of solvents. The zeolite has to be activated by heating in a microwave before use. In this study optimization of all the parameters involved in the conversion of ambradiol to (-) ambrafuran will be investigated.

3.4.1 Measuring the production of (-) ambrafuran in different ratios using zeolite CBV320

Zeolite CBV320 (1 g) was activated in a conventional microwave at power level of 70 W for 3 mins. The ratios of 1:5, 1:7 and 1:9 (ambradiol per zeolite CBV320) were tested for the conversion of ambradiol to ambrafuran using an orbital shaker incubator at 25˚C. This experiment served as the basis for the upcoming series of optimised experiments. The reaction for each ratio was conducted in triplicate as follows:

Ambradiol (5 mg) and 25 mg (1:5) or 35 mg (1:7) or 45 mg (1:9) of activated zeolite (CBV320) were weighed into McCartney bottles. Hexane (1 mL) was added to each McCartney bottle and they were tightly sealed to prevent evaporation of the solvent. The bottles were placed into an orbital shaker incubator at 25˚C and agitation at 180 rpm for 4 hours. At the end of the reaction, 1 mL of each sample was taken into Eppendorf tubes and centrifuged for 1 min at 13000 rpm. The supernatants were placed into vials and analysed by GC.

3.4.2 Determining the production of (-) ambrafuran in different solvents at 1:9 ratio using zeolite CBV320

In this experiment, different organic solvents were tested for the production of (-) ambrafuran in a ratio of 1:9 (ambradiol per zeolite). The zeolite (1g) was activated in a conventional microwave as previously described in 3.4.1. The purpose of this experiment was to examine solvents that can be used during the conversion of ambradiol to (-) ambrafuran and hexane was used as the reference solvent. The solvents tested included; Ethyl acetate, Hexane, Chloroform, Dichloromethane (DCM), 1,2-Butanediol, 1,2-Propanediol, t-Butanol, Isopropanol, Heptane, Toluene, Decane and Dimethylsulphoxide (DMSO). The experiment was conducted using all solvents mentioned above in separate reactions. The experiment was conducted in duplicate for each solvent at 25˚C as follows:

Ambradiol (5 mg) and 45 mg zeolite CBV320 were weighed into McCartney bottles; 1 mL of each solvent mentioned above was added. The bottles were placed into an orbital shaker incubator at 25˚C and agitation at 180 rpm for 4 hours. Sample (1 mL) from each reaction was taken into Eppendorf tubes following the 4 hour incubation reaction period and centrifuged for 1 min at 13000 rpm. The supernatants were collected into vials and analysed by GC.

3.4.3 Different zeolites for the determination of (-) ambrafuran production

Zeolites such as CBV500-X16, CBV8062, CBV100, CBV320 and an adsorbent, Alumina (1 g each) were all activated at 70 W power level in a conventional microwave before use for 3 mins. The solvent used for this set of reactions was hexane. The reactions were done in duplicate for each zeolite in a ratio of 1:5 at 25˚C temperature.

Ambradiol (20 mg) and 100 mg of each activated zeolites were weighed into McCartney bottles. Hexane (2 mL) was added into each bottle and tightly closed. The bottles were placed into an orbital shaker incubator at 25˚C and an agitation speed of 180 rpm for 6 hours. After the reaction, 1 mL of each sample was taken into Eppendorf tubes and centrifuged for 1 min at 13000 rpm. The supernatants were placed into vials and analysed using GC.

3.4.4 Evaluation of (-) ambrafuran production in a water bath sonicator, magnetic stirrer plate and orbital shaker incubator

The ratio was reduced in this experiment from 1:5, 1:7 and 1:9 to 1:3 ambradiol per zeolite. A water bath sonicator, magnetic stirrer plate and orbital shaker incubator instruments were used in this experiment for the reaction of ambradiol and zeolite CBV320 to produce ambrafuran. The CBV320 zeolite was initially activated in a conventional microwave at power level of 70 W for 3 mins. Reactions in an orbital shaker incubator and on magnetic stirrer plate were conducted at 25˚C. The reactions were executed in triplicate from each instrument mentioned above.

Ambradiol (5 mg) and 15 mg zeolite CBV320 were weighed into McCartney bottles and 1 mL hexane was added into each. The samples were placed either in an orbital shaker incubator or on a magnetic stirrer for 4 hours at 25˚C or in the sonicator water bath for 3 hours. Samples (1 mL) from each reaction were taken into Eppendorf tubes and centrifuged for 1 min at 13000 rpm. The supernatants were placed into vials and analysed by GC.

3.4.5 Measuring the production of (-) ambrafuran in a water bath sonicator using new zeolites

Two new zeolites with the identity of CFG-1 and ZD0614 received as a gift from Zeolyst International were tested for the production of (-) ambrafuran. The experiment was carried out in a water bath sonicator and new zeolites used were in an activated and non-activated form. The zeolites were activated by heating in a conventional microwave at 70 W for 3 min. Ratios of 1:3 and 1:6 were used in this experiment. The experiments were performed in duplicate for each ratio as follows.

Ambradiol (15 mg) and 45 mg activated zeolite CFG-1 (1:3) or 90 mg (1:6) or activated zeolite ZD06014 were weighed into McCartney bottles and 3 mL hexane was added into each. This was repeated for non-activated CFG-1 zeolite or zeolite ZD06014. The bottles

were sealed and placed in a water bath sonicator for 3.5 hours. Room temperature water was added into the sonicator in every hour to control the temperature. After 3.5 hours, 1 mL of each sample was collected into Eppendorf tubes and centrifuge at 13000 rpm for 1 min. The supernatants were placed into vials and analysed by GC.

3.4.6 Conversion of ambradiol to (-) ambrafuran at different temperature into an orbital shaker

Different temperatures of 50, 60 and 70˚C were tested for the conversion of intermediate ambradiol to ambrafuran into an orbital shaker incubator. Zeolites CFG-1, ZD06014 and CBV320 were used in this experiment. Zeolites CFG-1 and ZD0614 were not activated whereas CBV320 was activated in a conventional microwave at 70 W for 3 min. The 1:3 ratio was used and the solvent volume was reduced to 1 mL as from 3 mL used in 3.4.5. Reactions were conducted in triplicate for each zeolite.

Ambradiol (5 mg) and 15 mg zeolite CFG-1, or ZD0614 or CBV320 were weighed into McCartney bottles and 1 mL hexane was added into each. The bottles were sealed and placed into an orbital shaker incubator at 50˚C for 7 hours and agitation at 180 rpm. The same protocol was repeated for both 60˚C and 70˚C. Sample (1 mL) was taken into Eppendorf tubes at the end of the reaction and centrifuged for 2 mins at 13000 rpm. The supernatants were placed into vials and analysed by GC.

3.4.7 Determination of (-) ambrafuran production at low ratios using zeolites CFG-1, ZD0614 and CBV320 at 60˚C in orbital shaker incubator

Ratios of 1:1 and 1:2 were determined for (-) ambrafuran production in this experiment. Zeolite CBV320 required activation by heating in a microwave at 70 W power level before use while the other two zeolites (CFG-1 and ZD0614) were used without being activated.

Ambradiol (10 mg) and 10 mg zeolite (1:1) or 20 mg zeolite (1:2) CFG-1, ZD0614 or CBV320 were weighed into McCartney bottles and 1 mL hexane was added into each. The bottles were sealed and placed into an orbital shaker incubator at 60˚C and agitation at 180 rpm for 6 hours. Hexane (1 mL) was added into each sample after reaction to reconstitute and taken into Eppendorf tubes. The samples were centrifuged for 2 mins at 13000 rpm. The supernatants were collected into vials and analysed by GC.

3.4.8 Measuring the increased concentration of ambradiol for (-) ambrafuran production

Zeolite CFG-1 was used without activation as described in previous experiments. The experiments were conducted in triplicate for 7 hours.

Ambradiol (100 mg) and 200 mg zeolite CFG-1 were weighed into the McCartney bottles in and 1 mL hexane was added into each. The bottles were sealed and placed in an orbital shaker incubator at 60˚C for 7 hours and agitation at 180 rpm. Hexane (1 mL) was added after 7 hours reaction into each sample to reconstitute the sample and thoroughly mixed. Samples (1 mL) was taken into the Eppendorf tubes and centrifuged at 13000 rpm for 2 mins. The supernatants were taken into vials and analysed by GC.

3.4.9 Determination of the recycled zeolite CFG-1 for production of (-) ambrafuran

A reaction for the production of ambrafuran was done in higher concentration of ambradiol (100 mg) and 200 mg zeolite CFG-1 similar to the experiment above in 3.4.8. After the reaction, the used zeolite was washed with ethanol and incubated at 100˚C overnight. The zeolite (recycled) was cooled down at room temperature and used for ambrafuran production as explained below. The experiment was conducted in triplicate.

Ambradiol (10 mg) and 20 mg recycled zeolite CFG-1 washed with ethanol were both weighed into the McCartney bottles in duplicate and 1 mL hexane was added into each. The bottles were sealed and placed in an orbital shaker at 60°C for 7 hours, agitation at 180 rpm. Hexane (1 mL) was added into each sample after 7 hours reaction and thoroughly mixed. Sample (1 mL) was taken into Eppendorf tubes and centrifuged at 13000 rpm for 2 mins. The supernatants were taken into vials and analysed by GC.

3.4.10 Determining the production of (-) ambrafuran from impure ambradiol using zeolite CFG-1, CBV320 and ZD06014

The impure ambradiol containing the two contaminating peaks was tested for ambradiol production with the zeolite CFG-1, CBV320 and ZD06014. The experiments were carried out in duplicate as explained below.

Ambradiol (10 mg) and 20 mg zeolite CFG-1, CBV320 or ZD06014 were weighed into the McCartney bottles in and 1 mL hexane was added into each. The bottles were sealed and

placed in an orbital shaker incubator at 60˚C for 7 hours and agitation at 180 rpm. Hexane (1 mL) was added after 7 hours reaction into each sample to reconstitute the sample and thoroughly mixed. Samples (1 mL) was taken into Eppendorf tubes and centrifuged at 13000 rpm for 2 mins. The supernatants were taken into vials and analysed by GC.

3.4.11 Determining the effect of magnesium sulphate ($MgSO_4$) anhydrous and molecular sieves for production of (-) ambrafuran at 60˚C

This experiment involves the addition of $MgSO_4$ anhydrous and molecular sieves in a reaction for (-) ambrafuran production. All the reactions were conducted at 1:2 ratio using zeolite CFG-1 in an orbital shaker incubator. The reactions were conducted in triplicate as follows.

Ambradiol (10 mg), 20 mg zeolite CFG-1 and 3 spheres of molecular sieves or 1 micro spatula tip of $MgSO_4$ anhydrous were all placed into McCartney bottles and 1 mL hexane was added into each. The bottles were sealed and placed into an orbital shaker incubator at 60˚C for 7 hours and agitation at 180 rpm. Samples (1 mL) were each taken into Eppendorf tubes and centrifuged at 13000 rpm for 2 mins. The supernatants were placed into vials and analysed by GC.

3.4.12 Examining (-) ambrafuran production using anhydrous activated $MgSO_4$ & molecular sieves in combination with zeolite CFG-1

$MgSO_4$ anhydrous and molecular sieves were activated at 100˚C in an incubator for overnight for use in a reaction for (-) ambrafuran production. The molecular sieve spheres were crushed into a fine powder by pestle and mortar before activation. The reactions were performed in an orbital shaker incubator at 60˚C in triplicate as follows.

Ambradiol (10 mg), 20 mg zeolite CFG-1 and 2 micro-spatulas of activated molecular sieves or activated $MgSO_4$ anhydrous were all placed into McCartney bottles and 1 mL hexane was added into each. The bottles were sealed and placed into an orbital shaker incubator at 60˚C for 7 hours, agitation at 180 rpm. Hexane (1 mL) was added into each sample after reaction to reconstitute the reaction volume due to evaporation of the solvent and taken into Eppendorf tubes. The samples were centrifuged for 2 mins at 13000 rpm. The supernatants were placed into vials and analysed by GC.

3.4.13 Addition of activated $MgSO_4$ anhydrous in a test reaction for (-) ambrafuran production by zeolite CFG-1 at 60˚C

$MgSO_4$ anhydrous was initially heated at 100˚C for 24 hours in an incubator. The amount of dried $MgSO_4$ anhydrous added into the reaction was 1 g. The reaction was carried out in an orbital shaker incubator in triplicate at 1:2 ratios as follows.

Ambradiol (10 mg), 20 mg zeolite CFG-1 and 0.1 g activated $MgSO_4$ anhydrous were weighed into McCartney bottles and 1 mL hexane was added. The bottles were sealed and placed into an orbital shaker incubator at 60˚C for 7 hours and agitation at 180 rpm. Hexane (1 mL) was added into each sample after reaction to reconstitute and taken into Eppendorf tubes. The samples were centrifuged for 2 mins at 13000 rpm. The supernatants were placed into vials and analysed by GC.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Analytical

Analyses were done using an HP Gas chromatography 5890 Series II with a Restek column. At a later stage of the study an Agilent Technologies GC 6890N equipped with 7683B Series Injector was used. Samples were injected manually on an HP Gas chromatography 5890 Series II, which later broke down in the middle of the project and was substituted with an Agilent Technologies GC 6890N equipped with 7683B Series Injector. The different compounds present in the reaction mixtures as substrates or products could be separated with good peak resolution. As shown in Figure 16 the (-) ambrafuran, diol and sclareol were separated and eluted at 3.040, 4.457 and 5.209 mins retention times "respectively". The results are presented as area percentage of each compound as the triplicates showed good reproducibility (less than 2% difference) using these parameters.

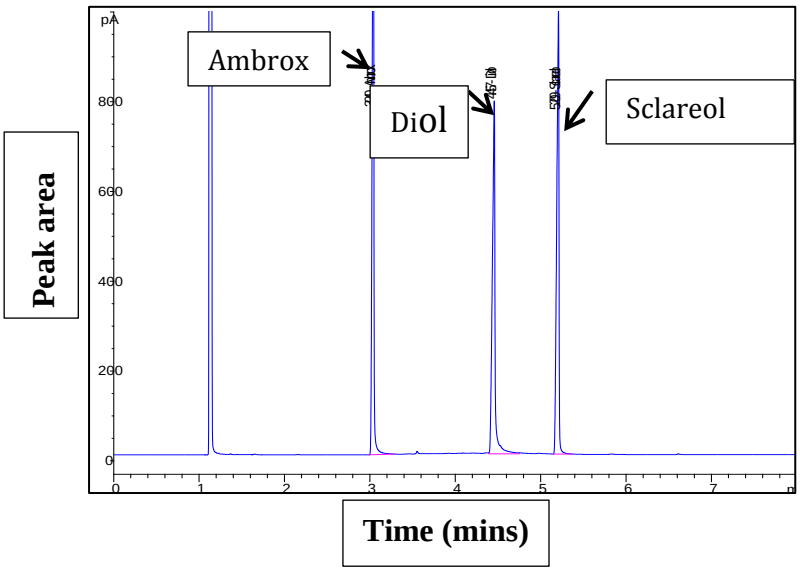


Figure 16: Illustrates the chromatogram for (-) ambrafuran, ambradiol and sclareol with their retention times separated by GC

The use of area percentage in GC analysis is well known and is defined as the area under a gas-chromatograph peak which is proportional to the amount (moles) of compound eluted.

Hence, the molar percentage composition of a mixture can be approximated by comparing relative peak areas. The calculation is done as illustrated in Figure 17 below.

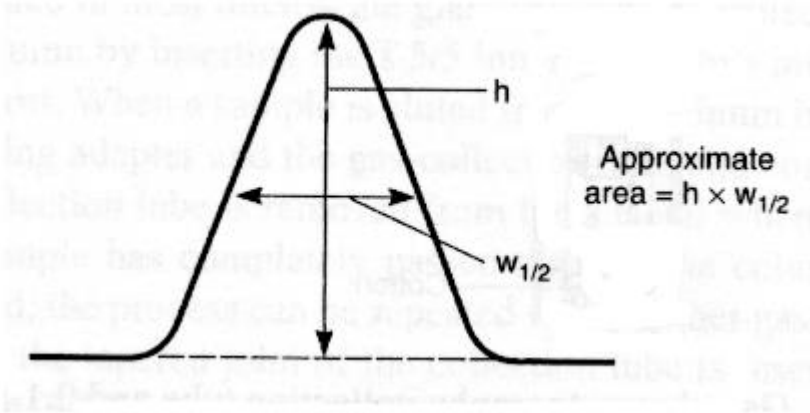


Figure 17: Calculation of the area percentage (http://quiz2.chem.arizona.edu/vip/chromatography/GC/analysis.htm accessed on 10/06/2015)

h - The height of the peak above the baseline and w1/2- the width of the peak at half of it height

It was difficult to obtain accurate amounts of product produced from the GC results as the injections were done manually and 1 µL injections were not found to be accurate (up to 38% difference). Final yields could be determined by isolating the products from the reaction mixture and were not based on masses obtained from a calibration curve. The area percentage results also indicated the presence of unwanted by-products which influenced the area percentage obtained for the product and starting material. The detection of unwanted by-products was crucial as the technology being developed should result in limited by-products as the final product down-stream processing should not be complicated by unwanted products requiring sophisticated separating techniques on large scale. UPLC-MS was also used to determine the accurate masses of the compounds to confirm the identities of the different compounds. A typical result of the separation of the compounds on UPLC-MS is shown in Figure 18.

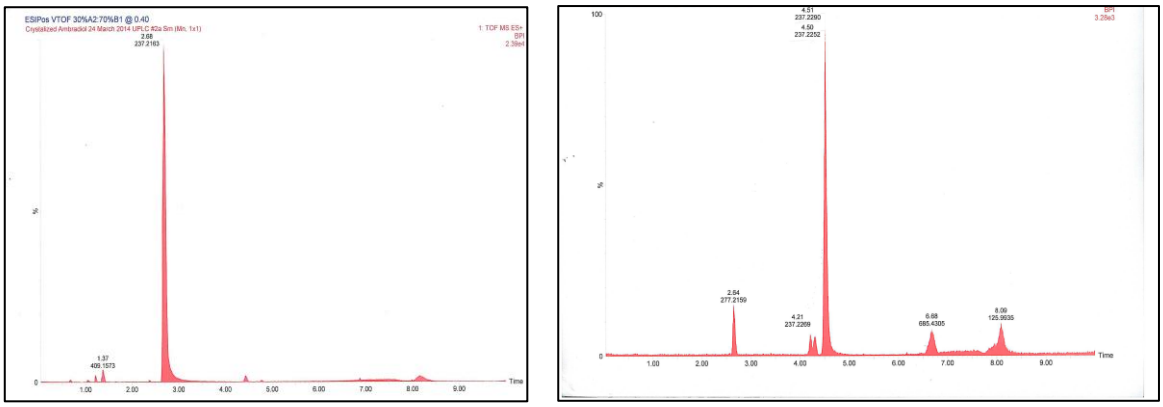


Figure 18: Chromatograms showing ambradiol (left) and (-) ambrafuran (right) measured by UPLC-MS

The chromatograms in Figure 18 above show the ambradiol and ambrafuran compounds being confirmed and separated by UPLC-MS.

4.2 CONVERSION OF SCLAREOL TO INTERMEDIATE AMBRADIOL

In the original patent by Farbood and Willis (1989) the conversion of sclareol to an intermediate diol using *H. roseoniger* was described. The methods used in this patent was used a base case and further optimisations were done as reported in the thesis.

4.2.1 Determining the growth of *H. roseoniger*

H. roseoniger from working cell bank as explained in 3.3.2 was used in this experiment for determining the growth phase of the organism. *H. roseoniger* (1 mL) was inoculated into 100 mL PDB and the samples were collected at 0, 8, 32, 48, 56 and 72 hours respectively. Figure 19 shows the growth phase of *H. roseoniger* optical density measured at 600 nm wavelength in three flasks.

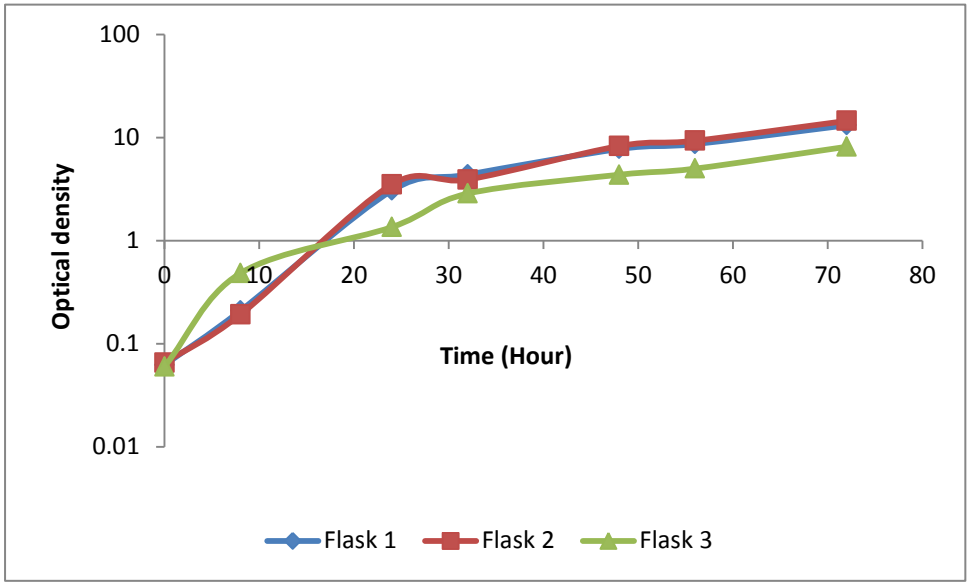


Figure 19: Results showing the growth curve for *H. roseoniger* in PDB media

The growth of the *H. roseoniger* started slowly as it took 8 hours to reach the optical density of 0.19 in flask 1 and 2 as seen in Figure 19. Cells in flask 3 show a rapid increase compared to flask 1 & 2 as it has reached the optical density of 0.48 in 8 hours. The microorganism took 24 hours to reach the stationary point as demonstrated by cells in flask 1 and 2. It was observed under the microscope that the cells in flask 3 were contaminated and it was confirmed by the decrease in optical density at 24 hours compared to cells in flask 1 and 2 as seen in Figure 19. A slight increase of cell growth in flask 3 at 24-32 hours proved that the cells may have been contaminated by other cells which inhibited the normal growth of *H. roseoniger* compared to cells in flask 1 and 2, as seen in Figure 19. It is imperative that the

cell cultures used in the experiments are pure and not contaminated as it may result in other products being produced by the contaminated microorganism.

4.2.2 Examination for conversion of sclareol to ambradiol using *H. roseoniger* pre-inoculums of 24, 48 and 72 hours at 25˚C without transferring to fresh medium

The starting material, sclareol, can be converted to the intermediate diol, ambradiol. The production of the ambradiol compound was examined by using *H. roseoniger* pre-inoculum cells grown for 24, 48 and 72 hours respectively. The pre-inocula were produced in 100 mL PDB medium and following the growth for 24, 48 or 72 hours, 20 mg of the sclareol was added to the same medium for induction of the microorganism. After 3 days of induction, the sclareol with Tween 80 were added and the growth followed 7 days and the results are presented in Table 1 below.

Table 1: The area percentage yield of ambradiol in potato dextrose media from 24, 48 and 72 hours pre-inoculums

Days of reaction	Pre-inoculum (hours)	Temperature (˚C)	Yield of ambradiol (Area %)
7	24	25	9.1
7	48	25	24.7
7	72	25	33.2

The yield of ambradiol was less than 50% in each reaction as shown in Table 2. *H. roseoniger* for the pre-inoculum for this experiment was grown from 1 cryovial in 100 mL of PDB and obviously did not give maximum growth compared to further experiments done in which 1 cryovial of the microorganism was grown in 25 mL of PDB. The difference can also be seen in the results from the growth curve shown under section 4.2.1. It is therefore very important to use the appropriate amount of cryovials per volume for maximum growth in the pre-inoculum. It could also be the result of diminishing nutrients in the PDB medium as the original PDB medium was used to produce the pre-inoculum and then the bioconversion was continued in the same PDB while in all other experiments, the pre-inoculum was prepared in a PDB medium and then 5 mL of the pre-inoculum was placed into fresh PDB medium for the bioconversion of the sclareol to the ambradiol. This means that the transfer of

microorganism to a fresh medium enhance the biotransformation of sclareol to ambradiol due to sufficient available nutrient.

4.2.3 An investigation for ambradiol production using *H. roseoniger* from 24, 48 and 72 hours pre-inoculums at 25˚C by transferring the pre-inoculum to fresh medium

This experiment described in 3.3.4 was to test the production of ambradiol from *H. roseoniger* pre-inoculum from a 1 mL cryovial for 24, 48 and 72 hours respectively in PDB (100 mL) at 25˚C. In this experiment, 5 mL each of pre-inoculum was transferred into a fresh (100 mL) PDB media and induced with 20 mg substrate (sclareol) for 3 days. The sclareol (1 g) and Tween-80 (1 mL) were added to the media and allowed to react for 7 days. The aim was to determine the optimum time for the pre-inoculum required for the biotransformation of the substrate (sclareol) to the intermediate diol. This investigation was also an attempt to demonstrate the effectiveness of the *H. roseoniger* in fresh media in comparison to the other experiment depicted in the previous section 4.2.2 above. The results for this investigation are listed in Table 2 below.

Table 2: Ambradiol area percentage yield obtained from reactions of 24, 48 and 72 hours with *H. roseoniger* pre-inoculums in fresh potato dextrose broth media at 25˚C

Pre-inoculums (hours)	Temperature (˚C)	Yield of ambradiol (%)
24	25	3.8
48	25	6.6
72	25	37.7

The biotransformation of sclareol to ambradiol with cells from 24 and 48 hours pre-inoculums were very low, both yielding less than 10% of ambradiol. The yield of 37.7% resulting from the 72 hour pre-inoculum as shown in Table 3 proved that 1 mL *H. roseoniger* from a cryovial in 100 mL PDB was not able to reach a specific biomass for optimum biotransformation of sclareol to ambradiol. This further suggested that biotransformation of sclareol to ambradiol by *H. roseoniger* was achievable due to more biomass produced from pre-inoculum and that the pre-inoculum should be grown for at least 72 hours. The increase

of the cryovial volume per volume of PDB was confirmed by the results from section 4.2.1 and for this reason all other experiments were done with pre-inoculums produced from 1 cryovial (1 mL) of *H. roseoniger* in 25 mL of PDB.

4.2.4 Testing different media for conversion of sclareol to ambradiol

Different aqueous nutrient media were tested namely. (1). 0.1% NH_4NO_3 (w/v), 0.1% KH_2PO_4 (w/v), 0.05% $MgSO_4.7H_2O$ (w/v) and Yeast extract; (2). 0.1% NH_4NO_3 (w/v), 0.1% KH_2PO_4 (w/v), 0.05% $MgSO_4.7H_2O$ (w/v), 0.5% (D+) Glucose (w/v) and Yeast extract; and (3). Difco™ Yeast nitrogen base without amino acids with 0.1% NH_4NO_3 (w/v). Figure 20 represents ambradiol area percentage yield obtained by GC from different media in terms of the ambradiol being produced with time.

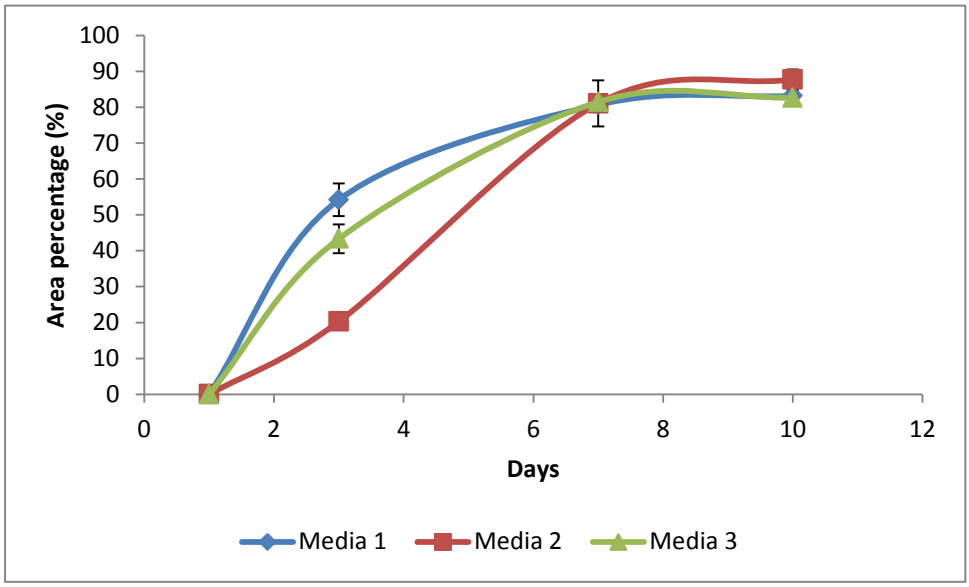


Figure 20: Area percentage yield of ambradiol obtained from different media using *H. roseoniger*

Figure legend: The media used are as follows; Media 1: 0.1% NH_4NO_3 (w/v), 0.1% KH_2PO_4 (w/v), 0.05% $MgSO_4.7H_2O$ (w/v) and Yeast extract; Media 2: 0.1% NH_4NO_3 (w/v), 0.1% KH_2PO_4 (w/v), 0.05% $MgSO_4.7H_2O$ (w/v), 0.5% (D+) Glucose (w/v) and Yeast extract; Media 3: Difco™ Yeast nitrogen base without amino acids with 0.1% NH_4NO_3 (w/v)

All data points were average values of triplicates (n=3) readings with standard deviations (±SD) shown.

The point converging at day 7 illustrated that the conversion rate was equivalent in all the media while medium 1 was initially faster and medium 2 had an initial lag phase. A new unidentified compound formed during the reaction which was unacceptable as it would have an influence on the final conversion of ambradiol to (-) ambrafuran. This unknown compound constitutes between 6 – 8% by reducing the area percentage of ambradiol. Furthermore in all

the media tested, sclareol was not entirely converted. media 3 which showed an increase in ambradiol production purity on the final day of the reaction and eventually yielded 87.69% while the other media had a slight increase from the point of convergence to both yield 82.64%. Farbood and Willis, (1989) used similar type of the media without yeast extract and dextrose and reported to have obtained 100% purity of ambradiol by GLC after recrystallization. The aim of the current work is "however" to obtain the ambradiol without unwanted by-products. If however by-products cannot be eliminated during scale-up of the technology, recrystallization will have to be considered. This suggests that ambradiol should undergo purification after biotransformation. The nutrient media containing glucose (media 2) showed high efficacy for ambradiol production but the product may require purification before the final cyclodehydration.

4.2.5 Recrystallization of ambradiol

Crude extracts were isolated by ethyl acetate in a reaction for ambradiol production which was carried out in NH_4NO_3 0.1 g, 0.1 g KH_2PO_4, 0.05 g $MgSO_4.7H_2O$, 0.5 g Dextrose and 0.2 g Yeast extract media as mentioned in the method section 3.3.6. The 3 g of starting material (sclareol) was available by combining material from the reaction done in triplicate. In this experiment, ambradiol was recrystallized using ethyl acetate and hexane solvents and the method was detailed in section 3.3.6. The aim of this experiment was to recrystallize and determine the purity and amount of ambradiol produce during the reaction. The results for this experiment are detailed in Table 3 below.

Table 3: Total yield of ambradiol recrystallized and from NH_4NO_3 0.1 g, 0.1 g KH_2PO_4, 0.05 g $MgSO_4.7H_2O$, 0.5 g Dextrose and 0.2 g Yeast extract media

Mass of crude extracts (g)	Mass of recrystallized diol (g)	Area % of ambradiol in crude extracts	Area % of recrystallized diol
2.28	1.51	87.51	95.85

The area percentage of ambradiol increased from 87.51% to 95.85% after recrystallization and the isolated crude extracts yield of 2.28 g resulted to 1.51 g as seen in Table 3. The unknown compound was observed which eluted close to ambradiol and constituted 8.63%. Sclareol was not completely converted in the reaction and 3.86% remained. After

recrystallization, sclareol was completely eliminated while the unknown compound was reduced to 4.15%. Farbood and Willis, (1989) used similar type of the media without dextrose and obtained 2.22 g ambradiol from 3 g sclareol, the sclareol was reported to have been recrystallized using hexane solvent, pulverized and passed through 50-sieve before undergoing biotransformation and the diol was reportedly confirmed with NMR spectroscopy. The purity of ambradiol was not determined in this case by Farbood and Willis. These results show 2% difference to that of Farbood and Willis in terms of the crude extracts obtained which was also found to be ambradiol with impurities by GC. This may suggest that recrystallization of ambradiol might be considered and that further purification techniques may be employed to improve the purity of ambradiol.

4.2.6 Determining cell biomass of *H. roseoniger* in different medium at different conditions for biotransformation of sclareol to ambradiol

Growing *H. roseoniger* at different temperatures and subjecting it to the biotransformation under different conditions such as doing the reaction in buffer and in medium was to determine the suitable conditions for both growth and conversion of sclareol to the intermediate ambradiol.

(i). Biotransformation of sclareol in 1 mM sodium phosphate buffer pH 7.2

The biotransformation of sclareol in 1 mM sodium phosphate buffer was to test whether the *H. roseoniger* would be capable to perform the biotransformation of sclareol to the intermediate ambradiol in a buffer instead of actively growing microorganism. The microorganism was initially grown in the different media as in the method section 3.3.7 for 3 days and then harvested. The harvested cells were placed in phosphate buffer and the sclareol added in the presence of Tween 80. The production of ambradiol was monitored up to 7 days. The results in Figure 21-24 show the production of ambradiol from the harvested cells of *H. roseoniger* in the buffer.

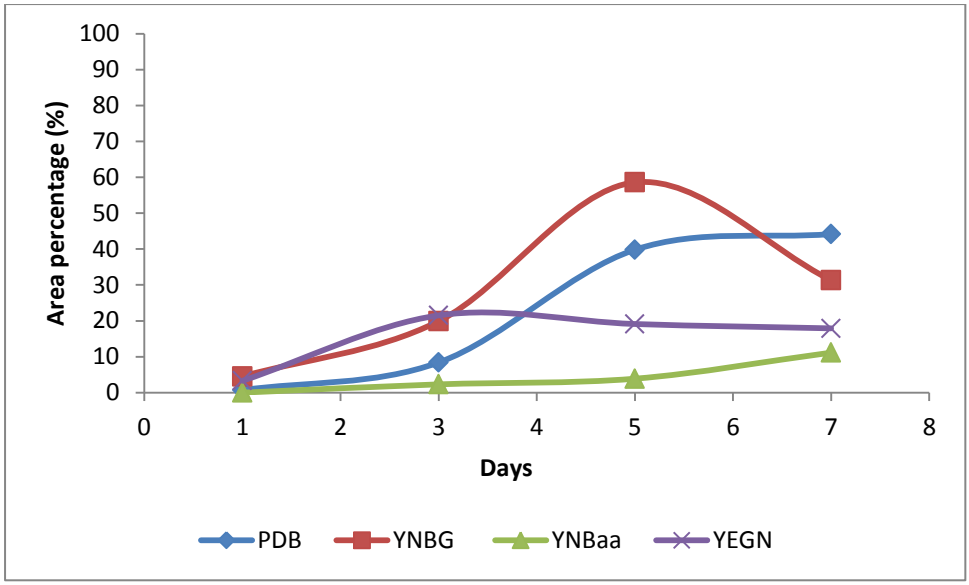


Figure 21: Area percentage yield of ambradiol produced by *H. roseoniger* harvested from different media at 20˚C in 1 mM sodium phosphate buffer

Figure legend: The media in which cells were harvested is as follows; - PDB: Potato dextrose broth; YNBG: Difco™ yeast nitrogen base without amino acids with glucose; YNBaa: Difco™ yeast nitrogen base without amino acids; YEGN: (0.2%) yeast extract, (1%) glucose, (0.2%) NH_4NO_3, (0.1%) KH_2PO_4 and (0.1%) $MgSO_4.7H_2O$.

H. roseoniger cells harvested from YNBG at 20˚C show a gradual increase in ambradiol productivity from day 1 until day 5 and began to decrease towards the end of reaction. The biotransformation of sclareol was not complete at the end of reaction. The resulting unknown compound, which cannot be identified with certainty using LC-MS, but have a similar structure to ambradiol and eluting next to ambradiol had increased with time. Cells from PDB also showed moderate activity on production of ambradiol in buffer at 20˚C, as seen in Figure 21 above. The results "therefore" indicate that at the chosen temperature of 20°C, the biotransformation does not proceed to completion in buffer and the results also indicate that the medium used for initial growth does play a role during the transformation reaction.

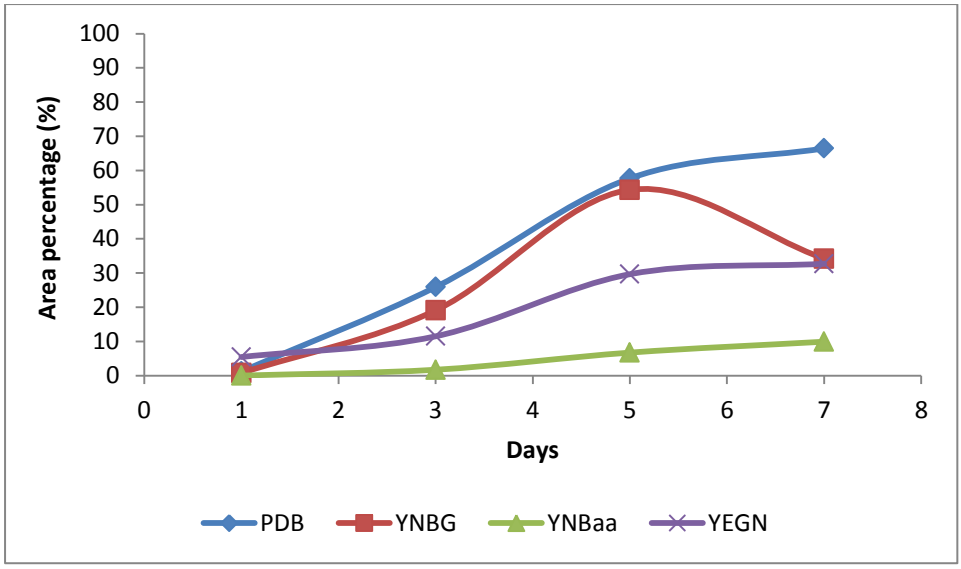


Figure 22: Area percentage yield of ambradiol produced by *H. roseoniger* harvested from different media at 25˚C in 1 mM sodium phosphate buffer

Figure legend: The media in which cells were harvested is as follows; PDB: Potato dextrose broth; YNBG: Difco™ yeast nitrogen base without amino acids with glucose; YNBaa: Difco™ yeast nitrogen base without amino acids; YEGN: (0.2%) yeast extract, (1%) glucose, (0.2%) NH_4NO_3, (0.1%) KH_2PO_4 and (0.1%) $MgSO_4.7H_2O$

H. roseoniger cells harvested from PDB and YNBG and the transformation done in buffer at 25°C showed increased potential in biotransformation of sclareol when compared to the results obtained at 20°C, as seen in Figure 22 above. In PDB the transformation continued up to day 7 and reached a conversion of approximately 66%. A decreased yield of ambradiol from day 5 to the end of reaction was seen specifically for cells harvested from YNBG. This may be due to the formation of the unknown compound that eluted very close to ambradiol and had a higher yield of 44.2% than that of ambradiol at the end of reaction. This tendency was also observed at 20°C as seen in Figure 21. Cells from (YNBaa) and YEGN had limited efficiency in the biotransformation of sclareol to ambradiol when harvested and transformed in buffer.

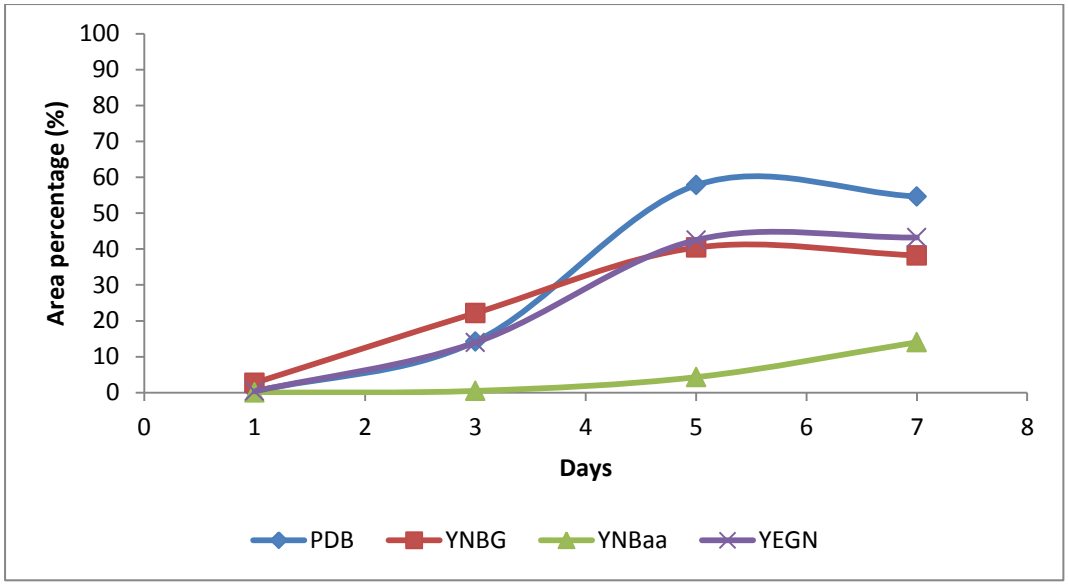


Figure 23: Area percentage yield of ambradiol produced from *Hyphozyma roseoniger* cell biomass harvested from different media at 28˚C in 1 mM sodium phosphate buffer

Figure legend: The media in which cells were harvested is as follows; PDB: Potato dextrose broth; YNBG: Difco™ yeast nitrogen base without amino acids with glucose; YNBaa: Difco™ yeast nitrogen base without amino acids; YEGN: (0.2%) yeast extract, (1%) glucose, (0.2%) NH_4NO_3, (0.1%) KH_2PO_4 and (0.1%) $MgSO_4.7H_2O$

Cells from PDB and the transformation done in buffer were effective for biotransformation from day 3 to 5 and decreased towards the end of reaction. The unknown compound eluting next to ambradiol yielding 23.6% also had an impact on the decrease in yield of ambradiol. This unknown compound is suggested to be the reducing factor for maximum production of ambradiol. The overall biotransformation of sclareol to ambradiol by *H. roseoniger* in buffer proves to be ineffective from all the media tested for initial growth.

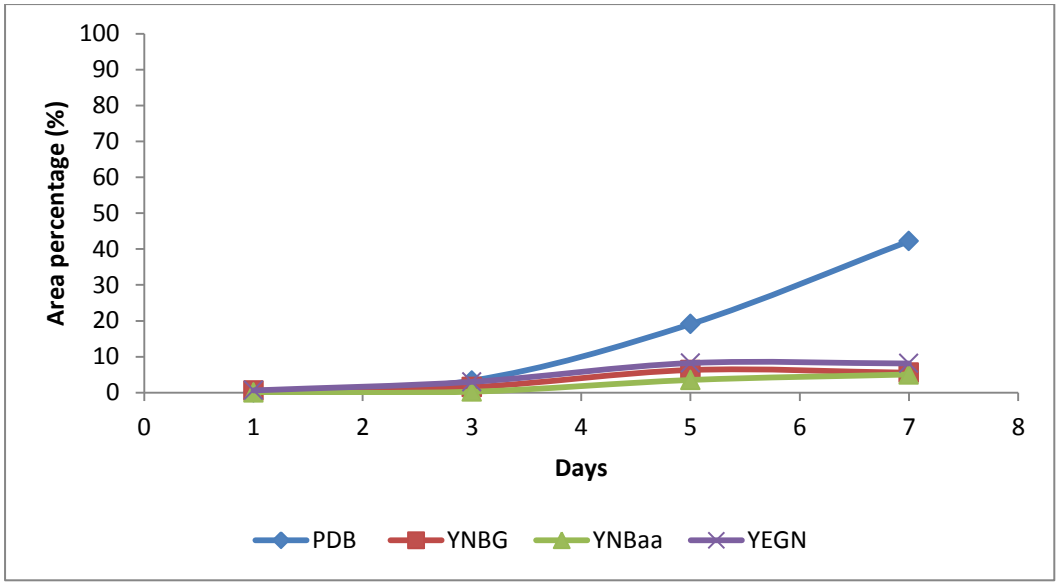


Figure 24: Area percentage yield of ambradiol produced by *H. roseoniger* cell biomass harvested from different media at 30˚C in 1 mM sodium phosphate buffer

Figure legend: The media in which cells were harvested is as follows; PDB: Potato dextrose broth; YNBG: Difco™ yeast nitrogen base without amino acids with glucose; YNBaa: Difco™ yeast nitrogen base without amino acids; YEGN: (0.2%) yeast extract, (1%) glucose, (0.2%) NH_4NO_3, (0.1%) KH_2PO_4 and (0.1%) $MgSO_4.7H_2O$

Cells harvested from all the media at 30˚C and the bioconversion done in buffer were not effective at performing the biotransformation when compared to reactions done at 20˚C, and 25°C. Figure 24 showed that only cells from PDB had a moderate activity on biotransformation of sclareol to ambradiol by yielding at least 42.2%. These suggested that the *H. roseoniger* microorganism is dependent on the temperature and type of media for effective biotransformation of sclareol to ambradiol

(ii). Biotransformation of sclareol in Difco™ yeast nitrogen base without amino acids following initial growth in different media

Biotransformation of sclareol in YNBaa and "therefore" in an actively growing state instead of using isolated cells in buffer as seen in the previous set of experiments and results, has been done before and has been reported to have yielded ≥98% of ambradiol by Steenkamp and Taka, (2010). Figure 25-28 below detailed the results for ambradiol obtained using isolated cells in YNBaa media.

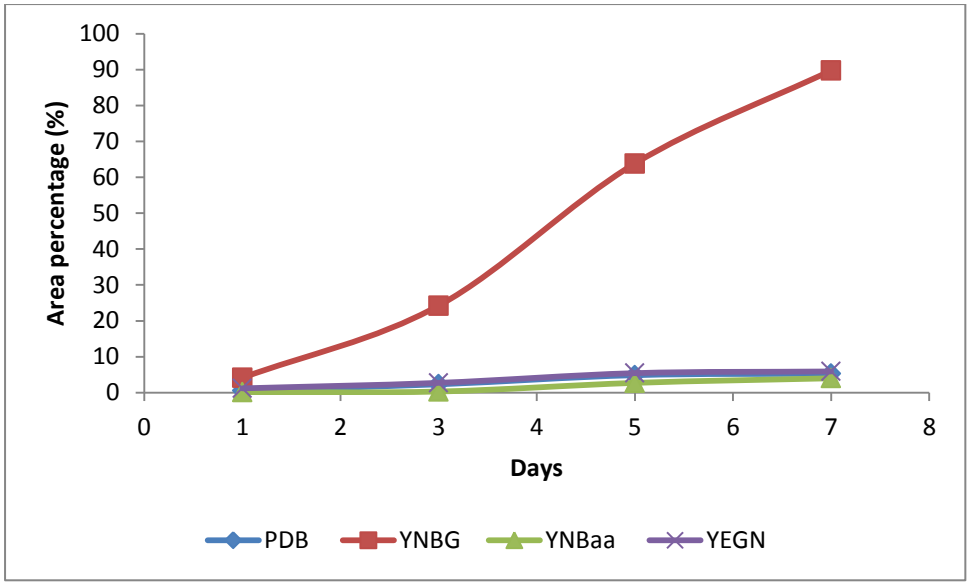


Figure 25: Shows the area percentage yield of ambradiol produced by *H. roseoniger* harvested (cell biomass) from different media at 20˚C in Difco™ yeast nitrogen base without amino acids

Figure legend: The media in which cells were harvested is as follows; PDB: Potato dextrose broth; YNBG: Difco™ yeast nitrogen base without amino acids with glucose; YNBaa: Difco™ yeast nitrogen base without amino acids; YEGN: (0.2%) yeast extract, (1%) glucose, (0.2%) NH_4NO_3, (0.1%) KH_2PO_4 and (0.1%) $MgSO_4$.7H2O

The microorganism was initially grown for 3 days in the different media as seen in the legend of the figure and then harvested and transferred to YNBaa for the biotransformation reaction. Biotransformation of sclareol to ambradiol in YNBaa yielded 89.74% of the diol using cells harvested from YNBG at 20˚C. The sclareol was not completely converted and an unknown compound has also appeared close to ambradiol peak with a peak area of 5.43%. There was no biotransformation of sclareol from the cells harvested from other media at 20˚C as shown in Figure 25 above.

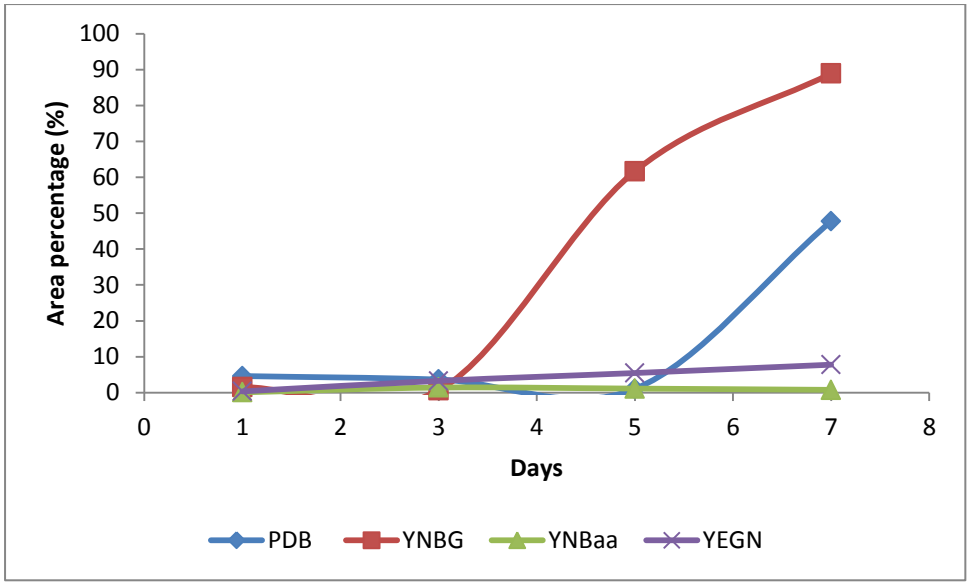


Figure 26: Shows the Area percentage yield of ambradiol produced by *H. roseoniger* harvested (cell biomass) from different media at 25˚C in Difco™ yeast nitrogen base without amino acids

Figure legend: The media in which cells were harvested is as follows; PDB: Potato dextrose broth; YNBG: Difco™ yeast nitrogen base without amino acids with glucose; YNBaa: Difco™ yeast nitrogen base without amino acids; YEGN: (0.2%) yeast extract, (1%) glucose, (0.2%) NH_4NO_3, (0.1%) KH_2PO_4 and (0.1%) $MgSO_4$.7H2O

The previous experiment was repeated at 25°C. Cells harvested from a pre-inoculum of YNBG media at 25˚C and then transferred to YNBaa, proved to be effective for ambradiol production as shown in Figure 26 by yielding 88.9% of the desired product. Only moderate biotransformation of sclareol to ambradiol occurred using cells harvested from PDB at 25˚C which yielded approximately 47.76% diol as seen in Figure 26 above. Cells harvested from YNBaa and YEGN were not effective for biotransformation in YNBaa. The unknown compound peak eluted close to ambradiol was regarded as a setback for the successful biotransformation of sclareol to ambradiol. The ambradiol formed from the cells harvested from the pre-inoculum in YNBG would require further purification to satisfy the requirements for the final conversion to (-) ambrafuran.

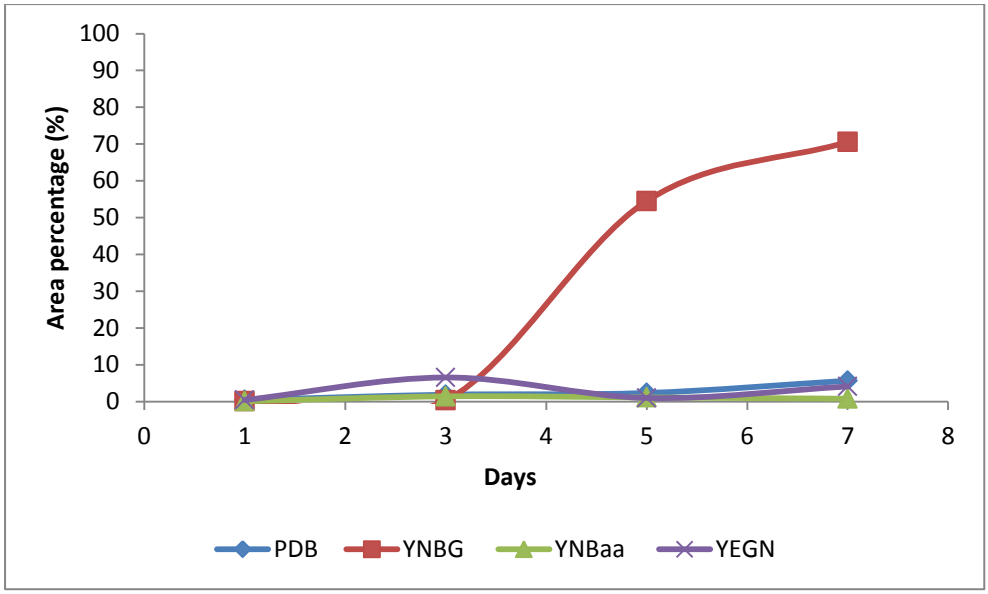


Figure 27: Demonstrates the area percentage yield of ambradiol produced by *H. roseoniger* harvested (cell biomass) from different media at 28˚C in Difco™ yeast nitrogen base without amino acids

Figure legend: The media in which cells were harvested is as follows; PDB: Potato dextrose broth; YNBG: Difco™ yeast nitrogen base without amino acids with glucose; YNBaa: Difco™ yeast nitrogen base without amino acids; YEGN: (0.2%) yeast extract, (1%) glucose, (0.2%) NH_4NO_3, (0.1%) KH_2PO_4 and (0.1%) $MgSO_4$.7H2O

The area percentage yield of 70.56% ambradiol was obtained from cells harvested from YNBG and the transformation done in YNBaa which were grown at 28˚C, as seen in Figure 27. There was 5.64% sclareol remaining after the reaction and an unidentified compound eluted close to ambradiol constituting 10.33% yield. The cells from other media were not effective for biotransformation of sclareol to ambradiol. The unidentified compound had a high area percentage yield 69.83% from cells in YEGN grown at 28˚C. These results here indicated that the biotransformation of sclareol to ambradiol by harvested cells of *H. roseoniger* and then transferred to YNBaa for the bioconversion is not feasible.

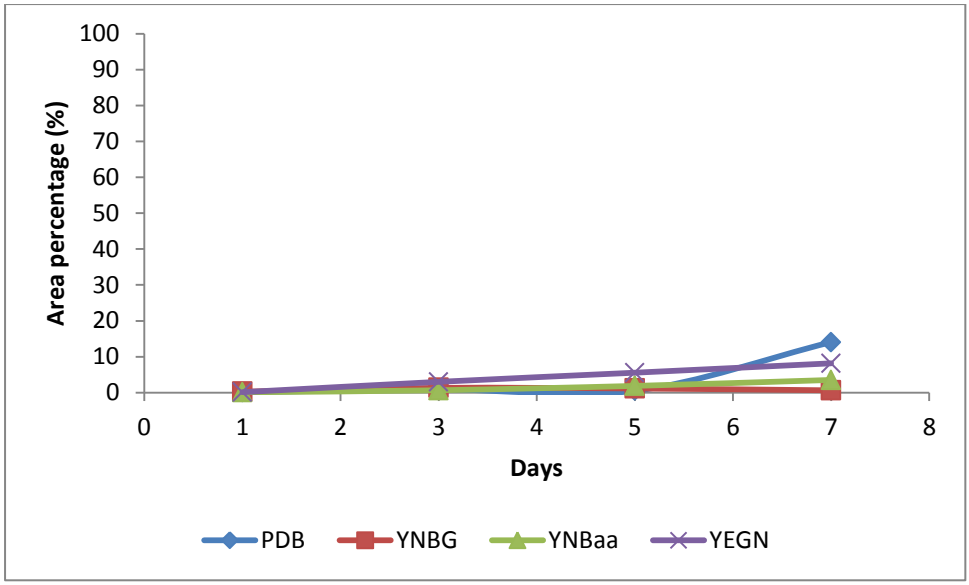


Figure 28: Shows the area percentage yield of ambradiol produced by *H. roseoniger* harvested (cell biomass) from different media at 30˚C in Difco™ yeast nitrogen base without amino acids

Figure legend: The media in which cells were harvested is as follows; PDB: Potato dextrose broth; YNBG: Difco™ yeast nitrogen base without amino acids with glucose; YNBaa: Difco™ yeast nitrogen base without amino acids; YEGN: (0.2%) yeast extract, (1%) glucose, (0.2%) NH_4NO_3, (0.1%) KH_2PO_4 and (0.1%) $MgSO_4.7H2O$

The production yield of ambradiol was low from all the cells harvested from different media and then biotransformed in YNBaa at 30˚C, as seen in Figure 28. Overall the results from this experimental section indicated that harvested microorganism *H. roseoniger* is not effective for biotransformation of sclareol to ambradiol in a buffer or YNBaa. In literature, Farbood and Willis, (1989) reported to have obtained 99% purity of ambradiol through isolated cells in 30 µM phosphate buffer pH 7.2. This may be as result of buffer concentration and pH used to produce low yield in this case.

4.2.7 Measuring the production of ambradiol in Difco™ yeast nitrogen base without amino acids & potato dextrose broth at 20˚C and 25˚C using *H. roseoniger*

The objective here was to detect and contrast the production of ambradiol at two different temperatures as well as different media.

Media such as yeast extract peptone and dextrose (YPD), yeast extract peptone and dextrose with 0.2% malt extract (YPD with Malt extract), and nutrient media were tested under different conditions for the conversion of sclareol to ambradiol using *H. roseoniger* but no conversion could be demonstrated and results are not shown.

Potato dextrose broth (PDB) and Difco™ yeast nitrogen base without amino acids were repeatedly tested at different temperatures and they were significantly better when compared to the other media. Optimization experiments were conducted using these two media. PDB and YNBaa were specifically tested for biotransformation of sclareol to ambradiol using *H. roseoniger* at both 20˚C and 25˚C respectively. The cells were also tested in an induced and non-induced form by sclareol for the production of intermediate ambradiol. The cells were pre-inoculated in a potato dextrose broth media at 25˚C for 3 days. Figure 29-32 below represent the results for the production of ambradiol from the experiment conducted in method section 3.3.8.

i. Non-induced reaction for ambradiol production in Difco™ yeast nitrogen base without amino acids (YNBaa) and potato dextrose broth (PDB) at 20˚C

The biotransformation of sclareol to ambradiol using *H. roseoniger* in both PDB and YNBaa were tested without initial induction with the starting material (sclareol). Figure 29 & 30 below represent the ambradiol production taken from day 1 to 10 in both YNBaa and PDB media at 20˚C following inoculation with the 3 day old pre-inoculum prepared in PDB.

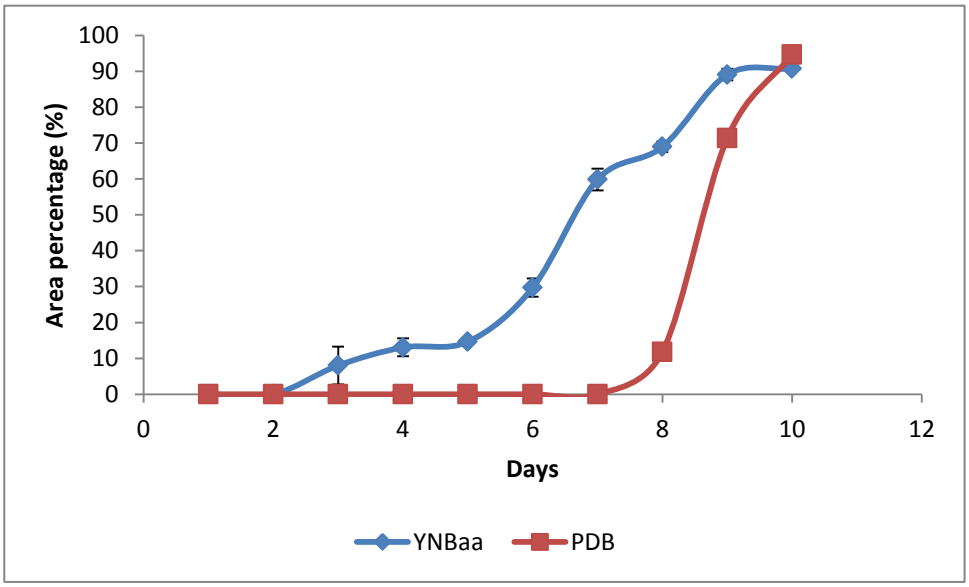


Figure 29: Area percentage yield of ambradiol from non-induced *H. roseoniger* cells in both Difco™ yeast nitrogen base without amino acids and potato dextrose broth at 20˚C

Figure legend: the media used for ambradiol production is as follows; PDB: Potato dextrose broth; YNBaa: Difco™ yeast nitrogen base without amino acids All data points were average values of duplicates (n=2) readings with standard deviations (±SD) shown.

The biotransformation of sclareol started on day 3 in YNBaa and gradually increases on a daily basis until the end of the reaction, as shown in Figure 29 above. The area percentage yield of 90.7% ambradiol from YNBaa was obtained in the end. There was 0.54% area percentage yield of sclareol remaining at the end of reaction and the formation of the unknown compound eluting close to ambradiol constituted 7.33%. Biotransformation of sclareol in PDB began from day 8 and rapidly increased until the final day and has yielded 94.6% ambradiol as seen in Figure 29 above. There was limited impurity of the two unknown compound constituting a total of 1.2% while a 2.2% of sclareol was left after the biotransformation in YNBaa. These results suggested that potato dextrose broth was a suitable media for growth of *H. roseoniger* as well as for biotransformation of sclareol to ambradiol. The temperature also plays a crucial role for growth of *H. roseoniger* and for biotransformation to take place.

ii. Non-induced reaction for ambradiol production in Difco™ yeast nitrogen base without amino acids & potato dextrose broth (PDB) at 25˚C

This experiment was related to the one above in Figure 29 and the only difference was the temperature which in this case was at 25˚C.

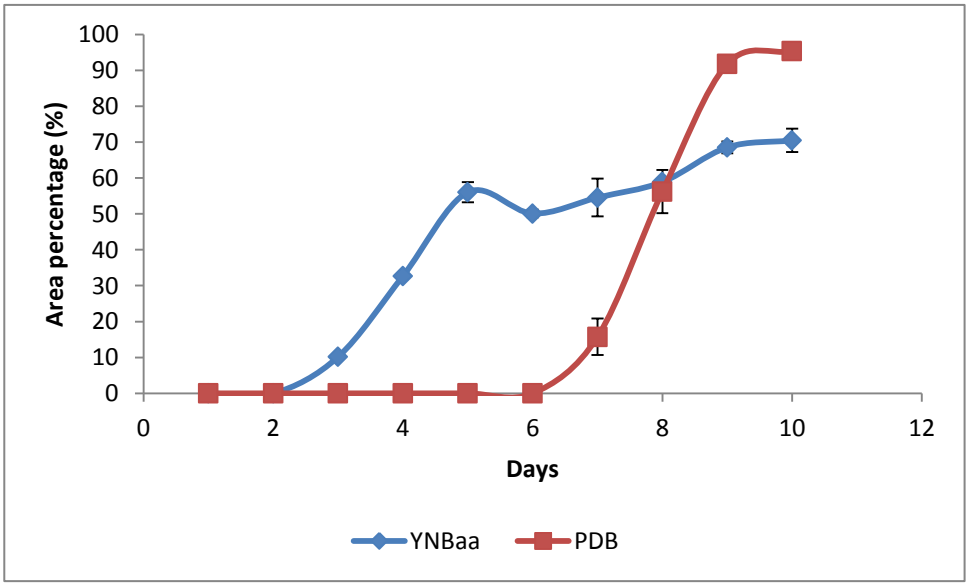


Figure 30: Area percentage yield of ambradiol from non-induced *H. roseoniger* cells in both Difco™ yeast nitrogen base without amino acids and potato dextrose broth at 25˚C

Figure legend: the media used for ambradiol production is as follows; PDB: Potato dextrose broth; YNBaa: Difco™ yeast nitrogen base without amino acids All data points were average values of duplicates (n=2) readings with standard deviations (±SD) shown.

The productivity of ambradiol in YNBaa rapidly increased from day 3 to day 5 and a slight drop on day 6 which could be due to a sampling error. The ambradiol production began to gradually increase from day 7 as shown in Figure 30 and in the end 70.46% ambradiol was produced. Production of ambradiol started late at 20˚C and 25˚C in PDB media as shown in Figure 29 and 30. The area percentage yield was slightly higher at 25˚C than at 20˚C yielding at least 95.3% ambradiol in PDB. The unknown compound eluting close to ambradiol had an area percentage yield of 21.5% in YNBaa and two unknown compounds appeared in PDB reactions with an overall yield of 3.6%. The sclareol was not completely converted in both media with 1.1% remaining in PDB and 3.4% in YNBaa at 25˚C. These results proved that ambradiol can be formed in PDB without *H. roseoniger* cells being induced. It does however require a longer time period before the conversion was completed.

iii. Induced reaction for ambradiol production in Difco™ yeast nitrogen base without amino acids & potato dextrose broth (PDB) media at 20˚C

The purpose of induction was to stimulate the cells to enhance the production of ambradiol and reduce the lag time before production start. Sampling was conducted from day one to day seven to monitor the production progress and is depicted in Figure 31 and 32 below.

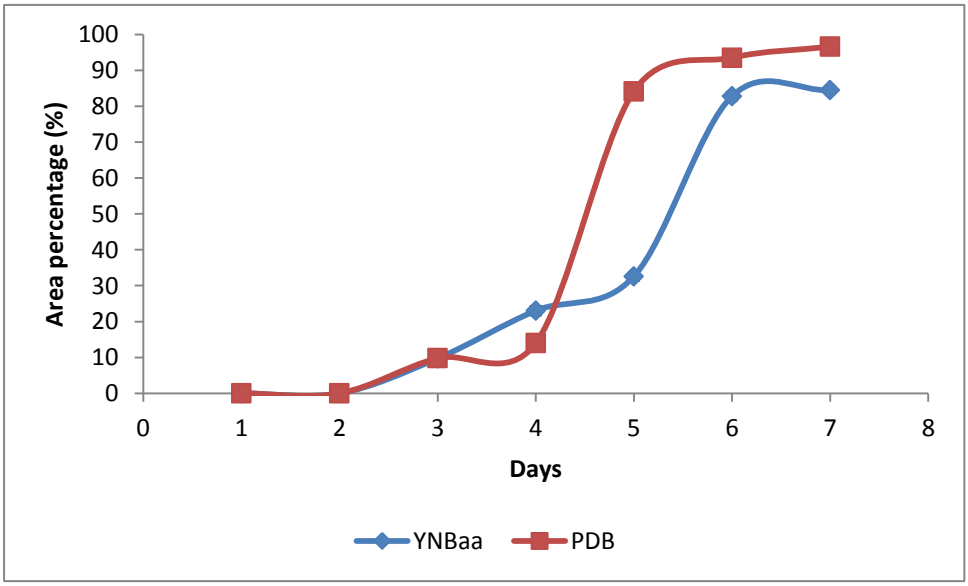


Figure 31: Area percentage yield of ambradiol from induced *H. roseoniger* cells in both Difco™ yeast nitrogen base without amino acids and potato dextrose broth at 20˚C

Figure legend: the media used for ambradiol production is as follows; PDB: Potato dextrose broth; YNBaa: Difco™ yeast nitrogen base without amino acids All data points were average values of duplicates (n=2) readings with standard deviations (±SD) shown.

There was a complete sclareol conversion in PDB media at the end of the reaction at day 7. The ambradiol productivity in PDB media shows a rapid increase from day 4 to 5 and the yield of 96.58% ambradiol at the end of the reaction. Small amounts of impurities were observed constituting 2.55% and 0.87% unknown compounds eluting just after (-) ambrafuran. From day 3-4, ambradiol production in YNBaa showed an increase compared to PDB as seen in Figure 31. The unidentified compound eluting close to ambradiol in YNBaa decreased the yield of ambradiol. The results demonstrate that ambradiol can be produced with high yield at 20˚C when *H. roseoniger* are induced especially in PDB media.

iv. Induced reaction for ambradiol production in Difco™ yeast nitrogen base without amino acids at 25˚C

In this experiment, the biotransformation of sclareol to ambradiol was conducted at 25˚C and the cells were induced. This experiment was similar to the one above (iii) but differed with incubation temperature. Ambradiol production was measured in area percentage from day 1-7 as shown in Figure 32 below.

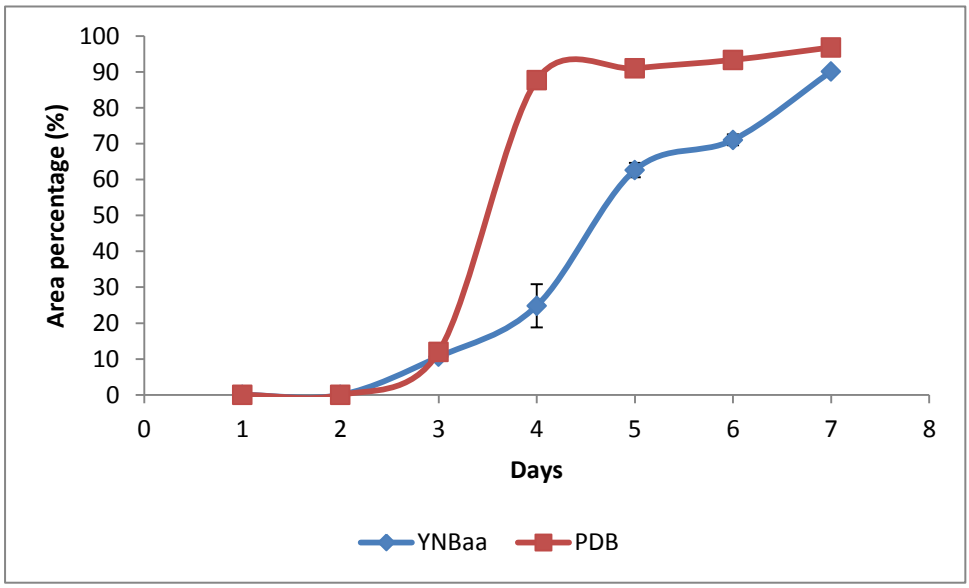


Figure 32: Area percentage yield of ambradiol from induced *H. roseoniger* cells in both Difco™ yeast nitrogen base without amino acids (YNBaa) and potato dextrose broth (PDB) at 25˚C

Figure legend: the media used for ambradiol production is as follows; PDB: Potato dextrose broth; YNBaa: Difco™ yeast nitrogen base without amino acids All data points were average values of duplicates (n=2) readings with standard deviations (±SD) shown.

The ambradiol production at 25˚C in PDB gave a final yield of 96.81%, as seen in Figure 32 above. Sclareol was completely converted in day 7, however the two unknown compounds

were observed eluting just before the ambradiol and constituted almost 3%. Ambradiol was also produced in YNBaa media yielding 90.09% with an unknown compound eluting next to it. Overall results in this experiment suggested that *H. roseoniger* was capable of performing the biotransformation of sclareol to ambradiol under aerobic condition in an aqueous nutrient medium. The induction reactions showed the highest yield of ambradiol in PDB media at both 20˚C and 25˚C temperature compared to the non-induction. After 7 days with the non-induced microorganism the conversion of the sclareol to ambradiol only reached 15.7% while it was 96.81% converted on day 7 following induction. The non-induced conversion took 10 days to reach a similar conversion.

4.2.8 Testing conversion of ambradiol on a larger scale in potato dextrose broth (PDB) at 25˚C using *H. roseoniger* in an orbital shaker incubator

This first scaled-up reaction was done in 1 L Fernbach flasks with a working volume of 700 mL. The purpose of this experiment was to determine the production of ambradiol in a large scale reaction. Table 4 below shows the production of ambradiol from a 7 day reaction in PDB by *H. roseoniger*.

Table 4: The area percentage yield of ambradiol produced from a larger scale reaction in potato dextrose broth media by *H. roseoniger*

Temperature (˚C)	Standard deviations	Yield of ambradiol (%)	Area percentage of the impurities (%)
25	1.5	96.2	3.8

The yield of ambradiol in these results was the average values calculated from the experiment being conducted in triplicate (n=3) with standard deviations (±SD).

The sclareol was totally transformed in all the flasks after 7 days reaction and the average yield of ambradiol was 96.2%. The impurities obtained were two unidentified compounds peaks with low percentage yield of 3.8% as indicated in Table 4, eluting just before and after the ambradiol peak at 3.552 & 4.531 retention times. These results substantiate that *H. roseoniger* was capable of transforming sclareol to ambradiol in PDB media at 25˚C at larger scale. It was evident that PDB can also be used as media in biotransformation of sclareol to ambradiol using the microorganism *H. roseoniger*. The production difference of ambradiol

between the flasks was marginal with the standard deviation of 1.5 shown in Table 4 above. This illustrated the consistency and reproducibility in terms of ambradiol production between the three flasks.

4.2.9 Test for ambradiol production in 2 L fermentation bioreactor using *H. roseoniger*

This experiment was conducted in a 2 L fermentation bioreactor at a scale of 1.2 L working volume at 25˚C. The pre-inoculum was grown for three days and then the microorganism (*H. roseoniger*) was induced for 3 days. The aim was to test the scalability using a fermenter resembling the equipment used on commercial scale and to assess the time for production. The following Table 5 outlined the results obtained during the experiment.

Table 5: Results for ambradiol production in area percentage yield taken at different times during fermentation process

Media type	Temperature (˚C)	Yield of ambradiol at 24 hours (%)	Yield of ambradiol at 31 hours (%)	Yield of ambradiol at 48 hours (%)
Potato dextrose broth	25	93.0	87.7	86.7

The ambradiol yield was high at 24 hours yielding at least 93.0% and decreased as fermentation process progresses as shown in the table above. Sclareol was not entirely converted during 24 hour and there was no any impurity at this stage. As it was close to completion samples were taken intermittently to determine the end point. At 31 hours, the ambradiol yield was 87.7% and sclareol (2.1%) was still available with the unknown compound peak noticed with 10.3% yield. The unknown compound eluting close to ambradiol was suspected to have caused the reduction due to its increasing yield. The sclareol was successfully converted at 48 hours and there was an increase of the unknown compound with a yield of 13.3% which resulted in slight decrease of ambradiol from 87.7% to 86.7% as shown in Table 5 above. The reaction was then stopped at 48 hours following the gradual reduction of ambradiol yield compared to 24 and 31 hours. The outcome of the fermentation indicated that biotransformation of sclareol to ambradiol was faster in a bioreactor as it already yielded 93% after 1 day. The reduction in yield of ambradiol with time suggested that

the microorganism was gradually converting the product (ambradiol) into the unidentified compound.

4.3 CYCLODEHYDRATION TO PRODUCE (-) AMBRAFURAN

Following conversion of the sclareol to ambradiol using the *H. roseoniger*, the final conversion of the ambradiol to (-) ambrafuran is required. As discussed in the literature review, chemical syntheses use very harsh chemicals such as pyridine for this reaction. Steenkamp and Taka (2010) reported and patented the use of zeolites for the conversion of the intermediate diol to (-) ambrafuran.

4.3.1 Measuring the production of (-) ambrafuran in different ratios using zeolite CBV320

The work done by Steenkamp and Taka (2010) was based on the use of zeolite CBV320 which was activated in a conventional microwave oven. The aim of this experiment was to examine the ability of zeolite CBV320 at different ratios of ambradiol per zeolite and also reducing the solvent for production of (-) ambrafuran at room temperature. Different power levels of 30 W, 50 W, 70 W and 90 W were tested for activation of zeolite (1 g) in a conventional microwave for 1, 2 & 3 mins and found that at 70 W for 3 mins the zeolite was necessary to activate the zeolite to catalyses the reaction for ambradiol conversion to ambrafuran. The ratios of 1:5, 1:7 and 1:9 (ambradiol per zeolite) as showed in the legends in Figure 34 were tested and compared for zeolite CBV320's ability to convert 5 mg of ambradiol and producing ambrafuran in 1 mL hexane solvent compared to the work previously done by Steenkamp and Taka (2010) which used 5 mg/20 mL solvent. Figure 33 below illustrates the results obtained from each ratio.

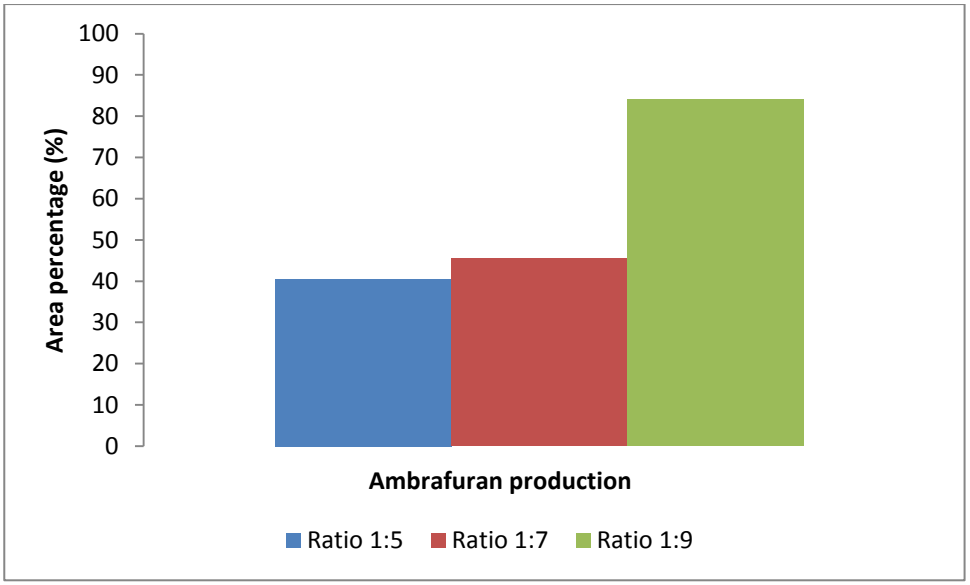


Figure 33: A bar graph representing area percentage of (-) ambrafuran from different ratio using zeolite CBV320 at 25˚C

The results show the mean values of experiments done in triplicates (n=3) with standard deviations (±SD).

A percentage yield of 84.03% (-) ambrafuran was obtained in a ratio of 1:9 (ambradiol per zeolite CBV320), as seen in Figure 33 above. Two unidentified compounds were detected and eluting after (-) ambrafuran in all the ratios at retention times of 3.372 and 3.545 mins respectively. Ambradiol was completely converted at 1:9 ratios compared to that at 1:7 and 1:5 ratios and the two unknown compound constituting 13.32% and the other 3.57% has reduced the production yield of ambrafuran. From the work done by Steenkamp and Taka, (2010) 98% (-) ambrafuran was obtained in a 1:9 ratio of ambradiol per zeolite CBV320 in 20 mL hexane. The results indicate that high amount of zeolite and high volume of solvent had more influence in the cyclodehydration of ambradiol to yield pure ambrafuran. This suggests that solvent reduction may have contributed in forming the unidentified compounds during the reaction. The zeolite (15 g) used by Steenkamp and Taka (2010) was activated in a microwave oven for 15 mins and at 50 W power level.

4.3.2 Determining the production of (-) ambrafuran in different solvents at 1:9 ratio using zeolite CBV320

Different solvents that are considered to be acceptable for production of (-) ambrafuran were tested in this experiment. Ethyl acetate, Hexane, Chloroform, Dichloromethane (DCM), 1,2-Butanediol, 1,2-Propanediol, t-Butanol, Isopropanol, Heptane, Toluene, Decane and Dimethylsulphoxide (DMSO) were all examined for ambradiol production and Figure 34

shows the production activity in area percent. The zeolite was activated in a conventional microwave at 70 W power level for 3 mins. Ambradiol (5 mg), 45 mg activated zeolite and 1 mL of the solvents mentioned was used in each reaction. The purpose of this experiment was to identify the potential solvents to be used for the cyclodehydration of ambradiol to form ambrafuran and the results are demonstrated in Figure 34.

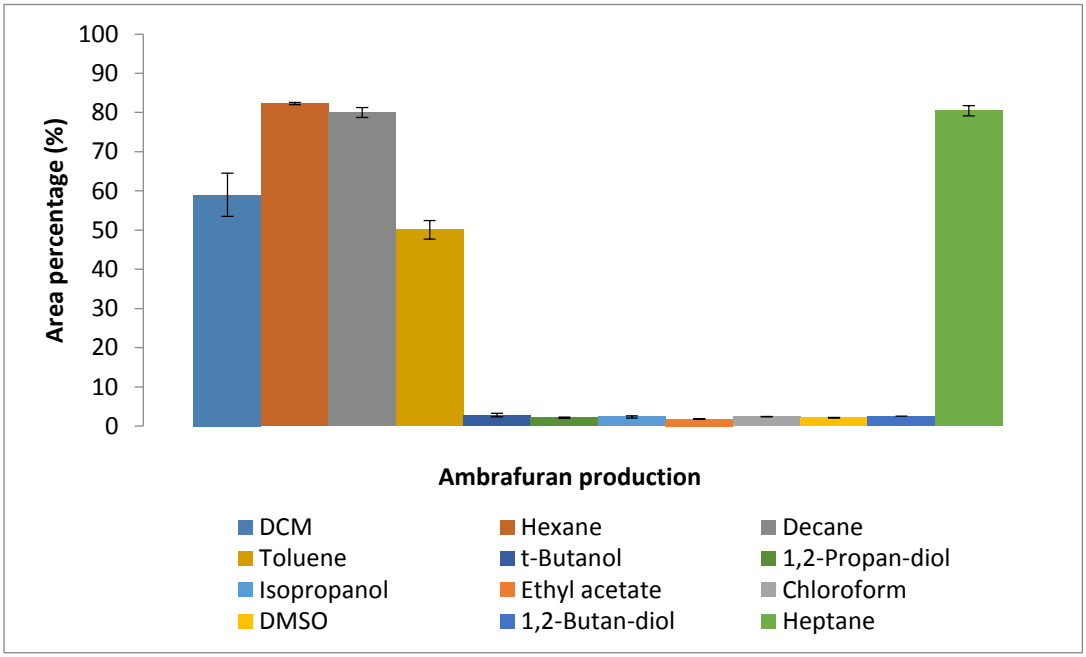


Figure 34: Production of ambrafuran results in area percentage obtained from different solvents

The results show the mean values of experiments done in duplicates (n=2) with standard deviations (±SD).

Reactions where t-Butanol, 1,2-propanediol, isopropanol, ethyl acetate, chloroform, Dimethylsulphoxide (DMSO) and 1,2-Butanediol solvents used, a low conversion of ambradiol was observed with a low yield of (-) ambrafuran as seen in Figure 34. The highest yield of (-) ambrafuran amongst all the solvents was in hexane, yielding 82.3%. Ambradiol conversion was complete in hexane, decane and heptane solvents with two unknown by-products forming after ambrafuran peak. Two unknown compounds before (-) ambrafuran peak eluted in a reaction involving decane with an overall yield of 2.3%. These results show a slight decrease by approximately 2% as compared to the results in Figure 33 at 1:9 ratio in hexane. This could be the effect of inconsistent injection volume as a result of manual injection in the GC. The overall results suggest that hexane was more effective than other solvents; however decane and heptane also proved to be valuable for the cyclodehydration of ambradiol to produce (-) ambrafuran.

4.3.3 Different zeolites for the determination of (-) ambrafuran production

Different zeolites such as CBV500-X16, CBV8062, CBV100, CBV320 found in-house from different suppliers as well as aluminium were tested for their ability to perform the cyclodehydration of ambradiol to produce (-) ambrafuran in hexane solvent. In this experiment, a ratio of 1:5 ambradiol (20 mg) per zeolite (100 mg) was used in 1 mL hexane solvent and the reaction took place at 25°C for 6 hours. The aim was to identify an effective zeolite for conversion of ambradiol to (-) ambrafuran and the method was explained in 3.4.3. Figure 35 below show the results for ambrafuran produced from each zeolites and aluminium.

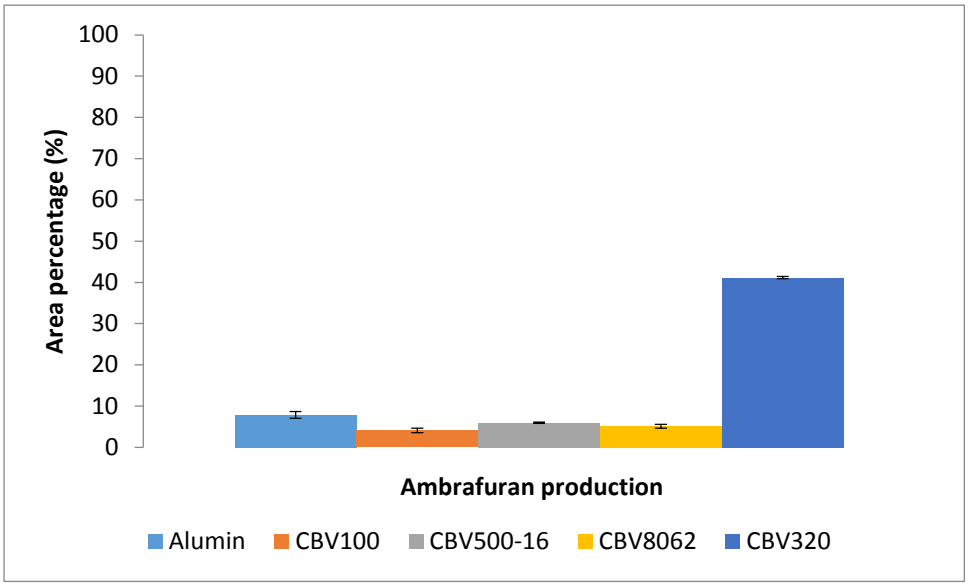


Figure 35: Show the production yield of (-) ambrafuran in area percentage from different zeolites

Figure legend: CBV100, CBV500-16, CBV8062 and CBV320 demonstrate the identity of each zeolite that was tested for production of ambrafuran. Alumin represent aluminium being tested for ambrafuran production in the legend entry.

The results show the mean values of experiments done in duplicate (n=2) with standard deviations (±SD).

Zeolite CBV320 yielded at least 41.15% (-) ambrafuran and 33.25% sclareol left after the reaction. The rest of the zeolites all yielded less than 10% (-) ambrafuran as seen in Figure 35 above. A number of unknown compounds formed constituting 25.6% in zeolite CBV320 and the results were related to the previous experiment in 4.3.1 for 1:5 ratio. Steenkamp and Taka, (2010) demonstrated that (-) ambrafuran was produced at 1:6 ratio at 25°C for 4-6 hours, however this was done in high volume of solvent. It can be suggested from Steenkamp and Taka that low volume of solvent had an impact on the low yield of ambrafuran.

4.3.4 Evaluation of (-) ambrafuran production in a water bath sonicator, magnetic stirrer plate and orbital shaker incubator

Equipment such as a sonicator water bath, magnetic stirrer plate and orbital shaker were used for running a reaction for (-) ambrafuran production in section 3.4.4. Zeolite CBV320 was used at 1:3 ratio in 1 mL hexane. Reactions on a magnetic stirrer plate and in an orbital shaker incubator were carried out at 25°C for 4 hours. It was difficult to control the temperature in the water bath sonicator as the water became hot which resulted in the evaporation of the solvent in the reaction. Therefore the experiment in the water bath sonicator was conducted for 3 hours while the hot water in the sonicator was replaced with fresh room temperature water every hour. Figure 36 below show the bar graph for ambrafuran production from each instrument. The purpose was to test for (-) ambrafuran production in the equipment mentioned and at low ratio of substrate to zeolite.

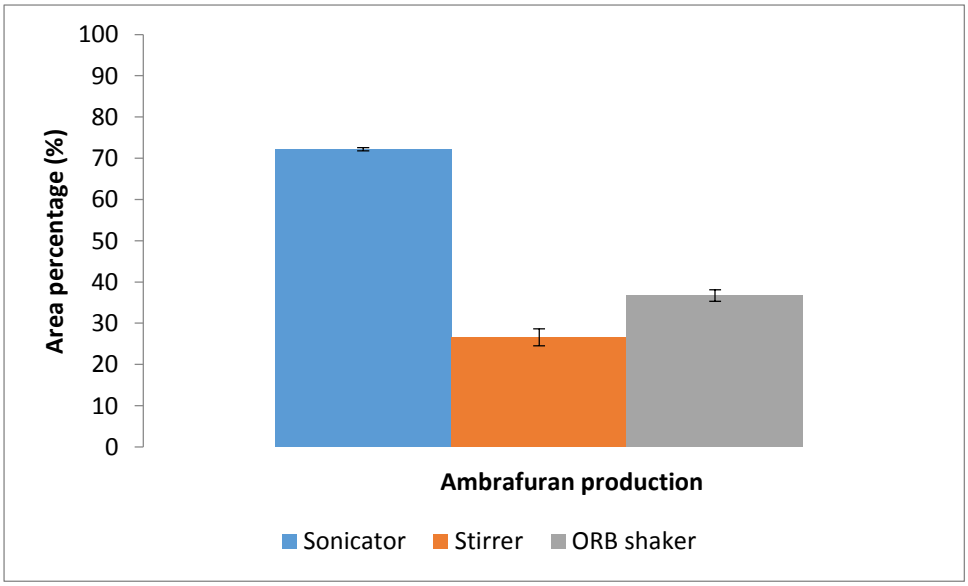


Figure 36: The results for (-) ambrafuran production from a reaction in a sonicator water bath, magnetic stirrer plate and orbital shaker incubator

Figure legend: ORB shaker: represent the orbital shaker incubator; Stirrer: represent the magnetic stirrer plate; Sonicator: For sonicator water bath.

The results show the mean values of experiments done in triplicates (n=3) with standard deviations (±SD).

The conversion of ambradiol was complete in a water bath sonicator reaction and produced 72.16% (-) ambrafuran with two unidentified compounds being formed eluting after the (-) ambrafuran peak yielding 15.30% and 12.54% respectively. These compounds could not be identified using the GC as no MS was available with this equipment. The LC-MS could also

not conclusively determine the identity of these two contaminating compounds. In a reaction with both a magnetic stirrer plate and orbital shaker incubator, the ambradiol was not entirely converted at 25°C and the yield of (-) ambrafuran was 26.59% and 36.69% as seen in Figure 36. Production of (-) ambrafuran in a water bath sonicator has not been reported before, and the increased temperature in the water bath or the improvement of mixing experienced in the sonicator may have influenced the conversion. The results from water bath sonicator show that (-) ambrafuran can be produced in a small ratio of ambradiol per zeolite and in low volume of solvent.

4.3.5 Measuring the production of (-) ambrafuran in a water bath sonicator using new zeolites

Two new zeolites were received as a generous gift from Zeolyst International and their identities were CFG-1 and ZD0614. They were both tested for the cyclodehydration process of ambradiol to produce (-) ambrafuran. The reaction was conducted in a water bath sonicator and the zeolites were in an activated and non-activated form. Activation of zeolites was conducted in a conventional microwave at power level of 70 W for 3 mins. The 1:3 and 1:6 ratios were used and the experiment was conducted for 3.5 hours. The main purpose for this experiment was to determine the ability of the new zeolites for (-) ambrafuran production. Figure 37 indicates the production of (-) ambrafuran for 1:3 ratio from both activated and non-activated new zeolites. The results for 1:6 ratio are not presented as there were multiple compounds eluting in a sequence with no (-) ambrafuran from both zeolites.

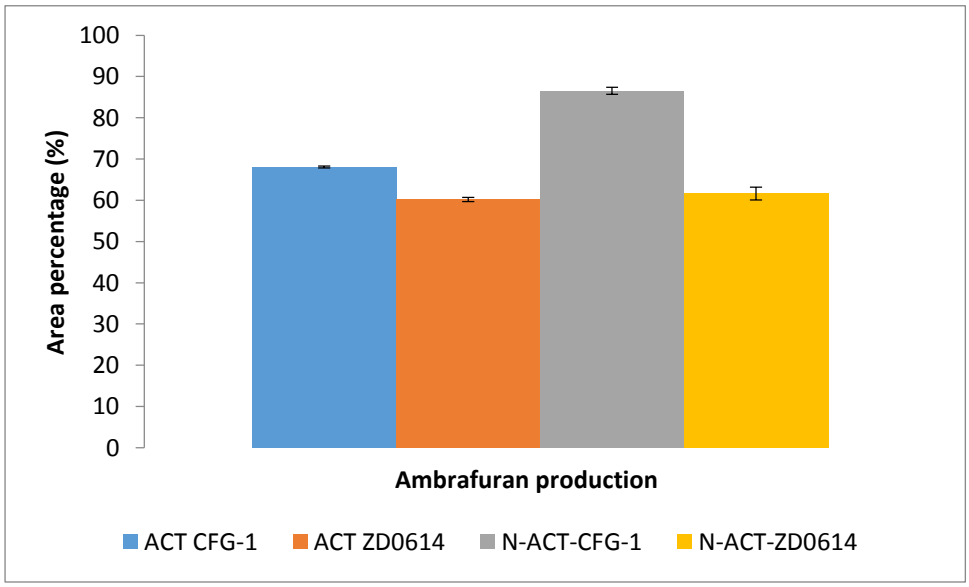


Figure 37: Shows the results for (-) ambrafuran production by zeolites CFG-1 and ZD0614 in an activated and non-activated form for 1:3 ratio

Figure legends: ACT-CFG-1: Activated zeolite CFG-1; ACT ZD0614: Activated zeolite ZD06014; N-ACT-CFG-1: Non-activated zeolite CFG-1: N-ACT-ZD0614: Non-activated zeolite ZD06014.

The results show the mean values of experiments done in duplicates (n=2) with standard deviations (±SD).

There was no (-) ambrafuran production at 1:6 ratio for both zeolites from activated and non-activated as explained above. Zeolite CFG-1 formed 86.5% (-) ambrafuran from a non-activated form at 1:3 ratio as seen in Figure 37 and ambradiol was completely transformed while two unknown compounds were observed. These unknown compounds constituted 9.3% and 4.2%. In a reaction with activated zeolite CFG-1, the yield of (-) ambrafuran was 68% and 5.4% ambradiol left after reaction. The two unknown compounds were observed in all the reactions. Ambradiol was not entirely converted by zeolite ZD06014 in both activated and non-activated form. The area percentages for ambradiol remaining in an activated and non-activated ZD06014 were 12.1% and 11.8%. Both zeolites were able to produce (-) ambrafuran at 1:3 ratio in a non-activated and an activated form. Zeolite CFG-1 was effective in a non-activated form compared to zeolites CBV320 and ZD06014 at 1:3 as seen in Figure 36 and 37. These results shows that the new zeolites CFG-1 and ZD06014 are able to perform the cyclodehydration of ambradiol to (-) ambrafuran without being activated compared to zeolite CBV320 which require activation before use. Both new zeolites were not able to produce ambrafuran at 1:6 ratio compared to CBV320 which was reported to have produced ±98% (-) ambrafuran at 25°C for 4-24 hours (Steenkamp and Taka, 2010; Carey, 2013b)

4.3.6 Conversion of ambradiol to (-) ambrafuran at different temperatures into an orbital shaker

The (-) ambrafuran production reactions were carried out using orbital shaker. The ratio of intermediate ambradiol and zeolites was 1:3 in 1 mL of hexane. Zeolites CBV320, CFG-1 and ZD0614 were used in this experiment. Zeolite CBV320 was activated at 70 W for 3 min in a microwave while the others were used without being activated. The aim was to determine the suitable condition and to find the effective zeolite for (-) ambrafuran production. Figure 38 demonstrates the production of ambrafuran at different temperatures by the three different zeolite mentioned above.

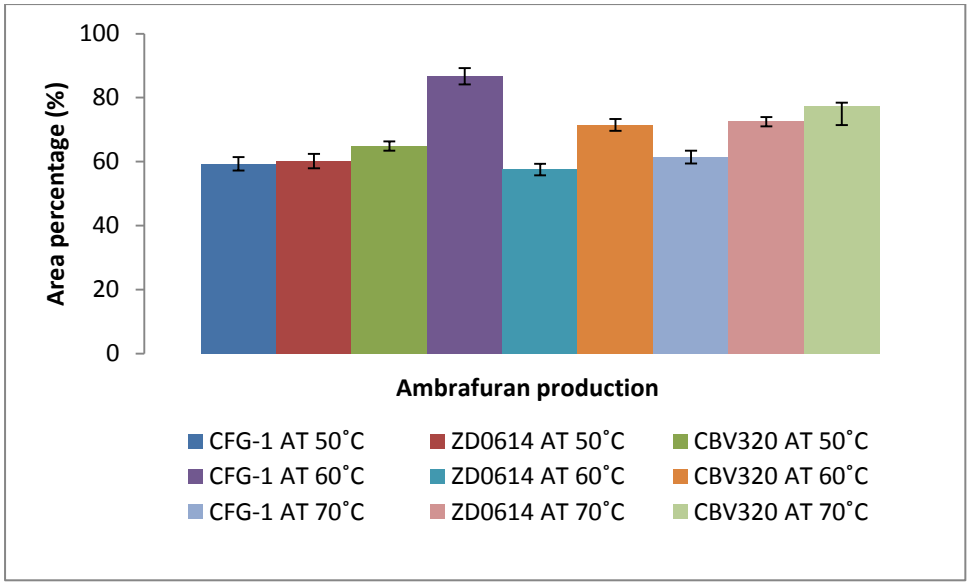


Figure 38: Illustrates the production of (-) ambrafuran at 50˚C, 60˚C and 70˚C by zeolites CBV30, CFG-1 and ZD06014

The results show the mean values of experiments done in triplicates (n=3) with standard deviations (±SD).

Temperatures of 30˚C, 35˚C, 40˚C and 45˚C were also done for the same 1:3 ratio using the three mentioned zeolites and (-) ambrafuran production was not achieved and therefore results are not shown. Ambradiol conversion was complete at 60˚C & 70˚C for each zeolite and the highest yield of (-) ambrafuran was obtained at 60˚C with a yield of 86.7% for CFG-1 zeolite as seen in Figure 38. Two unknown compounds were observed eluting just after the (-) ambrafuran compound with the combined yield of 13.3%. At 70˚C, zeolite CBV320 produced (-) ambrafuran with the highest yield of 77.4% and the lowest yield of 61.4% was produced by a zeolite CFG-1. All the zeolites were not able to completely transform ambradiol at 50˚C and CFG-1 zeolite again produced the lowest yield of (-) ambrafuran. The results indicated that CFG-1 was an effective zeolite for the transformation of ambradiol to (-) ambrafuran at 60˚C amongst the others by producing the highest yield.

4.3.7 Determination of (-) ambrafuran production at low ratios using zeolites CFG-1, ZD0614 and CBV320 at 60˚C in orbital shaker incubator

The lower ratios at 1:1 and 1:2 ambradiol per zeolite were tested in this experiment for the production of (-) ambrafuran in an orbital shaker incubator at 60˚C for a period of 7 hours. Zeolite CBV320 was activated as described before while the other two zeolites were used in a non-activated form. The aim of this test was to investigate whether the zeolites used are able

to transform ambradiol to (-) ambrafuran at lower ratios and Figure 39 shows the results obtained.

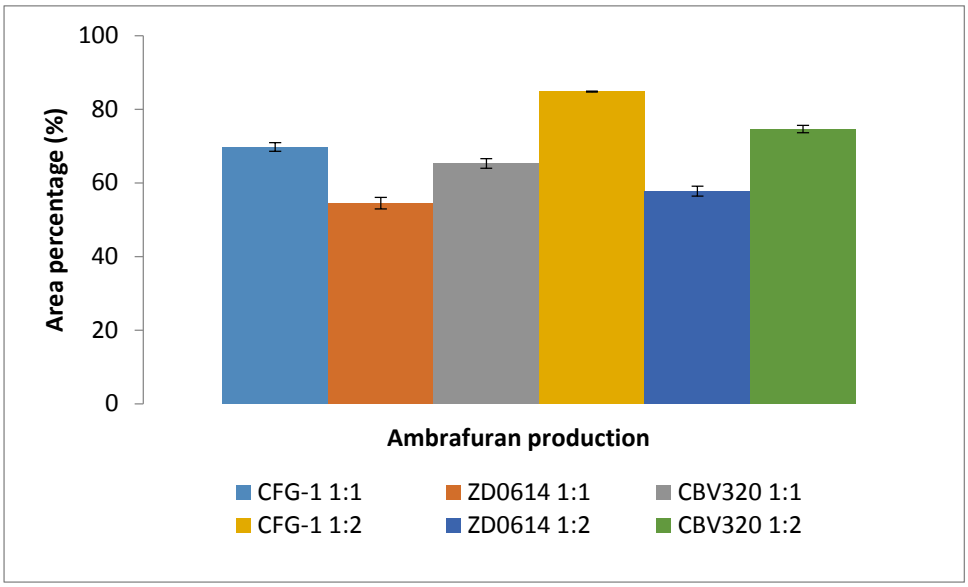


Figure 39: Results for ratios of 1:1 and 1:2 tested for production of (-) ambrafuran using zeolites CFG-1, ZD0614 and CBV320 at 60˚C

The legends entry represents the zeolites identity with the ratios used (ambradiol per zeolite).

The transformation of ambradiol was completed in all zeolites at 1:2 ratios and the highest yield of (-) ambrafuran was 84.81% obtained from the CFG-1 zeolite, while there was two unidentified peaks eluting at retention times of 3.372 and 3.545 mins. Zeolite ZD0614 formed an unidentified compound peak which eluted close to (-) ambrafuran and CBV320 zeolite yielded at least 74.6% (-) ambrafuran despite the two unidentified peaks. Ambradiol transformation was not complete in a 1:1 ratio for all the zeolites and the unidentified compounds peaks eluting at similar retention times as in 1:2 ratio were also noticed. Similar types of reaction were carried out for 24 hours and the results (not shown) were not much different from these. The two unidentified compounds peaks eluting at retention times of 3.372 and 3.545 were regarded as a major drawback as they reduced the productive yield of (-) ambrafuran.

4.3.8 Measuring the increased concentration of ambradiol for (-) ambrafuran production

The concentration of ambradiol was increase from 5 mg/mL to 100 mg/mL and zeolite CFG was used to catalyses the reaction. The aim was to determine the production of (-) ambrafuran at high concentration of the intermediate diol. The reaction was conducted at 60˚C for 7 hours

and the method was detailed in section 3.4.8 and Table 6 below shows the results for (-) ambrafuran produced.

Table 6: Illustrates the production of (-) ambrafuran at high concentration of intermediate diol in area percentage yield

Area % yield of (-) ambrafuran	Area % of unknown compounds	Standard deviations of (-) ambrafuran
85.0	15.0	0.36

The yields of (-) ambrafuran in these results are the average values of experiments conducted in triplicate (n=3).

Ambradiol was successfully converted; however the two unidentified compounds limited the production of (-) ambrafuran to 85% as seen in Table 6. The triplicate reaction showed a good variation as the standard deviation was 0.36. The results show a slight difference compared to the low concentration of ambradiol used in previous experiment in 4.3.7 which yielded 84.81%. This suggests that (-) ambrafuran may be produced at high ambradiol concentration at 1:2 ratio by zeolite CFG-1, however it may requires to undergo purification.

4.3.9 Determination of the recycled zeolite CFG-1 for production of (-) ambrafuran

The recycled zeolite CFG was tested for (-) ambrafuran production in this experiment at 1:2 ratios (10 mg diol and 20 mg zeolite) in 1 mL hexane. The zeolite was washed with ethanol and dried at 100˚C for overnight before used. Production of (-) ambrafuran was analysed by GC and the results are presented in Table 7 below. The purpose of the experiment was to determine the effectiveness of the recycled zeolite for production of (-) ambrafuran compared to the fresh zeolite.

Table 7: Demonstrates (-) ambrafuran production by recycled zeolite CFG-1 in area percentage

Area % yield of (-) ambrafuran	Area % of unknown compounds	Standard deviations of (-) ambrafuran
82.2	17.8	0.8

The area percentage yields of (-) ambrafuran are average values of the experiment conducted in triplicate (n=3).

The recycled zeolite CFG-1 was able to completely convert ambradiol but the two unknown compounds were appeared as in the previous experiments. The percentage yield of 82.2% (-) ambrafuran was obtained from the recycled zeolite CFG-1 as seen in Table 7. The unknown compounds inhibited the full production of ambrafuran by 17.8%. These results show a slight decrease of almost 3% compared to the fresh zeolite reaction. This proves that the recycled zeolite may be re-used for the production of (-) ambrafuran and purification may be required for high purity.

4.3.10 Determining the production of (-) ambrafuran from impure ambradiol using zeolite CFG-1, CBV320 and ZD06014

Ambradiol with unknown compounds eluting close to it was used in this experiment for (-) ambrafuran production. Zeolites CFG-1, CBV320 and ZD06014 were all tested with the contaminated intermediate ambradiol at 1:2 ratio in 1 mL (10 mg diol and 20 mg of each zeolites) as in previous experiment. Results are demonstrated in Table 8 below.

Table 8: : Show results for (-) ambrafuran production from contaminated intermediate diol by zeolite CFG-1, CBV320 and ZD06014

Area % of (-) ambrafuran from CFG-1	Area % of (-) ambrafuran from CBV320	Area % of (-) ambrafuran from ZD06014
52.1	33.8	31.9

The area percentage yields of (-) ambrafuran are average values of the experiment conducted in duplicate (n=2).

Zeolite CFG-1 proved to be the most effective compared to CBV320 and ZD06014 by yielding 52.1% (-) ambrafuran in an impure ambradiol reaction. The impure ambradiol was completely converted at zeolite CFG-1, however the unidentified compound eluting close to ambradiol remained with 24.2% yield. The two unknown compounds that were reported in previous experiments were also observed and contributed 16.3%. There were three additional compounds observed with each contributing ≤ 3%, see appendix in Figure 41. This was also observed in a CBV320 reaction, however the unidentified compound eluting close to ambradiol was 42.4% and the commonly two unknown compound with a combined yield of 16.8%. In ZD06014 reaction, ambradiol was not completely converted with 3.8% left while the unidentified compound eluting close to it contributed 41.5%. Lots of unknown

compounds were observed with the yields of ≤ 2% and the common two identified compounds contributing the total of 12%, see appendix in Figure 42. These results indicate that a pure ambradiol may be required for the cyclodehydration step to produce (-) ambrafuran with high purity.

4.3.11 Determining the effect of magnesium sulphate ($MgSO_4$) anhydrous and molecular sieves for production of (-) ambrafuran at 60˚C

During the reduction of the zeolite ratio, two unwanted peaks formed in the production of (-) ambrafuran and the hypothesis was that water which formed during the cyclodehydration may cause this. The use of the low ratios of zeolite to substrate compared to the initial work by Steenkamp and Taka (2010) might not absorb the water formed, giving rise to the unwanted compounds. This warranted the addition of a small amount of magnesium sulphate anhydrous or molecular sieves in the reaction. In this experiment, we demonstrated how the addition of magnesium sulphate anhydrous and molecular sieves can enhance the purity of ambrox produced. The purpose of this experiment was to trap the formed water which is produced as a result of the cyclodehydration to increase the purity of (-) ambrafuran and Table 9 below outlined the outcome of the experiment.

Table 9: The percentage yield of (-) ambrafuran from a reaction with addition of non-activated magnesium sulphate anhydrous and molecular sieves

Reaction with added:	Reaction temperature (˚C)	Total reaction time (Hours)	Yield of (-) ambrafuran (%)	Area percentage of the two unknown peaks (%)
MgSO4 anhydrous	60	7	87.9	12.1
Molecular sieves	60	7	87.9	12.1

The yields of (-) ambrafuran in these results are the average values of experiments conducted in triplicate (n=3).

There was a slight decrease of yield for the unidentified compounds from both reactions with either magnesium sulphate anhydrous and molecular sieves. This confirmed that the

unidentified compounds were formed due to the water effect from the cyclodehydration. The area percentage yield of (-) ambrafuran was similar for both reactions and yielded at least 87.9% from the 84.81% in a reaction without the adsorbed as seen in Figure 39 above. This proved that both magnesium sulphate anhydrous and molecular sieves may contribute in trapping off water from the cyclodehydration reaction. The overall reduced yield of unidentified compounds was almost 3% from both reactions with magnesium sulphate and/or molecular sieves and warranted further investigation.

4.3.12 Examining (-) ambrafuran production using anhydrous activated $MgSO_4$ & molecular sieves in combination with zeolite CFG-1

The reaction for production of (-) ambrafuran involving the magnesium sulphate anhydrous and molecular sieves was to minimise water content in the reaction. The magnesium sulphate and molecular sieves were activated in an oven before addition of the reaction. Molecular sieves spheres were crushed into fine powder form before activated as mentioned in method section 3.4.12. The results for production of (-) ambrafuran are shown in Table 10 below.

Table 10: The percentage yield of (-) ambrafuran from a reaction with addition of activated magnesium sulphate anhydrous and molecular sieves

Reaction with added:	Reaction temperature (˚C)	Total reaction time (Hours)	Yield of (-) ambrafuran (%)	Area percentage of the two unknown peaks (%)
Activated $MgSO_4$ anhydrous	60	7	90.7	9.3
Activated molecular sieves	60	7	90.6	9.4

The yields of (-) ambrafuran in these results are the average values of experiments conducted in triplicate (n=3).

The (-) ambrafuran yield increased to ≥90% in both reactions containing dried magnesium sulphate anhydrous and molecular sieves respectively. The unknown compounds were not

completely separated during the reaction. These results suggest that either magnesium sulphate anhydrous and/or molecular sieves absorbed water that was suspected to have been the cause of forming the unknown compounds. The ambradiol was successfully transformed.

4.3.13 Addition of dried $MgSO_4$ anhydrous in a test reaction for (-) ambrafuran production by zeolite CFG-1 at 60˚C

The amount of dried magnesium sulphate anhydrous was increased in this experiment and the activation time was extended to 24 hours. The intention of this investigation was to absorb more water coming from the cyclodehydration and to eliminate the formation of the unknown compounds from the process for production of (-) ambrafuran. Table 11 illustrates the yield of ambrafuran obtained during the experiment.

Table 11: The results of (-) ambrafuran produced from a test reaction with added magnesium sulphate anhydrous activated for 24 hours at 100˚C

Reaction with added: (0.1 g)	Reaction temperature (˚C)	Total reaction time (Hours)	Yield of ambrafuran (%)	Area percentage of the two unknown compounds (%)
Activated magnesium sulphate anhydrous	60	7	92.6	7.4

These results are presented by the average values of experiments conducted in triplicates (n=3).

The unknown compounds were very difficult to completely eliminate and their combined yield was 7.4%. The (-) ambrafuran yield was increased gradually and still required some treatment to be complete pure, see appendix Figure 44. This result implied that more magnesium sulphate anhydrous needed to be added into the reaction for actual reduction of the unidentified compounds. The unfortunate thing is that these unknown compounds were not reported in studies by Steenkamp and Taka (2010) and Carey (2013). Steenkamp and Taka, (2010) have produced (-) ambrafuran with the highest yield of 98% and this might be as a results of higher amounts of zeolite used which could had absorb more water during the reaction.

5. CONCLUSION

The aim of the overall project was to develop technology for a client, Teubes cc for the commercial production of (-) ambrafuran. An initial two-step process was developed by Steenkamp and Taka (2010) and patented. The downside of the process was the use of a high ratio of zeolite to substrate as well as the volume of hexane required. This two-step patented method was a significant improvement on the current commercial production of (-) ambrafuran which is done in at least 8 steps and require very harsh chemicals and elevated temperatures. The use of pyridine in the last step during current commercial production has an influence on the final fragrance and has been overcome by the use of zeolites in the new patented process. Optimisation of the process which included testing different new zeolites was necessary to give a commercially viable process.

Conversion of sclareol to intermediate ambradiol using microorganism *H. roseoniger* was successfully achieved in a potato dextrose media following several optimisation experiments. This intermediate ambradiol served as the starting material for production of (-) ambrafuran with the use of zeolite. Two unwanted products formed during the fermentation step using PDB as the medium for the conversion of sclareol to ambradiol. This impure ambradiol product was used with zeolites to determine if it has an influence on the final (-) ambrafuran, but resulted in new unwanted compounds. A downstream method was also tested to separate unreacted sclareol from ambradiol should a large scale run not go to completion. It was possible by extracting the reaction mixture with ethyl acetate followed by hexane to separate the substrate from the product. Production of ambradiol was further scaled-up in a fermentation bioreactor and the yield was 93% at 24 hours of reaction. This will be a significant improvement in the technology as the complete biotransformation of sclareol to ambradiol was achieved in 13 days as opposed to the previously achieved 16 days.

Following the production of ambradiol from sclareol in a single biotransformation reaction using *H. roseoniger* compared to the 7 steps required by chemical synthesis of the intermediate diol, the diol has to be converted to the (-) ambrafuran. The previous work by Steenkamp and Taka (2010) identified the use of a zeolite, CBV320 which required initial activation for this final conversion.

Two new zeolites (CFG-1 and ZD06041) were identified which did not require activation before use and the ratio of substrate per zeolite was reduced. The most effective zeolite was

CFG-1. The yield of two unknown compounds formed during the process for production of (-) ambrafuran was reduced by approximately 8% through the addition of activated magnesium sulphate anhydrous and molecular sieves respectively. Further investigation such as addition of higher concentrations of magnesium sulphate, recrystallization and small increases in the zeolite concentration may be warranted to achieve high purity of (-) ambrafuran and eventually eliminating the unknown compounds. The work also illustrated that higher concentrations (more than 5 mg/mL and up to 100 mg/mL) can be converted successfully. This will also contribute to the feasibility of the technology as it resulted in a significant reduction of the volume of hexane required. Lower volumes of solvent will be a positive outcome on commercial scale.

The process developed during this work illustrated that the production of (-) ambrafuran should be viable on larger scale and that the final product should carry a "natural" label as the initial step is done via biocatalysis using the *H. roseoniger* microorganism, while the second step uses the benign zeolites which can be re-used. Through the re-use of the zeolites as well as recycling of the hexane solvent, the process will result in an environmentally friendly process as it will have limited impact on the environment in terms of waste disposal. This is in sharp contrast with the current commercial process requiring the waste disposal of very harsh chemicals such as pyridine, tosylchloride and butyllitium to name but a few.

The aim of the project was therefore achieved and scale-up of the complete process to approximately 10 L scale should give the data necessary to finalise a technology demonstrator which will enable the client to commercialise the process for the production of (-) ambrafuran.

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APPENDIX

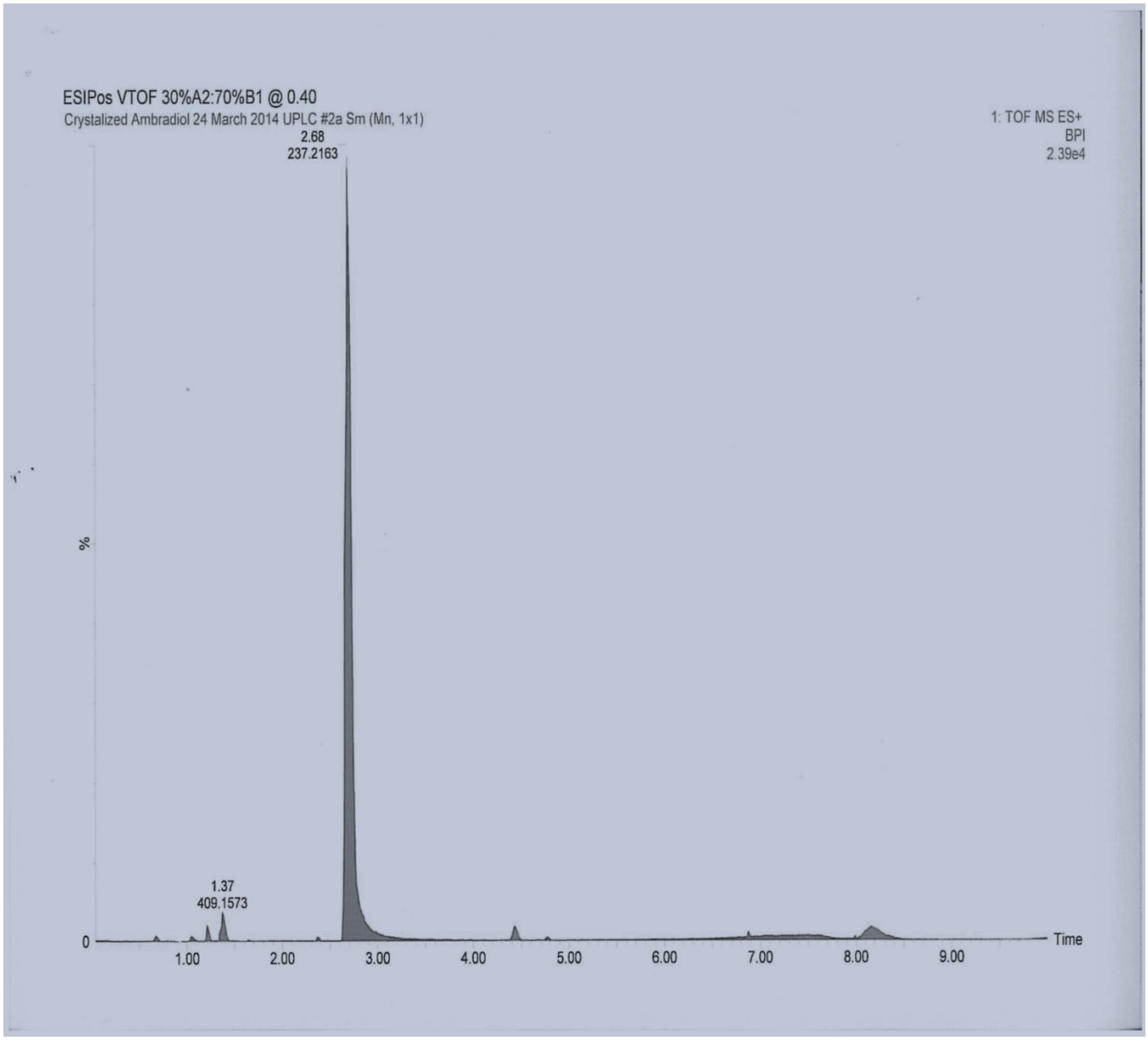

Figure 40: A chromatogram for recrystallized ambradiol analysed by UPLC-MS

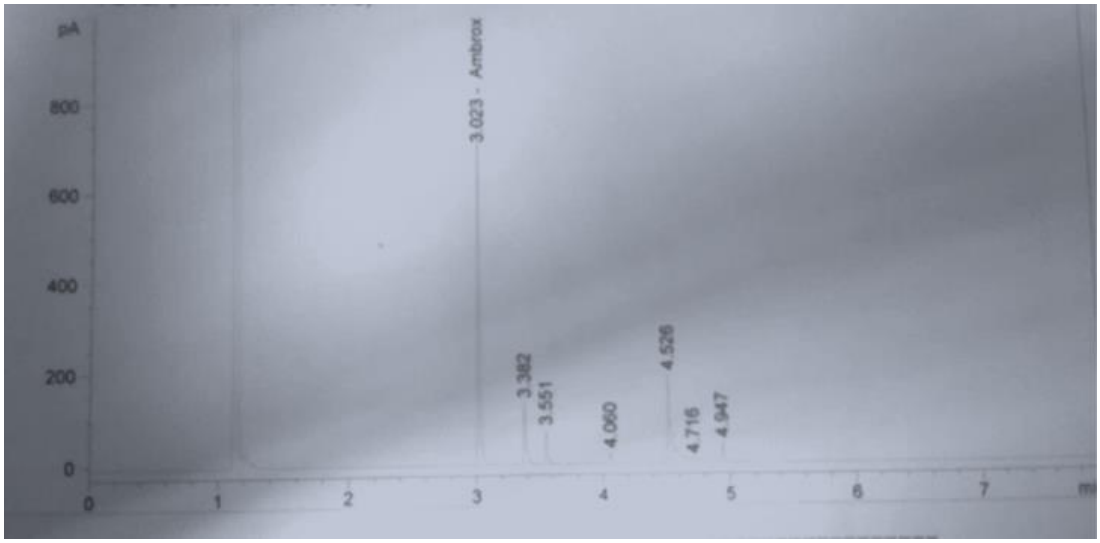

Figure 41: A picture of chromatogram showing (-) ambrafuran peak produced from impure/contaminated ambradiol by zeolite CFG-1

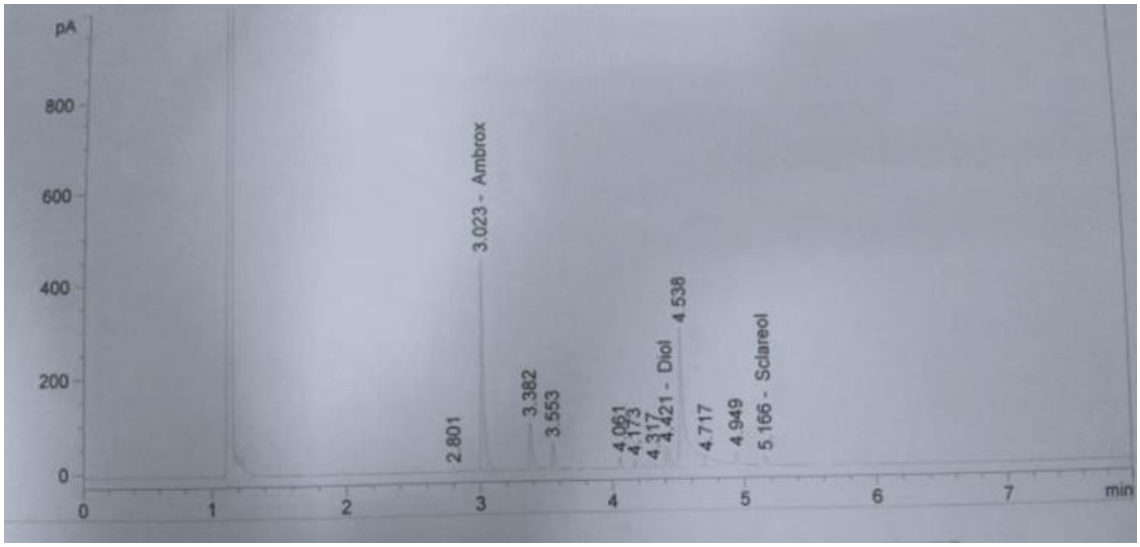

Figure 42: A chromatogram showing (-) ambrafuran peak produced from impure ambradiol by zeolite ZD06014

Figure 43: (-) Ambrafuran peak produced in a reaction without $MgSO_2$ anhydrous by zeolite CFG-1

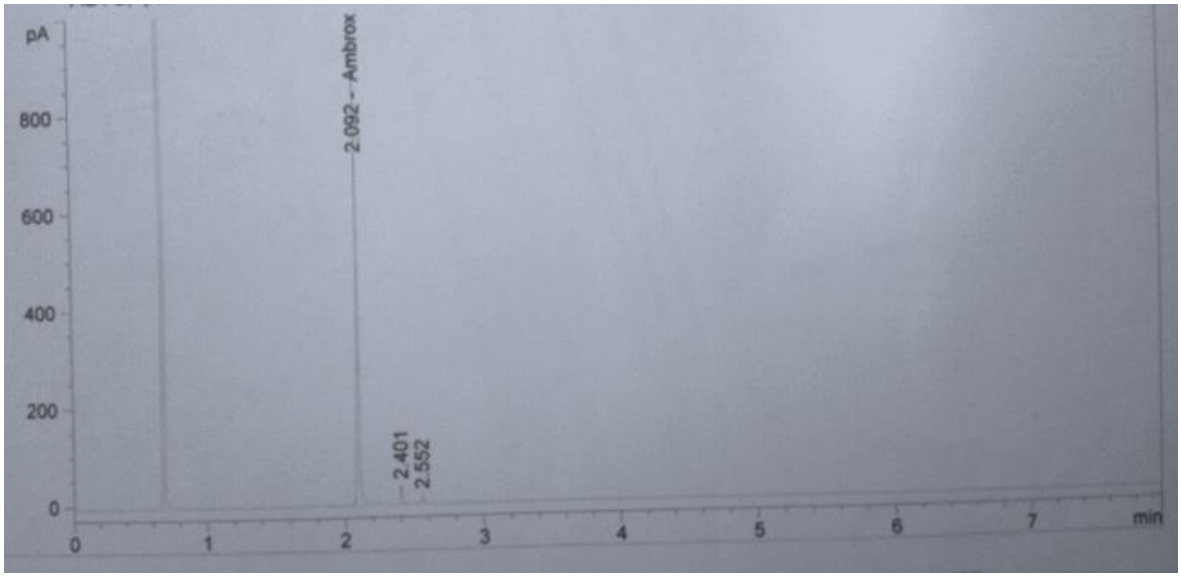

Figure 44: (-) Ambrafuran peak produced in a reaction with $MgSO_2$ anhydrous by zeolite CFG-1