EXTRACTION OF VAULE-ADDED CHEMICALS FROM BIOREFINERY RESIDUES

by

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B.S., Agricultural University of Hebei, China, 2013

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2015

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Abstract

Large quantities of byproducts are generated during the biomass processing, which leads to under-utilization of resources and concomitant waste disposal problem. Typically, these byproducts still contain considerable amounts of high-value compounds that have important industrial applications. However, in current time, most of these byproducts are used for low-value applications, including as boiler fuel. These byproducts are potential sources for many valuable chemicals such as antioxidants, flavors, colorants, preservatives, and biocides. Therefore, the reuse and recycle of these biomass residues is very important for the bioeconomy. By some additional and necessary processing steps, we can transfer those biomass residues from a low-value level to a higher value status and apply the final products to various fields such as food industry, and pharmaceutical industry, etc. Till date, limited research has been reported in the production of important specialty chemicals from biomass residues. This thesis is focused on the solvent extraction and isolation of valuable chemicals from bioprocessing byproducts. While choosing different solvents and techniques, "environmental friendly" green solvents were also evaluated. Also some new techniques, such as thin-layer chromatography plates making and laboratory-made lignin are developed to make the research more economically feasible. Even though conventional extraction method such as solid-liquid extraction was evaluated, we tried to minimize the solvent/biomass ratio and also augmented additional processes to the conventional process to obtain higher yield of compounds of interest (COI).

In this research, different biomass resources were evaluated for valuable specialty chemicals. These resources include: lignocellulosic biomass and raw biomass. Lignocellulosic biomass is a sustainable feedstock for the production of biofuels and chemicals. The potential chemicals from the resources were extracted using various organic solvents and analyzed by gas

chromatography-mass spectrometry (GC-MS). The results indicated that the selected biomass residues contain relatively higher amounts of three valuable compounds: vanillin, apocynin, and phytol. Different types of organic solvents and extraction techniques were tested to optimize the extraction process. Ultra-sonication was considered as an efficient extraction method and ethanol was chosen as the final solvent. Commercially viable isolation methods such as thin-layer chromatography (TLC) and column chromatography were also studied in this research. A solvent system of hexane, dichloromethane, ethyl acetate, and chloroform with 1:1:1:0.1 v/v ratio gave us a good separation of the COI. Biomass-derived lignin was made in the laboratory to compare with commercially available lignin. The results show that the laboratory-made lignin contains similar bioactive compounds and gives us a good quantity of target compounds.

In conclusion, instead of letting the byproducts being discarded or used as low-value applications or become a threat to the environment, the decision to select them as raw materials to produce valuable specialty targeted compounds for industries has been demonstrated in our research. The future research will focus on optimization and scale-up study of the extraction process. In addition, the application and production of bioactive compounds will be further evaluated.

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Acknowledgements

I want to express my sincerely gratitude to my advisor Dr. Praveen V. Vadlani for his constant advice and thorough guide during the whole project. Dr. Vadlani is an excellent advisor, he is always there to listen to what I thought and encourage me to try new ideas. He taught me how to be a good graduate student and how to start something new. He is also a good friend, always be willing to help me out and asking about my daily life. I feel so lucky to have him as my advisor.

I also want to give my truthfully thanks to my committee members, Dr. Sun and Dr. Ambrose. Dr. Sun works on various areas that includes biological materials and kinetic properties, and Dr. Ambrose also worlds on process modeling. I took their courses and learned a lot from them. Also thanks Dr. Sun provided lab facilities for my research.

I wish to express my gratitude to Krishna Bastola. As a post doctor in our lab, he taught me a lot of lab techniques hand by hand and gave me a lot of suggestions during my experiments. Every time I had questions, I went to talk to him and discussed what next we should do. He helped me to stay in the right track.

Also many thanks to all my lab mates who gave me many supports and help whenever I needed. They brought a lot of happiness to me and I feel honored to work with them. Thanks for all. Specially, I want to thank all my families for understanding and supporting me all the time. They are the most important person in my life and I appreciate all they did. Thanks for always being there for me.

Finally, I am grateful to DOE-ARPA-E for funding this Project through Plants Engineered to Replace Oil (PETRO) program.

Preface

This thesis is the result of a master research project "Extraction of value-added chemicals from biorefinery residues". The thesis consists of 6 chapters.

Chapter 1 provides an introduction of the main subjects involved in this thesis. It covers the background, objectives and results. This research consists of three major objectives: 1) Evaluate value-added chemicals from lignocellulosic biomass and raw biomass materials; 2) Investigate different extraction methods for optimization of compounds of interest (COI) extraction from aforementioned biomass; 3) Investigate an efficient method for COI isolation and raw biomass samples.

Chapter 2 describes the extraction and analysis of lignocellulosic biomass and raw biomass materials. Seven different biomasses were screened, and the extract constituents were qualitatively and quantitatively evaluated using gas chromatography mass spectroscopy (GC-MS). The GC-MS chromatogram was analyzed for the detection of value added compounds. The selection of the compound as value added product was based on its commercial value and its concentration in the extract. The chromatogram indicated the presence of many compounds of high commercial value but at low concentration. Such compounds were not considered as the commercially viable value added products. Based on the above selection method, we identified three compounds (vanillin, apocynin, and phytol) as the value added compounds of interest (COI) extracted from the lignocellulosic biomass material.

In Chapter 3, different extraction methods for COI were investigated for solvent and extraction procedure optimization. The different extraction methods used were bead beating and ultra-sonication. Bead beating was rapid and large number of samples could be extracted in shorter

period of time (15 minutes). The extraction by ultra-sonication required more time (45 amplitude@ 1hour), but was more efficient when compared to bead beating method.

Chapter 4 illustrates the qualitative and quantitative isolation techniques used for the separation of COI from the biomass extract. Thin-layer chromatography (TLC) technique was used to evaluate the tentative number of extract constituents, determine the retardation factor (Rf) value of the COI using standards, optimize the mobile phase for the good separation of COI components in the TLC plate, and use the optimized phase solvent composition in the preparative scale isolation of COI by silica column chromatography.

Chapter 5 discusses the conversion of biomass into lignin rich material. It is anticipated that large quantity of lignin will be generated during biomass processing for biofuels and chemicals. Evaluation of these cost-effective lignin sources for COI is essential to generate additional revenue streams for the biofuels industry.

In Chapter 6, the main conclusions of this research are presented and as well as some suggestions for future work are provided.

Chapter 1 - Introduction

Bioactive compounds

Bioactive compounds are secondary plant metabolites synthesized by plants that can elicit pharmacological or toxicological effects in man and animals (Herbert, 1989; Aksel. 2010). Some of the well-known bioactive compounds are terpenoids (Rohdich et al., 2000), phenolic compounds (Stafford, 1967), and alkaloids (Leete, 1965). Based on certain criteria, bioactive compounds in plants are classified into groups such as glycosides, flavonoids, etc. Bioactive volatiles play a vital role in nature as signaling compounds for communication between species (Buchs et al., 2011). For instance, plants release volatiles to attract insects or protect themselves from herbivore attack (Kessler et al., 2006; Heil & Bueno, 2007). They also play important role in the plant growth process and affect plants in various ways. For example, some compounds in tomatoes such as aldehydes and esters are responsible for the flavor of tomatoes (Sucan et al., 2002), some of the bioactive compounds are components of the plant biomolecules (carbohydrates, proteins, lipids, etc.), and some of them are not directly needed for daily functioning even if they are products of plant cells. However, these bioactive compounds have huge applications as functional ingredients in the pharmaceutical, food and feed industries (Sacchetti et al., 2005). As shown in Figure 1.1 (Christaki et al., 2012), the food industry uses the compounds as flavors, and fragrances; and the pharmaceutical industry uses them for their functional properties (Lubbe & Verpoorte, 2011); while the feed industry utilizes them for antioxidants, the cosmetic industry uses them as perfumes, and skin products. Essential oils are important in the food and cosmetic industry because of their characteristic taste and odor (Arce A et al., 2007).

Different extraction methods

Several considerations should be taken into account during the extraction process, including the polarity and stability of the extractives and the solvent, the volatility, viscosity, and toxicity of the extraction solvent (Bucar et al., 2013). Many methods have been reported for extraction, such as solid phase extraction (Smith, 2013; Lin et al., 2013; Sigma-Aldrich Co., 1998), distillation (Lin et al., 2013; Navarrete et al., 2011; Mashkatalsadat et al., 2011; Teixeira et al., 2007), liquid-liquid extraction (Furniss et al., 1989; Skoog & West, 1986; Kennedy, 1984) and solid-liquid extraction (Jerman, 2010; Yu et al., 2010), etc. However, these methods also have some drawbacks. Distillation is energy intensive and the final composition may still contain significant impurities (Hornburg & Cruver, 1977). Liquid-liquid extraction might cause the loss or degradation of the COI due to the solvent evaporation (Hernanz et al., 2008).

Several new extraction methods have been developed, to add to the conventional extraction methods, to improve the extraction efficiency. Methods include ultrasonic extraction (Jerkovic et al., 2011, Jerman, 2010; Leblanc et al., 2009; Trusheva et al., 2007), microwave assisted extraction (Lin et al., 2013; Cui et al., 2008; Trusheva et al., 2007), and physical methods such as bead beating (Hansen et al., 2014; Kim et al., 2014). These techniques can reduce the consumption of solvents as well as simplify and increase the speed of the extraction process (Biesage & Pyrzynska, 2013). In this research, all the extraction methods are based on the conventional solid-liquid extraction along with assisted methods such as bead beating and ultrasound-assisted methods. Solid-liquid extraction is a classic extraction method that allows the soluble compounds to be removed from solids to solvents. Very common applications of solid-liquid extraction are isolation of oil or phenolic compounds from the plant materials (Jerman et al., 2010). The basic principle of solid-liquid extraction is the diffusion of solute molecules from the solid matrix surrounded by the

extracting liquid (Naviglio et al., 2014). The interaction between the molecules of solvent and solute can be explained by "like dissolves like" principles (Reichardt & Welton, 2011). Table 1.1 shows the relationship between solubility and polarity of the solvents and solutes. It allows us to predict solubility in designing experiments. Polar compounds have large interactions with each other, while nonpolar compounds have small interactions (Reichardt & Welton, 2011).

Solvent and green chemistry

From sustainable chemistry perspective, organic solvents are not that favored. The best solvent would be no solvent based on the "environmental friendly" rule. Jiao et al. (2014) tried microwave-assisted aqueous enzymatic method to extract the oil from pumpkin seeds, making the process more environmental-friendly. However, enzymatic methods cost more than conventional methods of extractions. Therefore, organic solvents are still widely used for extraction of bioactive compounds (Reichardt & Welton, 2011).

Researchers are still trying to find "greener solvents" as alternative to organic solvents in order to reach a balance between the economy and the environment. The green chemistry concept—the reduction of the use of hazards substances in the manufacture, design and application of chemical products—has been developed since the 1990s (Anastas & Warner, 1998; Anastas & Eghbali, 2010). In addition, reducing the use of organic solvent is also very important to the green chemistry endeavor. Many solvents are volatile compounds that can evaporate easily in the environment (Smallwood, 1993). This can cause acute toxicities as well as greenhouse gas emissions (Jimenez-Gonzalez et al., 2004). However, it is not always possible to reduce solvent use. In this case, it is necessary to choose the solvents that are less hazardous to the environment. Too many solvents have been considered as "green solvents" without having a very convinced claim. Usually, ethanol is commonly considered as a green solvent, but other solvents are still

needed to be verified. A green solvent should be “green” based on the environmental effects, including their synthesis, use and disposal (Jessop, 2011). The Pfizer “traffic light” system (Table 1.2) is a widely used tool to give an overall green solvent selection (Alfonsi et al., 2008). In this selection system, “Preferred” solvents are more safer to the environment and are preferred; “Usable” solvents are of some environmental concern but still can be considered when there is no other suitable solvents from the preferred list; “Undesirable” solvents are more hazardous to the environment and their use should be replaced by other safer solvents or non-solvent-based extraction methods.

Gas chromatography and mass spectrometry

GC-MS is an analytical method that combines the features of gas-liquid chromatography (GC) and mass spectrometry (MS) to identify different substances within a test sample. It is the ubiquitous analytical technique for the identification and quantitation of organic substances in complex matrices (Sparkman, 2012). James and Martin (1952) originated the use of a combination of mass spectrometer and gas chromatography, after that the use of GC-MS started to be developed. These comparatively sensitive devices were originally limited to laboratory settings. Nowadays, GC-MS is widely used as an identifying and structure-analyzing tool. For instance, it can obtain the compositions of volatile compounds of the oils (Brenes & Roura, 2010). The combination of GC and MS is becoming the definitive analytical tool in the research and commercial analytical laboratory. This combination offers several advantages (Abian, 1999). The most important is that it separates components of the analyte and provide unique information about the components; second, it has high sensitivity for volatile compounds and can be used for quantitative purposes. GC isolates the components of the samples, and MS is a direct method for the identification of bioactive compounds (Tashiro & Imoto , 2011). The configurations of GC-

MS might varies, but them all share common types of components (Marvin, 2008). For instance, an injector is needed for getting the simple into the chromatogram; a packed column with a stationary phase has to be there for separation; and there must be a gas chromatography to connect the injector to the column and out to the mass spectrometer interface. In addition, a data/control system is required for data processing (Figure 1.2).

Thesis Motivation and Objectives

Large quantities of byproducts are produced during the bioprocessing of renewal resources. These byproducts are mainly utilized for low value applications or even become environmental problems if not treated properly. This thesis comprises three research parts and each part has an objective. The first objective of this research is to find value addition of the biomasses that are commonly used for fermentation to produce biofuels and different platform chemicals, including butane-2,3 diol, D-lactic acid, ethanol. The left over solid residues, which are usually discarded or used for low value applications, are good sources for isolation of value added compounds. These value-added compounds include natural oils, antioxidants, colorants, fragrances, preservatives, biocides and other bioactive substances (Seabra, 2010). The second objective of this research is to find an efficient extraction method for valuable compounds from the solid residues. Different organic solvents and extraction methods were investigated to obtain optimum yield of the target compounds. The third objective is the isolation of the target compounds from the constituent mixtures of the extract by thin-layer chromatography and column chromatography. In the last objective, biomass-derived lignin was made in the lab and compared with commercial lignin for COI extraction.

Table 1.1 Solubility and polarity (Pimentel, 1963)

Solute A	Solvent B	Interaction			Solubility of A in B
		A…A	B…B	A…B	
Nonpolar	Nonpolar	Weak	Weak	Weak	Can be high [a)]
Nonpolar	Polar	Weak	Strong	Weak	Probably low [b)]
Polar	Nonpolar	Strong	Weak	Weak	Probably low [c)]
Polar	Polar	Strong	Strong	Strong	Can be high [a)]

a) Not much change for solute or solvent

b) Difficult to break up B…B

c) Difficult to break up A…A

Table 1.2 The Pfizer "traffic light" solvent preference system (Alfonsi et al., 2008)

Preferred	Usable	Undesirable
Water	Cyclohexane	Pentane
Acetone	Heptane	Hexane (s)
Ethanol	Toluene	Di-isopropyl ether
2-Propanol	Methyl cyclohexane	Diethyl ether
1-Propanol	t-Butyl methyl ether	Dichloromethane
Ethyl acetate	Isooctane	Carbon tetrachloride
Isopropyl acetate	Acetonitrile	Dimethyl formatmide
Methanol	2-Methyl-THF	*N*-Methylpyrrolidinone
Ethyl methyl ketone	Tetrahydrofuran	N-Methylpyrrolidin-2-one
1-Butanol	Xylenes	Pyridine
t-Butanol	Dimethyl sulfoxide	Dimethyl acetate
	Acetic acid	Dioxane
	Ethylene glycol	Dimethoxyethane
		Benzene

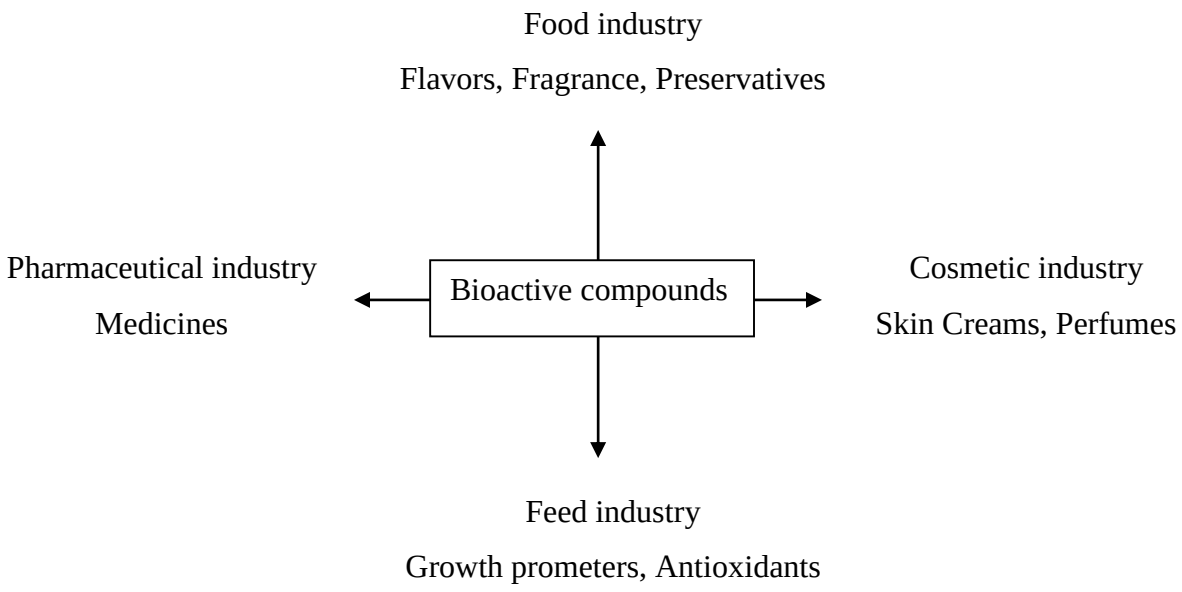


Figure 1.1 Common applications of bioactive compounds

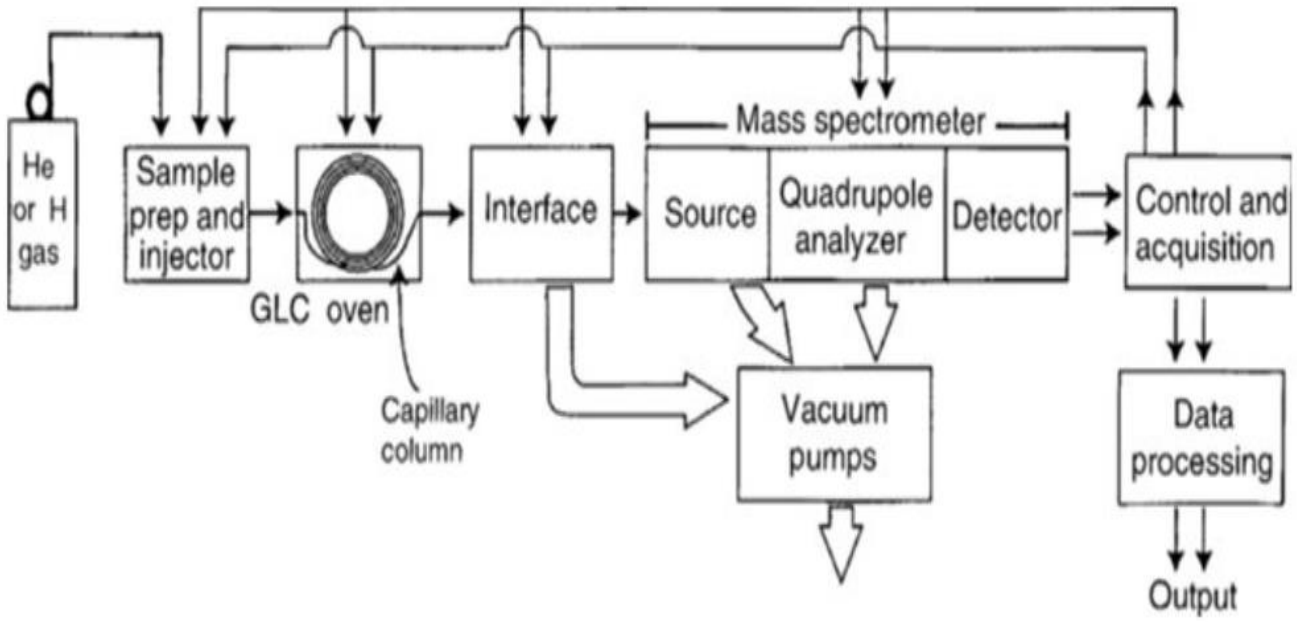


Figure 1.2 A typical GC-MS system diagram

Chapter 2 - Screening of value-added bioactive compounds of different lignocellulosic biomass.

Introduction

Different biomass residues

Seven types of lignocellulosic biomass were evaluated for the production of valuable bioactive compounds. Lignocellulosic biomass is one of the most important resources for sustainable development due to its abundance, low cost, and renewable character (Parajo et al., 2008). Lignocellulosic biomass is a complex network of structure consisting of mainly three polymeric components: hemicellulose, cellulose, and lignin. The polymeric carbohydrate (cellulose and hemicellulose) of lignocellulosic is a good source of sugars, which can be converted into different platform chemicals and biofuels by fermentation process. The complex network structure of biomass is not easily fermentable by microorganisms. Therefore, pretreatment is required to remove lignin from the biomass materials (Cheng et al., 2011). Lignin is a polymer which consists of phenyl propane molecular units (Freudenberg, 1965; Nada et al., 1998) and it is the major composition of lignocellulosic biomass; other compositions exist as waxes, minerals and chemical compounds (Chowdhury, 2014). Lignin is abundant in nature; however, they are used either as a fuel source or for other low-value applications. Demand of full utilization of lignocellulosic biomass continues to increase due to the production of high-value chemicals (Zhang & Vadlani, 2013). Some strategies have been developed so far towards finding proper ways of the lignin utilization, such as converting lignin to biofuels or other value-added products; depolymerizing the lignin to simple units that can be regarded as useful resources for higher value chemicals (Chowdhury, 2014). In this research, seven types of lignocellulosic biomass were evaluated for the production of valuable bioactive compounds. These include raw biomass,

biomass residue after fermentation, commercially available lignin called protobind lignin (Protobind-1000, 2400 and 5000). Protobind series are commercially available byproducts of bioprocessing.

Solvent extraction

The objective of this chapter is to screen different types of bioactive compounds from different lignocellulosic biomasses. Methanol, ethanol and dichloromethane were selected as the screening solvents due to their different polarities. According to "like dissolves like" principle (Reichardt & Welton, 2011), various types of solvents can help us extract wide range of nonpolar and polar bioactive compounds. Dichloromethane is a non-polar solvent, while methanol and ethanol's polarities are much higher. Dichloromethane is used to extract nonpolar and medium polar compounds. Methanol and ethanol being polar solvents extract polar bioactive compounds. The combination of the non-polar and polar solvent are suitable to extract wide range of non-polar and polar compounds.

Materials and methods

Lignocellulosic biomass

Four types of lignin materials and three types of raw biomass were used for the extraction study of valuable compounds. Commercial lignin includes Lignin Protobind-1000, Protobind-2400 and Protobind-5000 (A.L.M. Pvt. Ltd. India) and alkali lignin (Sigma Aldrich, Batch #: MKBB6413). Raw biomass includes sorghum stalk (Texas A&M University), switch grass and corn stover (Kansas State University).

Extraction methods

The commercial lignin was used as received for extraction. The pretreated biomass (corn stover, switch grass, sorghum stalk) was first grinded (Laboratory mill 3033, Perten Instruments. Sweden) and then used for extraction. Extraction was performed using a bead beater (Mini Bead Beater-16, Model 607, Bartlesville). Three types of solvents were used for extraction, namely, Dichloromethane (DCM, Cat No. AC354800025), Methanol (MeOH, Cat No. A456-212), and ethanol (EtOH, Cat No. BP2818-500). All the solvents were of ACS grades and were purchased from Fisher Scientific.

One gram of lignin was mixed with six steel beads (3.2 mm dia. Chrome Steel, Cat No. 11079132c, BioSpec Products) and 4 mL of solvent (DCM, MeOH or EtOH) (Table 2.1) into a plastic vial. The vial was fixed to the Bead beater, and bead beating was performed for 30 seconds (x 3 times). The supernatant was collected and centrifuged (Centrifuge 5415 R, Eppendorf) at 24x3,75g for 10min. The supernatant of centrifuged sample was collected and filtered through a syringe filter of 0.45µm pore size to obtain clear solution for GC-MS analysis.

GC-MS analysis

GC-MS analysis was performed using a GCMS-QP2010SE (Shimadzu, Cat No. 226-20013-42) consisting of a gas chromatograph interfaced to a mass spectrometer instrument using following conditions: SHRXI-5MS capillary column (stationary phase: 5% diphenyl siloxane and 95%dimethyl siloxane) of length 30 m, 0.25 mm inner diameter, and 0.25 µm particle size. The MS analysis was performed using electron impact mode (70 eV). Helium was used as the carrier gas at constant total flow of 6.9 mL/min and 0.1mL/min column flow, and injection volume of 1µL was employed (split ration 6:1). Injector temperature and ion-source temperature were programmed at 250°C and 230°C, respectively. The oven temperature was programmed from 70°C

(isothermal for 2 min), with an increase of 5°C/min, to 250°C (isothermal for 2 min). Solvent cut time was set at 6 minute. Mass spectra were taken at 70 eV; a scan interval of 0.5s and fragments from 35 to 700 Da.

Results and discussion

Biomass selection

GC-MS is an excellent tool for the analysis of value added chemicals for lignin biomass. As evident from the GC-MS spectra (Figure 2.1-2.21), all the extracts of different lignin biomass samples have a complex composition. Using MS library data search, it was found that almost all the lignin biomass extracts contain considerable amount of vanillin, apocynin, Homovanillic acid, beta-amyrin, and phytol (Figure 2.1-2.21). GC-MS spectra of alkali lignin (Sigma Aldrich) extract indicated the presence of 47 identified compounds (Figure 2.2). Based on the yields, it was found that some of these compounds such as phenol, vanillin, apocynin, homovanillic acid, and benzene, are present in relatively higher amount. GC-MS spectra (Figure 2.4-2.12) of the three protobind lignin (Protobind-1000, Protobind-2400 and Protobind-5000) extracts, based on the yields, indicated that they contain more number of compounds than alkali lignin. Protobind-1000 and Protobind-2400 both contain three valuable compounds: vanillin, apocynin, and phytol. The yield of the chemicals from Protobind-1000 are relatively higher than yields from Protobind-5000. GC-MS spectra of Protobind-2400 (Figure 2.7-2.9) indicated the presence of almost 50 compounds. Based on the yields, it was found that some COI, such as 2-methoxy-4-vinylphenol, vanillin, tetraethylene glycol, and hexaethylene glycol are present in relatively higher amount. GC-MS spectra of raw biomass (Figure 2.13-2.21) indicated the presence of less number of compounds with relatively low concentration compared to alkali and protobind lignin.

The mass spectra of different biomass residues indicated the presence of number of valuable compounds. Some of the compounds are detected in relatively higher amount while others are present in very low amount. It is not feasible to classify all this compounds as value added products or commercially viable valuable chemicals. Only chemicals that are present in relatively high amount, and have high market value and wide application, are considered as the commercially viable compounds of interest (COI). Based on this concept, we identified three main compounds in the extract that can high market value. The three compounds are phytol, vanillin, and apocynin. The concentration of these compounds in all the aforementioned extract were compared (Table 2.3), and it was found that protobind-1000 is the most promising material for the economic extraction of the three compounds. It contains all the three target compounds and gives us encouraging yields. Other biomass, either they don't contain the the three compounds, or have low amount in the extract.

Selection of commercially viable valuable chemicals

Based on the aforementioned concept, we identified three main compounds in the extract that has high market value (Table 2.2). The three compounds are phytol, vanillin, and apocynin.

Vanillin is the most popular flavor compound in the world, and it has extensive applications in the food, fragrance and cosmetic industry (Sinha et al., 2008). It is also active against Germ-positive and Germ-negative food spoilage bacteria, yeasts and mounds in laboratory growth medias and fruit purees; thus has potential to be used as a food preservative (Dnyaneshwar et al., 2009). Because of its phenolic characteristic, vanillin can also be used as a chemical intermediate in the production of pharmaceuticals and fine chemicals, such as biocides (Hocking, 1997; Noubigh et al., 2010). Various extraction methods can be used to obtain vanillin from many plants.

Generally, vanillin is produced from vanilla bead pods. It can also be obtained from many other plants including tobacco, fruits, and fruit derived products (Dnyaneshwar et al., 2009).

Apocynin is a natural organic compound structurally related to vanillin. The difference in the chemical structure between vanillin and apocynin is that apocynin has a methyl group that is connected to the carbonyl carbon, whereas vanillin has a hydrogen atom instead of methyl group. Due to this small structure difference, vanillin and apocynin have altogether different physical and chemical properties, and differences in their applications. Apocynin can inhibit NADPH oxidase activity by blocking the formation of NADPH oxidase complex (Stolk et al., 1994). It is also commonly used as a standard NOX inhibitor for research purposes (Bedard et al., 2007). Shugo et al. (2013) studied the effect of apocynin on prostate carcinogenesis and found the percentages and numbers of carcinomas in both the ventral and lateral prostate were significantly reduced by apocynin treatment.

Unlike vanillin and apocynin, which contain aromatic rings, phytol is an aliphatic carbon chain with 4 methyl side chain group and 1 hydroxyl group. Phytol is an acyclic diterpene alcohol which can be used in cosmetics, fine fragrances, shampoos, toilet soaps as well as in non-cosmetic products such as household cleaners and detergents (Mcginty et al., 2010). It can also be applied to the synthesis of medicine, such as vitamin K1, health products, functional foods (Ping et al., 2011).

The concentration of three compounds in all the aforementioned extract were compared, and it was found that protobind-1000 is the most promising material for the economic extraction of the three compounds.

Optimum solvent selection

Different types of solvents were tested to optimize the efficient extraction of the three target compounds. Three different types of solvents, viz. MeOH, EtOH, and DCM were used for extraction. GC-MS chromatogram of protobind-1000 (Figure 2.5-2.7) extracted with different solvents indicated that methanol, dichloromethane and ethanol are potential solvents for the efficient extraction of vanillin and apocynin. Dichloromethane is a better solvent than methanol, ethanol, and mixed solvent for the extraction of phytol, respectively. Different parameters were considered to select the optimum solvent for extraction. The different parameters include extraction efficiency, environmental hazards, and solvent cost. Dichloromethane is environmental hazard solvent. Exposure to DCM affects nervous system and increases the chance of developing liver and lung cancer, and benign mammary gland tumors. Methanol is highly toxic and when consumed effects the central nervous system and may cause blindness, coma, and death. Ethanol is less toxic compared to DCM and MeOH and it is regarded as the green solvent. Based on the yields of COI from GC-chromatogram, it was found that ethanol is a suitable solvent for the efficient extraction of vanillin, and apocynin, but extraction of phytol by ethanol was slightly less efficient. However, this factor is negligible in comparison to the green solvent property of ethanol, and therefore, ethanol was finally selected as the optimum solvent for the extraction of the three target compounds.

Solvation of target compounds with ethanol solvent

The choice of solvent for extraction often follows the principle “like extracts like”. The selection of solvent system depends on the specific nature of the target compound in the plant material. Polar solvent is used for the extraction of polar compounds, and non-polar solvent for non-polar compounds. Ethanol is a polar solvent and it polarity index is 3.9. Vanillin is a phenolic

aldehyde, and it has three functional groups attached to the phenyl ring, namely methyl ether, aldehyde, and phenolic hydroxyl (-OH) group. The presence of –OH group in vanillin makes it a polar compound. Phytol is a diterpene alcohol, and the compound is to certain extend polar due to the presence of hydroxyl group at the end of the chain. Apocynin is a Phenolic compound with one benzene ring containing there functional groups, namely methyl ketone, methyl ether, and phenolic hydroxyl (-OH) group. Apocynin is also a polar compound due to the presence of the hydroxyl group. The polarity of the three target compounds based on the types of the functional groups can be arranged in the order:

Decreasing order of polarity: Vanillin> Apocynin> Phytol.

Phytol is least polar when compared to the polarity of the vanillin and apocynin. It is therefore expected that ethanol would extract phytol to lesser extend compared to vanillin and apocynin.

Extraction of the three compounds with ethanol is based on the solvation process. In this process, first a cavity is formed in the solvent to accommodate the solute (target compound). In the solvation process, the –OH functional group of the target compounds forms hydrogen bond with the –OH group of ethanol which leads to the stabilization of the solute species in the solution (Figure 2.23).

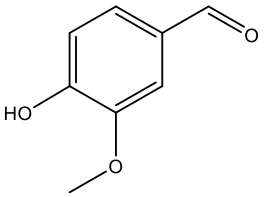
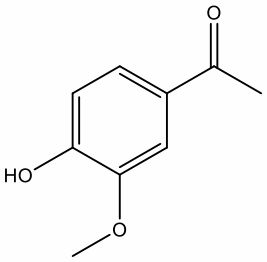
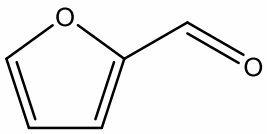
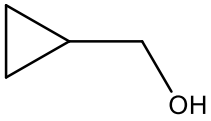
Conclusions

Three bioactive compounds—vanillin, apocynin, and phytol were selected as the target compounds based on the commercial viability. Protobind-1000 was selected as the final lignocellulosic biomass material, since larger amount of the three target compounds could be extracted from this material when compared to the other lignocellulosic biomass.

Table 2.1 Solvents and solvent/biomass ratio (v/w) for screening compounds

Solvent	Solvent/biomass ratio (v/w, mL/g)
Methanol	4
Ethanol	4
Dichloromethane	4

Table 2.2 Structures and main commercial uses of some interest compounds

Compound Name	Structure	Price & Uses
Vanillin		$43.30/100g Food, fragrance, and cosmetic industries; chemical intermediate
Apocynin		$62/100g Anti-arthritic; bowel disease; anti-asthmatic; atherosclerosis; familial ALS; inhibitor
Furfural		$31.1/100mL Renewable, non-petroleum based, chemical feedstock, make other furan chemicals
Cyclopropyl carbinol		$136.5/25mL A primary alcohol, can be used as solvents, fuels and chemical raw materials.

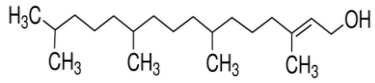
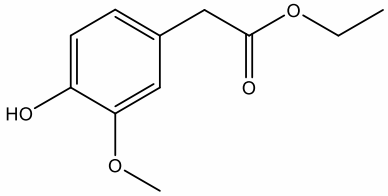
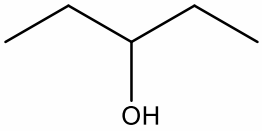
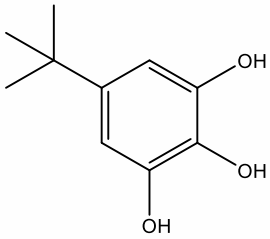
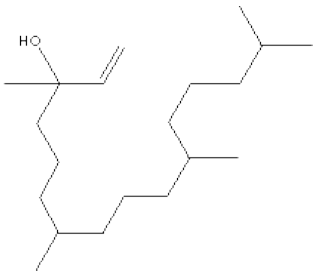
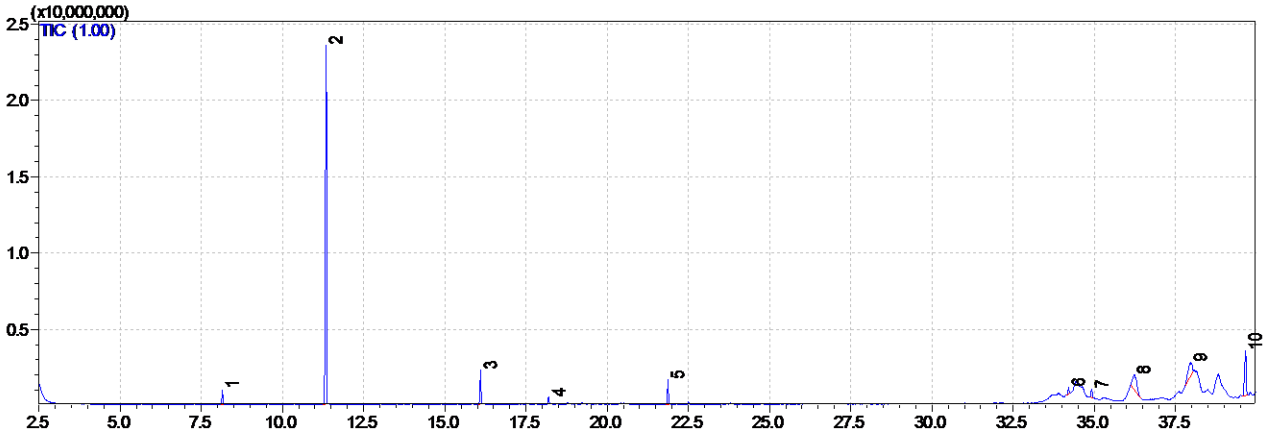
Compound Name	Structure	Price & Uses
Phytol		$103/100g Fragrance industry and used in cosmetics, shampoos, toilet soaps, household cleaners, and detergents
Ethyl homovanillate		$55.4/1g No obvious commercial use
3-pentanol		$72/100mL One of the isomers of amyl alcohol
5-tert-butylpyrogallol		$58.1/1g No obvious commercial uses
Isophytol		$43/5mL No obvious commercial uses

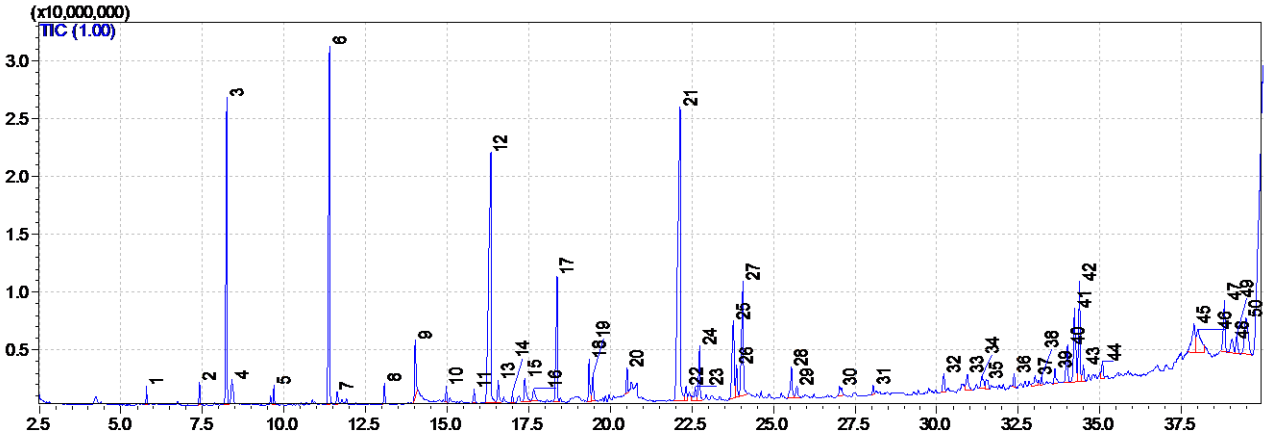
Figure 2.1 GC-MS chromatogram of DCM extract of alkali lignin



Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	8.15	1962860	1.98	Phenol	0.03
2	11.35	57086085	57.5	1,3,5-trichloromethane	0.88
3	16.10	5094965	5.13	Vanillin	0.08
4	18.19	982825	0.99	Apocynin	0.02
5	21.87	3684251	3.71	Homovanillic acid	0.06
7	34.92	1329543	1.34	9-Octadecenamide	0.02
9	37.98	8081437	8.14	beta-Amyrin	0.12

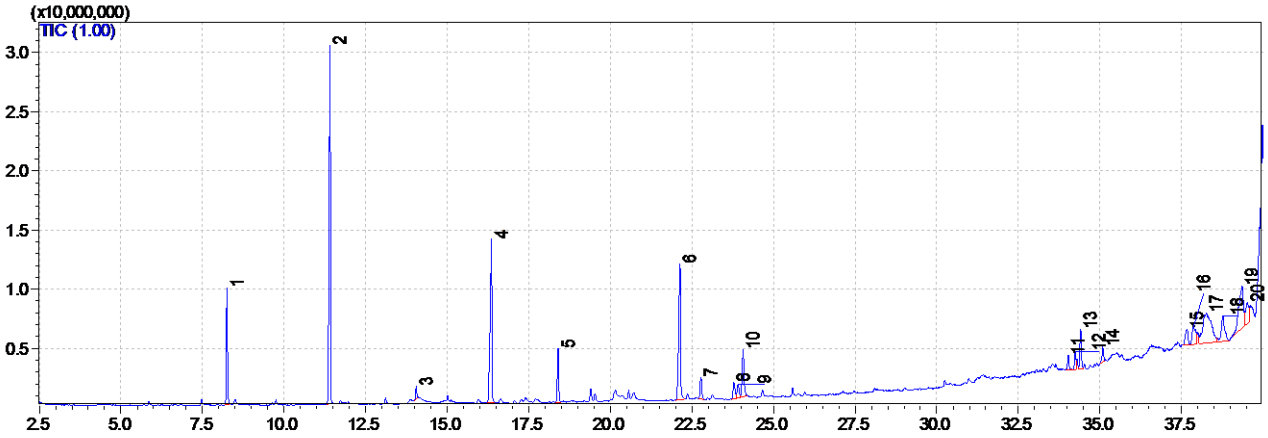
*Samples highlighted in yellow are target chemicals.

Figure 2.2 GC-MS chromatogram of methanolic extract of alkali lignin



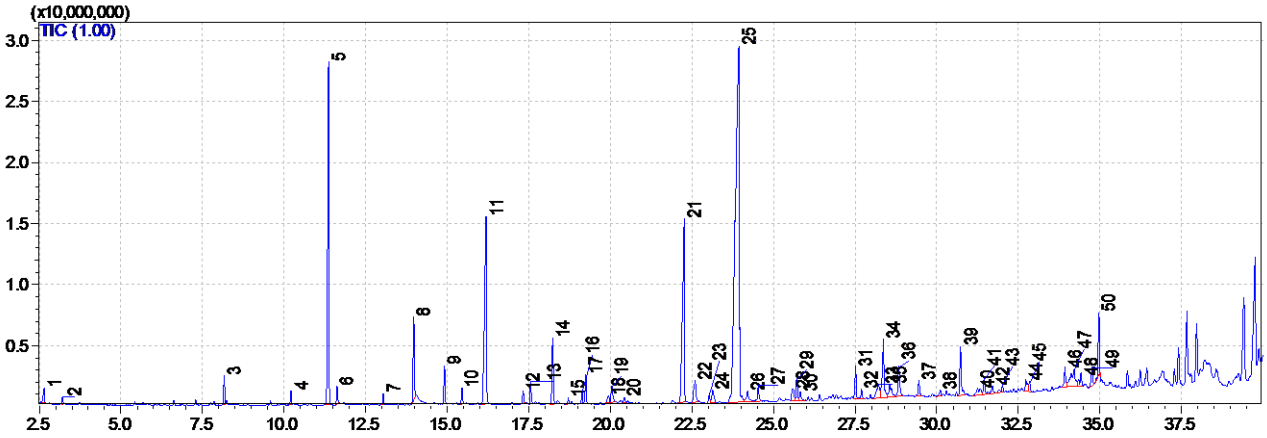
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	5.78	3084100	0.35	Phenol	0.61
2	7.41	4452405	0.51	2-Cyclopenten-1-one	0.88
3	8.25	68209977	7.84	Phenol	13.48
4	8.402	8385674	0.96	Cyclopropyl carbinol	1.66
6	11.39	86870600	9.98	1,2,3-trichloromethane	17.17
7	11.626	3757550	0.43	Catechol	0.74
8	13.08	4245554	0.49	Phenol	0.84
9	14.02	13963821	1.60	4-Hydroxy-3-methylacetophenone	2.76
10	14.97	3099571	0.36	Phenol	0.61
11	15.82	2850477	0.33	Benzaldehyde	0.56
12	16.33	1.15E+08	13.27	Vanillin	22.82
13	16.56	6383011	0.73	1,4-Benzenediol	1.26
14	17.00	2169192	0.25	1,4-Benzenediol	0.43
15	17.38	8932911	1.03	trans-Isoeugenol	1.77
16	17.65	5341671	0.61	4-Hydroxybenzamide	1.06
17	18.37	34413090	3.95	Apocynin	6.80
18	19.35	11290550	1.30	2-Propanone	2.23
19	19.47	6654891	0.76	Homovanillyl alcohol	1.32
20	20.51	5330621	0.61	2,4'-Dihydroxy-3'-methoxyacetophenone	1.05
21	22.14	1.37E+08	15.71	Homovanillic acid	27.01
22	22.32	4768683	0.55	Benzaldehyde	0.94
24	22.73	14453177	1.66	2,4'-Dihydroxy-3'-methoxyacetophenone	2.86
25	23.77	30628952	3.52	Benzeneacetic acid	6.05
37	33.02	4318923	0.50	Benzene	0.85
44	35.08	5304645	0.61	9-Octadecenamide	1.05
46	38.00	19171664	2.20	Tetratetracontane	3.79
47	38.82	17105110	1.97	Benzene	3.38

Figure 2.3 GC-MS chromatogram of ethanolic extract of alkali lignin



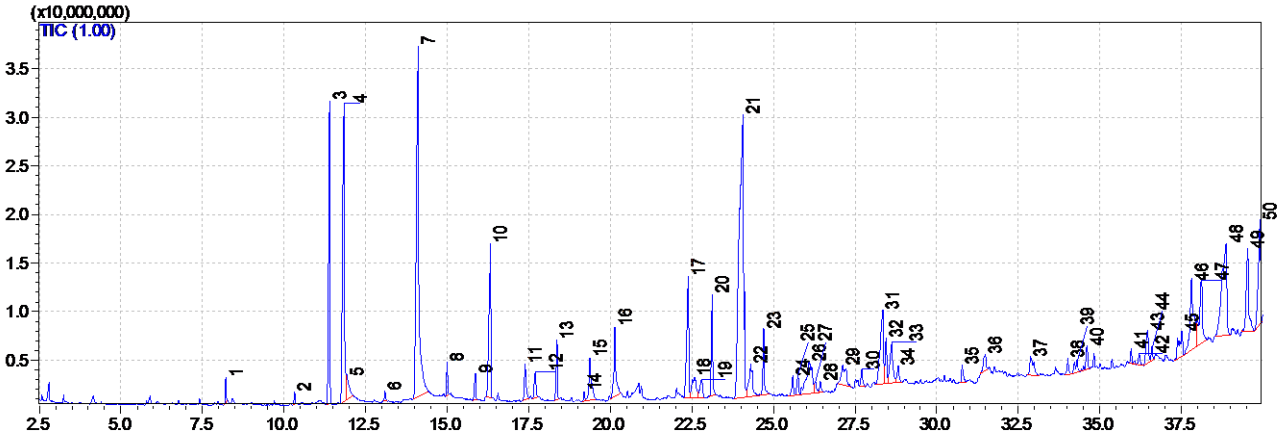
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	8.25	23052760	5.64	Phenol	0.24
2	11.41	84419231	20.64	1,2,3-trichloromethane	0.88
3	14.05	2816799	0.69	4-Hydroxy-3-methylacetophenone	0.03
4	16.36	53697349	13.13	Vanillin	0.56
5	18.40	13179277	3.22	Apocynin	0.14
6	22.13	45104042	11.03	Homovanillic acid	0.47
8	23.79	5669396	1.39	Benzeneacetic acid	0.06
9	23.92	4896505	1.20	Ethanone	0.05
14	35.09	4217645	1.03	9-Octadecenamide	0.04

Figure 2.4 GC-MS chromatogram of DCM extract of Protobind-1000



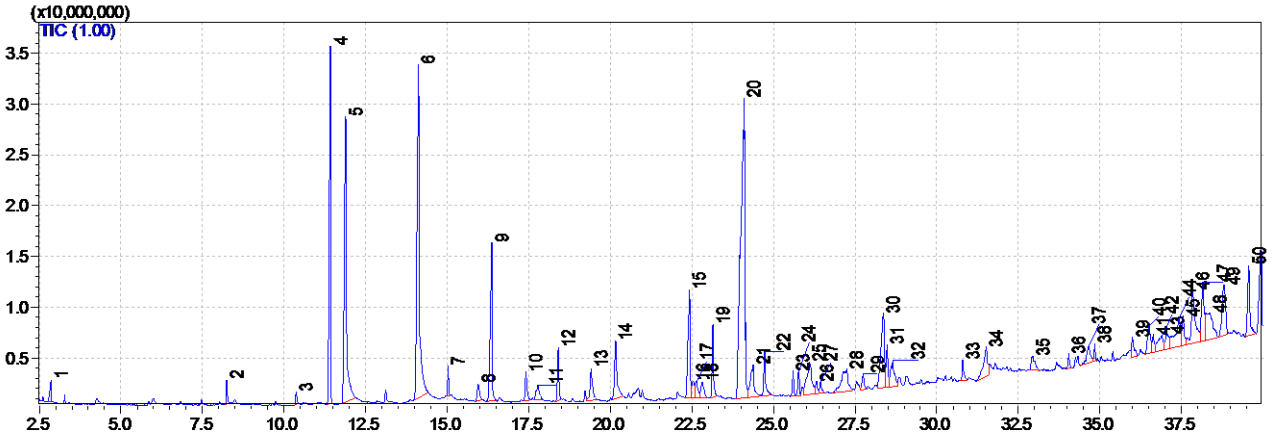
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	2.65	2705765	0.39	Butanoic acid	0.03
3	8.17	4725851	0.67	Phenol	0.06
5	11.37	75611911	10.79	1,2,3-trichloromethane	0.88
6	11.62	3781522	0.54	Benzofuran	0.04
8	13.97	17508510	2.50	4-Hydroxy-3-methylacetophenone	0.20
9	14.92	6705695	0.96	Phenol	0.08
11	16.20	64998724	9.27	Vanillin	0.76
12	17.33	2752177	0.39	Acetophenone	0.03
13	17.56	4642404	0.66	Phenol	0.05
14	18.24	14655797	2.09	Apocynin	0.17
16	19.13	2254066	0.32	5-tert-Butylpyrogallol	0.03
17	19.25	6267319	0.89	2-Propanone	0.07
18	19.95	2183999	0.31	Dodecanoic acid	0.03
20	20.43	1187942	0.17	1,2-Dimethoxy-4-n-propylbenzene	0.01
21	22.26	63976979	9.13	Benzaldehyde	0.74
25	23.93	2.41E+08	34.41	Ethanone	2.81
26	24.21	4983232	0.71	Tetradecanoic acid	0.06
28	25.58	4966460	0.71	Acetate	0.06
29	25.72	5184747	0.74	2-Pentadecanone	0.06
31	27.51	7305891	1.04	Ethanone	0.09
33	28.22	7493784	1.07	n-Hexadecanoic acid	0.09
34	28.37	19076834	2.72	9,10-Anthracenedione	0.22
37	29.455	4316826	0.62	Cyclic octaatomic sulfur	0.05
39	30.733	12843803	1.83	Phytol	0.15
41	31.456	3823535	0.55	Oxacycloheptadec-8-en-2-one	0.04
42	31.71	3192851	0.46	9-Octadecenamide	0.04
48	34.433	4431558	0.63	Bibenzyl	0.05
50	34.973	13392532	1.91	9-Octadecenamide	0.16

Figure 2.5 GC-MS chromatogram of methanolic extract of Protobind-1000



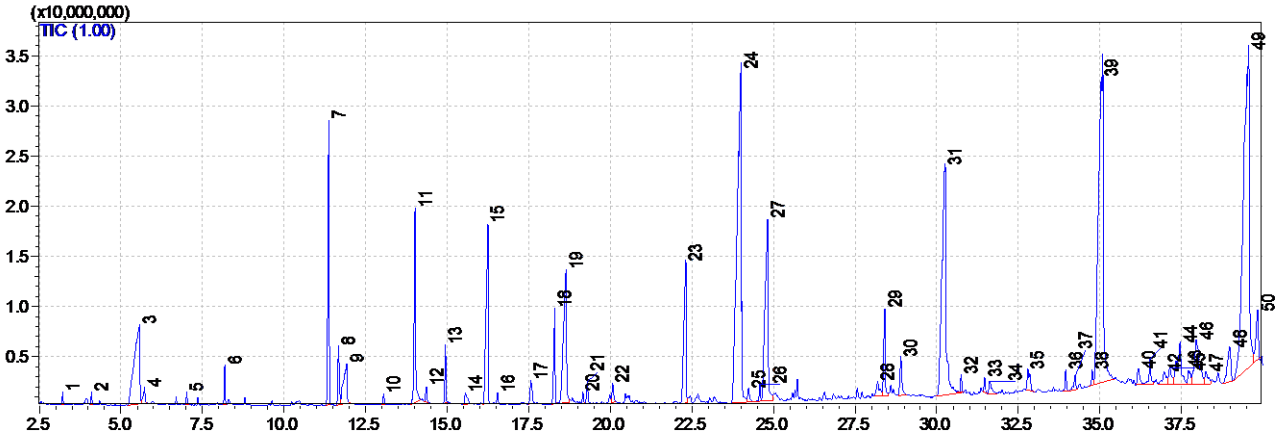
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	8.22	5355088	0.32	Phenol	0.05
2	10.32	2873510	0.17	Phenol	0.03
3	11.40	87262171	5.25	1,2,3-trichloromethane	0.88
4	11.84	1.32E+08	7.69	Benzofuran	1.33
6	13.10	2370895	0.14	Phenol	0.02
7	14.11	2.06E+08	12.42	2-Methoxy-4-vinylphenol	2.08
8	15.00	7442934	0.45	Phenol	0.08
9	15.87	8727738	0.53	Benzaldehyde	0.09
10	16.33	61715158	3.72	Vanillin	0.62
11	17.39	12872532	0.78	trans-Isoeugenol	0.13
12	17.70	10156674	0.61	Acetophenone	0.10
13	18.37	17213342	1.04	Apocynin	0.17
14	19.20	2612010	0.16	Benzene	0.03
15	19.37	23239465	1.40	2-Propanone	0.23
17	22.39	52584127	3.17	Benzaldehyde	0.53
18	22.58	18886310	1.14	Benzenepropanoic acid	0.19
20	23.12	30506324	1.84	Phenol	0.31
21	24.07	2.75E+08	16.55	Ethanone	2.77
25	25.75	7893714	0.48	2-Pentadecanone	0.08
27	26.29	5079109	0.31	Pentadecanoic acid	0.05
30	27.704	6284966	0.38	Ethanone	0.06
31	28.349	53899096	3.25	n-Hexadecanoic acid	0.54
32	28.453	14699513	0.89	9,10-Anthracenedione	0.15
33	28.623	25823230	1.55	Mandelic acid	0.26
35	30.783	6688622	0.40	Phytol	0.07
36	31.492	9932282	0.60	Z,E-2,13-Octadecadien-1-ol	0.10
40	34.606	10239430	0.62	Phenol	0.10

Figure 2.6 GC-MS chromatogram of ethanolic extract of Protobind-1000



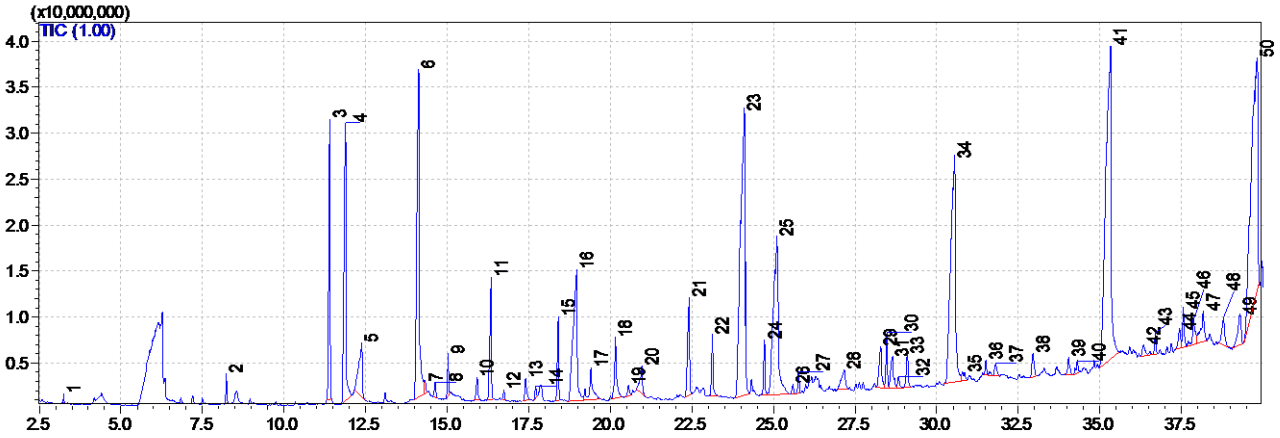
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	2.86	5688218	0.37	Butanoic acid	0.05
2	8.24	5617380	0.37	Phenol	0.05
4	11.42	1.05E+08	6.88	1,2,3-trichloromethane	0.88
5	11.89	1.38E+08	8.99	Benzofuran	1.15
6	14.12	1.67E+08	10.87	2-Methoxy-4-vinylphenol	1.39
7	15.03	6705539	0.44	Phenol	0.06
8	15.95	7829829	0.51	Benzaldehyde	0.07
9	16.37	64861024	4.23	Vanillin	0.54
10	17.41	10599370	0.69	Phenol	0.09
11	17.78	10048811	0.66	Acetophenone	0.08
12	18.41	16018009	1.05	Apocynin	0.13
13	19.41	16373569	1.07	2-Propanone	0.14
14	20.16	29885140	1.95	3',5'-Dimethoxyacetophenone	0.25
15	22.43	49052923	3.20	Benzaldehyde	0.41
16	22.54	8528831	0.56	Phenol	0.07
18	22.81	10373404	0.68	Vanilic acid hydrazide	0.09
19	23.14	23456550	1.53	Phenol	0.20
20	24.10	2.47E+08	16.14	Ethanone	2.06
21	24.38	25016162	1.63	Tetradecanoic acid	0.21
24	25.77	8195700	0.53	2-Pentadecanone	0.07
26	26.32	5171725	0.34	Pentadecanoic acid	0.04
28	27.25	31280183	2.04	2-Propenoic acid	0.26
30	28.37	53371704	3.48	n-Hexadecanoic acid	0.45
31	28.48	16319482	1.06	9,10-Anthracenedione	0.14
32	28.66	20491442	1.34	Mandelic acid	0.17
33	30.80	7265312	0.47	Phytol	0.06
34	31.52	25925616	1.69	Z,Z-3,13-Octadecadien-1-ol	0.22
37	34.66	13681048	0.89	Bibenzyl	0.11

Figure 2.7 GC-MS chromatogram of DCM extract of Protobind-2400



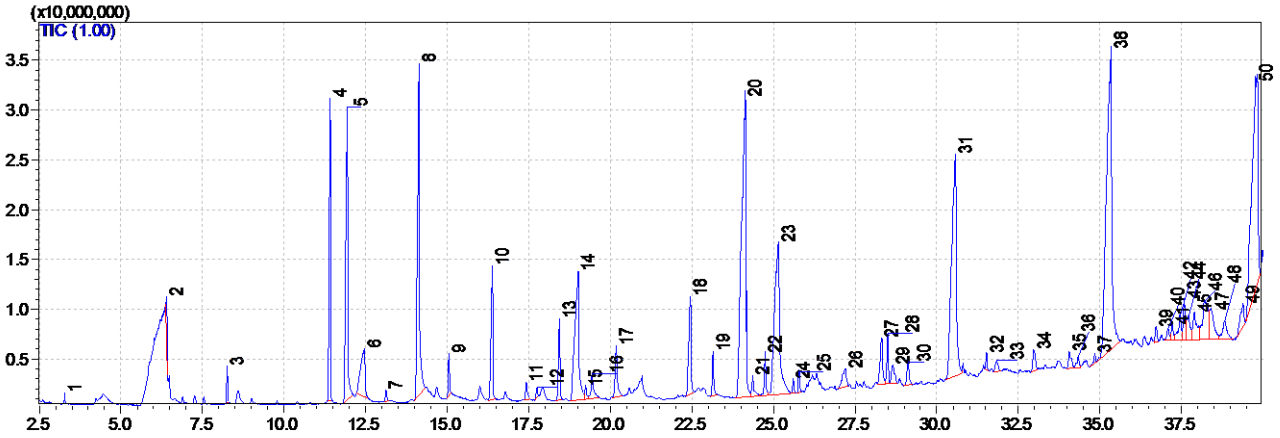
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	3.22	1899026	0.08	Furfural	0.02
2	4.10	1988549	0.09	1,4-Dioxan-2-ol	0.02
3	5.57	93118172	4.08	Ethanol	1.09
4	5.72	6183348	0.27	5-Hexyn-3-ol	0.07
5	7.02	2970562	0.13	Ethane	0.03
6	8.18	8100788	0.36	Phenol	0.10
7	11.37	75011299	3.29	1,2,3-trichloromethane	0.88
8	11.67	18023911	0.79	Benzofuran	0.21
9	11.92	30277607	1.33	Triethylene glycol	0.36
10	13.06	2395776	0.11	Phenol	0.03
12	14.36	4303570	0.19	Triethylene glycol	0.05
13	14.95	13001171	0.57	Phenol	0.15
14	15.56	5869739	0.26	Benzaldehyde	0.07
15	16.25	79837141	3.50	Vanillin	0.94
16	16.54	2793775	0.12	Acetic acid	0.03
18	18.30	29399092	1.29	Apocynin	0.34
19	18.64	86528721	3.80	Tetraethylene glycol	1.02
21	19.29	5835724	0.26	2-Propanone	0.07
23	22.31	67222117	2.95	Benzaldehyde	0.79
24	24.00	3.09E+08	13.56	Ethanone	3.63
25	24.24	5666232	0.25	Tetradecanoic acid	0.07
27	24.82	1.2E+08	5.29	Pentaethylene glycol	1.41
28	28.19	9953216	0.44	n-Hexadecanoic acid	0.12
29	28.41	30263183	1.33	9,10-Anthracenedione	0.36
31	30.25	2.04E+08	8.96	Hexaethylene glycol	2.40
32	30.75	4332455	0.19	Phytol	0.05
33	31.472	3823447	0.17	Oxacycloheptadec-8-en-2-one	0.04
46	37.958	45860153	2.01	Heneicosane	0.54

Figure 2.8 GC-MS chromatogram of methanolic extract of Protobind-2400



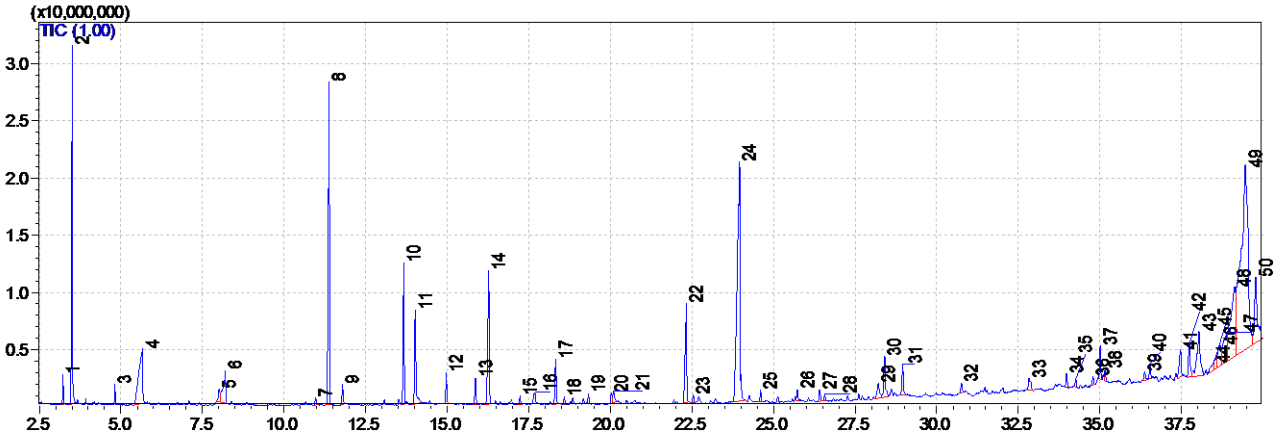
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	3.24	1386877	0.05	Furfural	0.01
2	8.23	6844678	0.26	Phenol	0.07
3	11.40	87005135	3.30	1,2,3-trichloromethane	0.88
4	11.89	1.36E+08	5.14	Benzofuran	1.37
5	12.38	43968166	1.67	Triethylene glycol	0.44
6	14.13	1.87E+08	7.08	2-Methoxy-4-vinylphenol	1.89
7	14.33	5293617	0.20	Dimethyl diglycolcarbonate	0.05
8	14.63	5413634	0.21	Triethylene glycol	0.05
9	15.03	11233442	0.43	Phenol	0.11
10	15.92	8288683	0.31	Benzaldehyde	0.08
11	16.35	49569304	1.88	Vanillin	0.50
13	17.40	9179063	0.35	Phenol	0.09
14	17.71	3617143	0.14	Phenol	0.04
15	18.41	26394927	1.00	Apocynin	0.27
16	18.97	1.23E+08	4.65	Tetraethylene glycol	1.24
17	19.41	24904075	0.94	2-Propanone	0.25
21	22.42	45786785	1.74	Benzaldehyde	0.46
22	23.13	22552003	0.86	Phenol	0.23
23	24.11	2.96E+08	11.22	Ethanone	2.99
25	25.10	1.83E+08	6.93	Pentaethylene glycol	1.85
28	27.17	17645501	0.67	3-Hydroxy-4-methoxycinnamic acid	0.18
29	28.29	25032130	0.95	n-Hexadecanoic acid	0.25
30	28.48	18168717	0.69	9,10-Anthracenedione	0.18
31	28.64	20417605	0.77	Mandelic acid	0.21
34	30.55	2.47E+08	9.38	Hexaethylene glycol	2.50
36	31.51	4855275	0.18	Oxacycloheptadec-8-en-2-one	0.05
41	35.33	3.98E+08	0.34	Hexaethylene glycol	4.02

Figure 2.9 GC-MS chromatogram of ethanolic extract of Protobind-2400



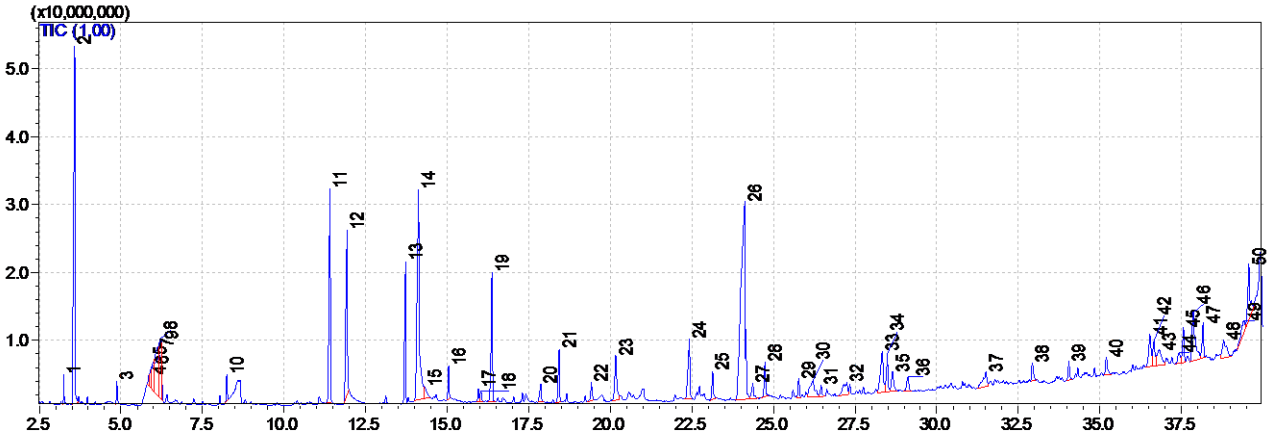
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	3.28	1725845	0.07	Furfural	0.02
3	8.26	7733709	0.32	Phenol	0.08
4	11.41	86086536	3.57	1,2,3-trichloromethane	0.88
5	11.94	1.28E+08	5.31	Benzofuran	1.31
6	12.46	44871692	1.86	Triethylene glycol	0.46
7	13.12	3386844	0.14	Phenol	0.03
8	14.14	1.54E+08	6.40	2-Methoxy-4-vinylphenol	1.58
9	15.06	11481328	0.48	Phenol	0.12
10	16.39	50043014	2.07	Vanillin	0.51
11	17.42	6772602	0.28	trans-Isoeugenol	0.07
12	17.75	3044560	0.13	Phenol	0.03
13	18.44	26439033	1.10	Apocynin	0.27
14	19.02	1.14E+08	4.73	Tetraethylene glycol	1.17
15	19.25	5012066	0.21	Benzene	0.05
16	19.44	13974157	0.58	2-Propanone	0.14
17	20.18	24054037	1.00	3',5'-Dimethoxyacetophenone	0.25
18	22.45	45999161	1.91	Benzaldehyde	0.47
19	23.15	15080970	0.63	Phenol	0.15
20	24.14	2.78E+08	11.51	Ethanone	2.84
21	24.37	9994505	0.41	Tetradecanoic acid	0.10
23	25.15	1.7E+08	7.06	Pentaethylene glycol	1.74
25	25.78	6708784	0.28	2-Pentadecanone	0.07
26	27.21	16382679	0.68	3-Hydroxy-4-methoxycinnamic acid	0.17
27	28.31	24758788	1.03	n-Hexadecanoic acid	0.25
28	28.50	16821036	0.70	9,10-Anthracenedione	0.17
29	28.66	13098698	0.54	Mandelic acid	0.13
31	30.57	2.21E+08	9.16	Hexaethylene glycol	2.26
32	31.53	4618583	0.19	Oxacycloheptadec-8-en-2-one	0.05

Figure 2.10 GC-MS chromatogram of DCM extract of Protobind-5000



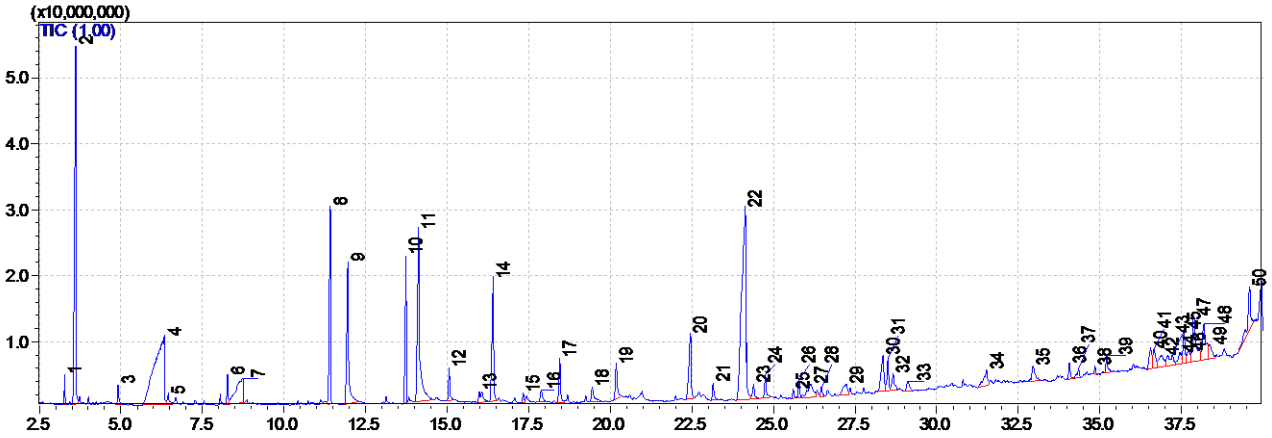
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	3.22	3856862	0.39	Furfural	0.05
2	3.51	59993454	6.05	2-Furanmethanol	0.74
3	4.82	3026345	0.31	2(5H)-Furanone	0.04
4	5.66	40081003	4.04	Ethanol	0.50
5	8.01	3274609	0.33	Furan	0.04
6	8.19	15274917	1.54	Ethanone	0.19
8	11.38	70991990	7.16	1,2,3-trichloromethane	0.88
9	11.79	4547875	0.46	Benzofuran	0.06
10	13.67	28977535	2.92	Furan	0.36
12	14.98	5833085	0.59	Phenol	0.07
14	16.27	36866406	3.72	Vanillin	0.46
16	17.69	3896448	0.39	Acetophenone	0.05
17	18.33	10044489	1.01	Apocynin	0.12
19	19.33	2544166	0.26	2-Propanone	0.03
22	22.33	28961545	2.92	Benzaldehyde	0.36
24	23.96	1.24E+08	12.55	Ethanone	1.54
26	25.73	2314876	0.23	2-Pentadecanone	0.03
29	28.21	6747443	0.68	n-Hexadecanoic acid	0.08
30	28.41	12483659	1.26	9,10-Anthracenedione	0.15
32	30.76	2507083	0.25	Phytol	0.03
37	35.01	7408104	0.75	9-Octadecenamide	0.09
38	35.16	2564790	0.26	Octanoic acid	0.03
49	39.46	2.47E+08	24.94	Hexaethylene glycol monododecyl ether	3.06

Figure 2.11 GC-MS chromatogram of methanolic extract of Protobind-5000



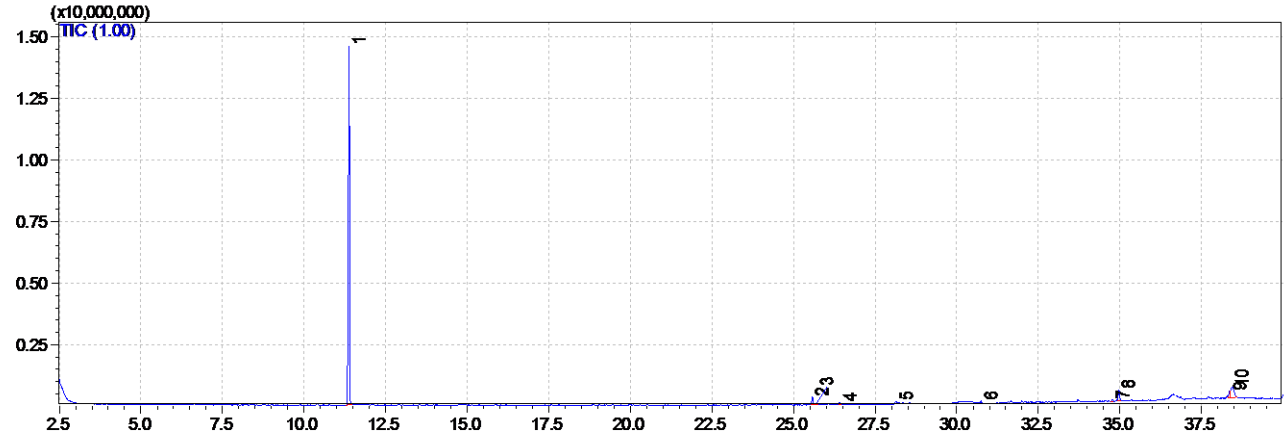
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	3.25	6214743	0.39	Furfural	0.06
2	3.59	1.54E+08	9.58	2-Furanmethanol	1.52
3	4.88	6971648	0.43	2(5H)-Furanone	0.07
4	5.86	4621905	0.29	Phenol	0.05
6	5.95	25951924	1.61	2-Propanol	0.26
7	6.10	46242949	2.87	Ethanol	0.46
10	8.25	7699489	0.48	Phenol	0.08
11	11.41	89418918	5.55	1,2,3-trichloromethane	0.88
12	11.93	84017490	5.22	Benzofuran	0.83
13	13.72	53407028	3.32	Furan	0.53
14	14.12	1.49E+08	9.27	4-Hydroxy-3-methylacetophenone	1.47
16	15.05	12021078	0.75	Phenol	0.12
17	15.96	4888096	0.30	2,4-Pentadienoic acid	0.05
18	16.04	5615826	0.35	Benzaldehyde	0.06
19	16.37	57150536	3.55	4-Hydroxy-2-methoxybenaldehyde	0.56
20	17.87	10395142	0.65	Acetophenone	0.10
21	18.43	22399954	1.39	Apocynin	0.22
22	19.42	8884280	0.55	2-Propanone	0.09
24	22.42	36313704	2.25	Benzaldehyde	0.36
25	23.13	13149682	0.82	Phenol	0.13
26	24.12	2.66E+08	16.54	Ethanone	2.62
27	24.37	10431660	0.65	Tetradecanoic acid	0.10
32	27.25	16893892	1.05	2-Propenoic acid	0.17
33	28.34	36847900	2.29	n-Hexadecanoic acid	0.36
34	28.49	19177850	1.19	9,10-Anthracenedione	0.19
37	31.51	18615613	1.16	Oxacycloheptadec-8-en-2-one	0.18

Figure 2.12 GC-MS chromatogram of ethanolic extract of Protobind-5000



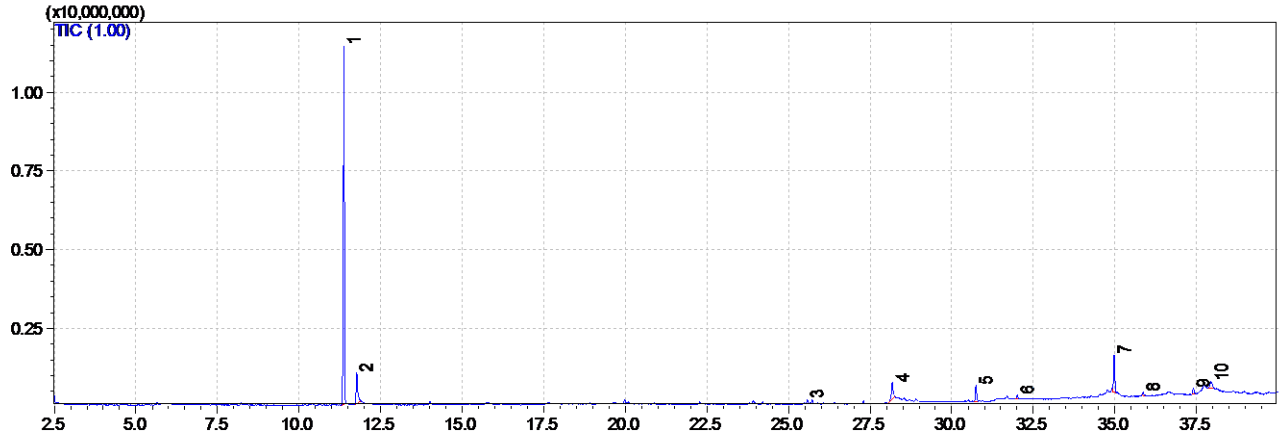
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	3.28	7524193	0.41	Furfural	0.08
2	3.62	1.72E+08	9.45	2-Furanmethanol	1.76
3	4.91	8256666	0.45	2(5H)-Furanone	0.08
4	6.33	2.13E+08	11.70	Ethanol	2.18
5	6.46	6602152	0.36	3-Pentanol	0.07
6	8.27	11184954	0.61	Phenol	0.11
7	8.73	55307177	3.04	Pentanoic acid	0.57
8	11.42	85724462	4.71	1,2,3-trichloromethane	0.88
9	11.96	93904838	5.16	Benzofuran	0.96
10	13.74	60025220	3.30	Furan	0.62
11	14.13	1.33E+08	7.29	4-Hydroxy-3-methylacetophenone	1.36
12	15.07	14584119	0.80	Phenol	0.15
13	15.99	13566002	0.75	2,4-Pentadienoic acid	0.14
14	16.41	68753577	3.78	4-Hydroxy-2-methoxybenaldehyde	0.71
16	17.89	9284028	0.51	Acetophenone	0.10
17	18.45	22807386	1.25	Apocynin	0.23
18	19.45	12804845	0.70	2-Propanone	0.13
20	22.46	46610394	2.56	Benzaldehyde	0.48
21	23.15	9450147	0.52	Phenol	0.10
22	24.13	2.53E+08	13.9	Ethanone	2.60
25	25.61	4077018	0.22	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.04
30	28.363	35018648	1.93	n-Hexadecanoic acid	0.36
31	28.511	17119794	0.94	9,10-Anthracenedione	0.18
34	31.528	19304567	1.06	Oxacycloheptadec-8-en-2-one	0.20
39	35.211	9786619	0.54	Octanoic acid	0.10

Figure 2.13 GC-MS chromatogram of DCM extract of sorghum stalk



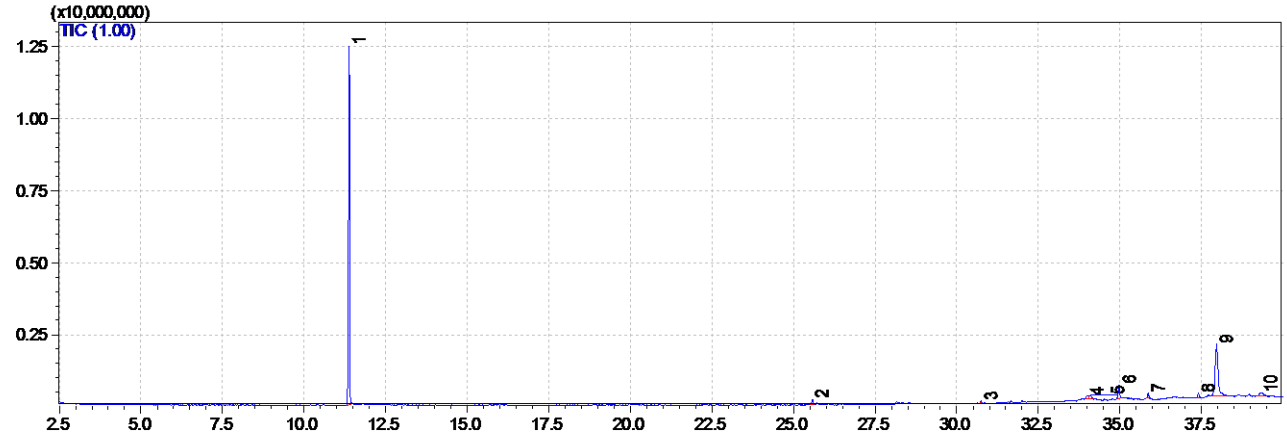
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	11.38	36685955	83.51	1,2,3-trichloromethane	0.88
2	25.57	916318	2.09	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.02
3	25.72	232826	0.53	2-Pentadecanone	0.01
4	26.41	284255	0.65	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.01
5	28.14	189785	0.43	n-Hexadecanoic acid	0.01
6	30.74	262365	0.60	Phytol	0.01
7	34.77	233843	0.53	4,8,12,16-Tetramethylheptadecan-4-olide	0.01
8	34.97	1200879	2.73	9-Octadecenamide	0.03
10	38.43	3079933	7.01	2-methyloctacosane	0.07

Figure 2.14 GC-MS chromatogram of methanolic extract of sorghum stalk



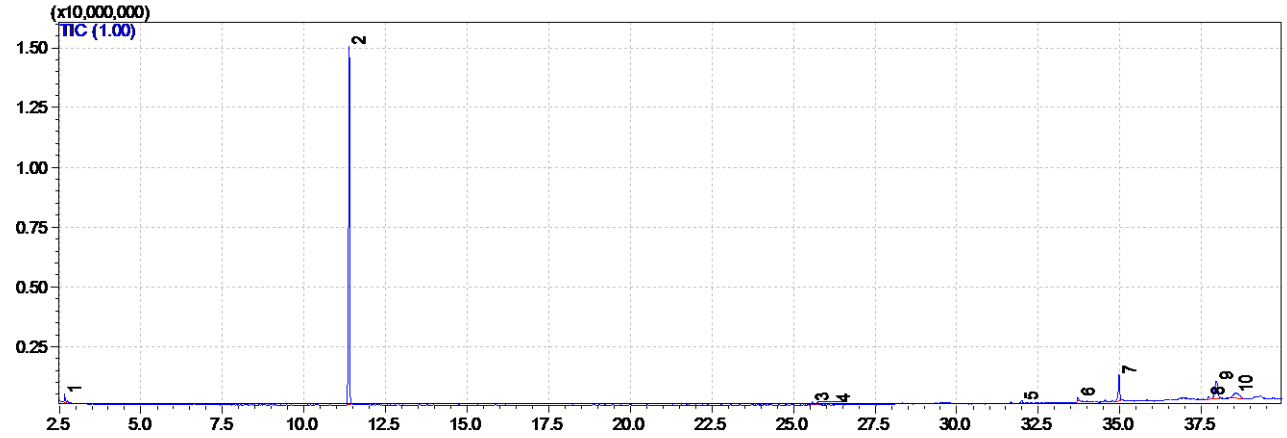
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	11.38	26387556	68.51	1,2,3-trichloromethane	0.88
2	11.76	3299444	8.57	Benzofuran	0.11
3	25.57	297597	0.77	Acetate	0.01
4	28.18	2028748	5.27	n-Hexadecanoic acid	0.07
5	30.74	1350216	3.51	Phytol	0.05
6	32.01	304948	0.79	Hexadecanamide	0.01
7	34.98	3056880	7.94	9-Octadecenamide	0.10
8	35.86	289533	0.75	Octadecanal	0.01
9	37.40	460565	1.20	Pentadecanal	0.02
10	37.95	1041757	2.70	Pentacosane	0.03

Figure 2.15 GC-MS chromatogram of ethanolic extract of sorghum stalk



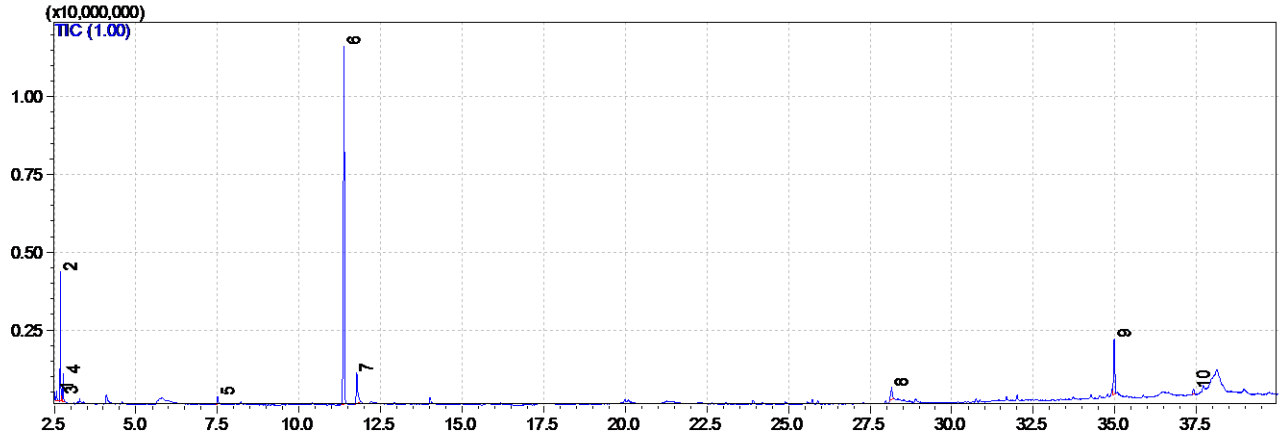
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	11.39	30599388	65.06	1,2,3-trichloromethane	0.88
2	25.57	560373	1.19	Phytol	0.02
3	30.74	216347	0.46	Phytol	0.01
4	33.99	412125	0.88	2-methylhexacosane	0.01
5	34.09	651425	1.39	Tetratetracontane	0.02
6	34.97	1273968	2.71	9-Octadecenamide	0.04
7	35.87	511464	1.09	Pentadecanal	0.01
8	37.40	497276	1.06	Pentadecanal	0.01
9	37.97	11229609	23.88	Hentriacontane	0.32
10	39.32	1079850	2.30	Octadecanal	0.03

Figure 2.16 GC-MS chromatogram of DCM extract of corn stover



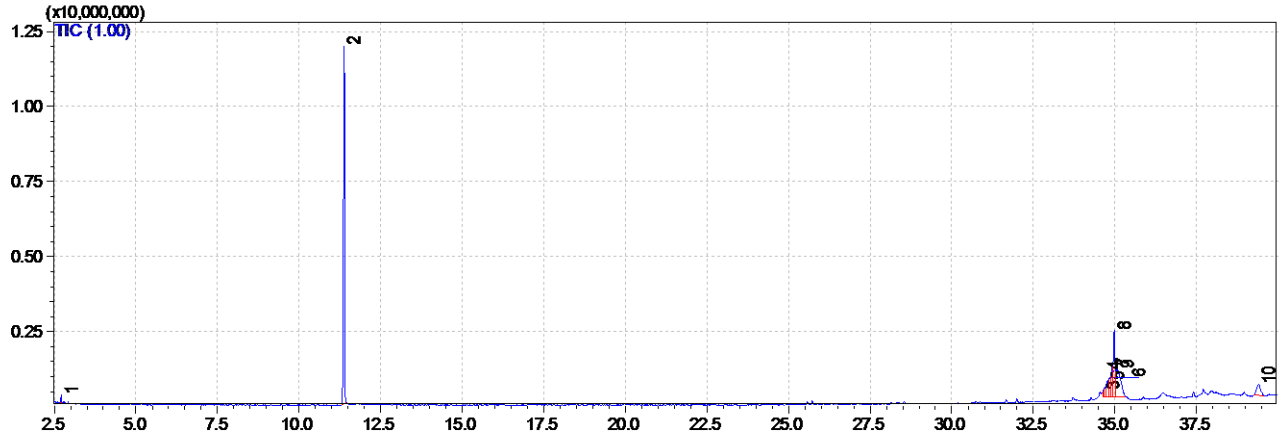
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	2.66	580584	1.15	2,3-Butanediol	0.01
2	11.39	37461196	74.02	1,2,4-trichloromethane	0.88
3	25.57	309947	0.61	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.01
4	25.73	292370	0.58	2-Pentadecanone	0.01
5	31.99	320063	0.63	Hexadecanamide	0.01
6	33.72	393577	0.78	Butanoic acid	0.01
7	34.98	3332647	6.58	9-Octadecenamide	0.08
8	37.71	409767	0.81	7-Hexadecenal	0.01
9	37.95	4714645	9.32	Pentacosane	0.11
10	38.54	2795199	5.52	Hentriacontane	0.07

Figure 2.17 GC-MS chromatogram of methanolic extract of corn stover



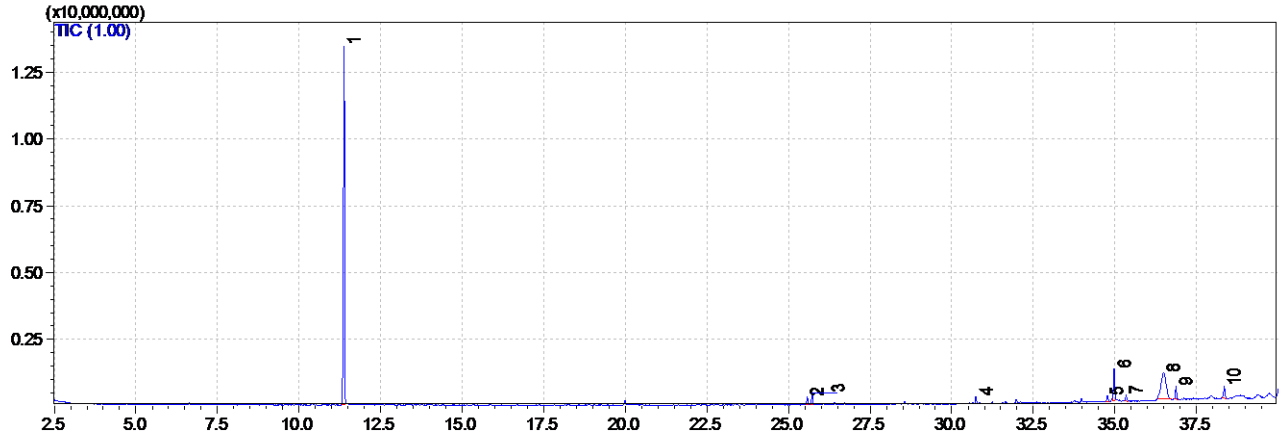
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	2.57	449557	1.03	Glycerin	0.01
2	2.69	4444268	10.17	2,3-Butanediol	0.14
3	2.73	347331	0.80	Propanoic acid	0.01
4	2.77	1026525	2.35	2,3-Butanediol	0.03
5	7.51	521391	1.19	4-Isobutoxy-2-butanone	0.02
6	11.38	27211994	62.29	1,2,3-trichloromethane	0.88
7	11.77	3175561	7.27	Benzofuran	0.10
8	28.16	1261055	2.89	n-Hexadecanoic acid	0.04
9	34.98	4783874	10.95	9-Octadecenamide	0.15
10	37.41	463383	1.06	Pentadecanal	0.01

Figure 2.18 GC-MS chromatogram of ethanolic extract of corn stover



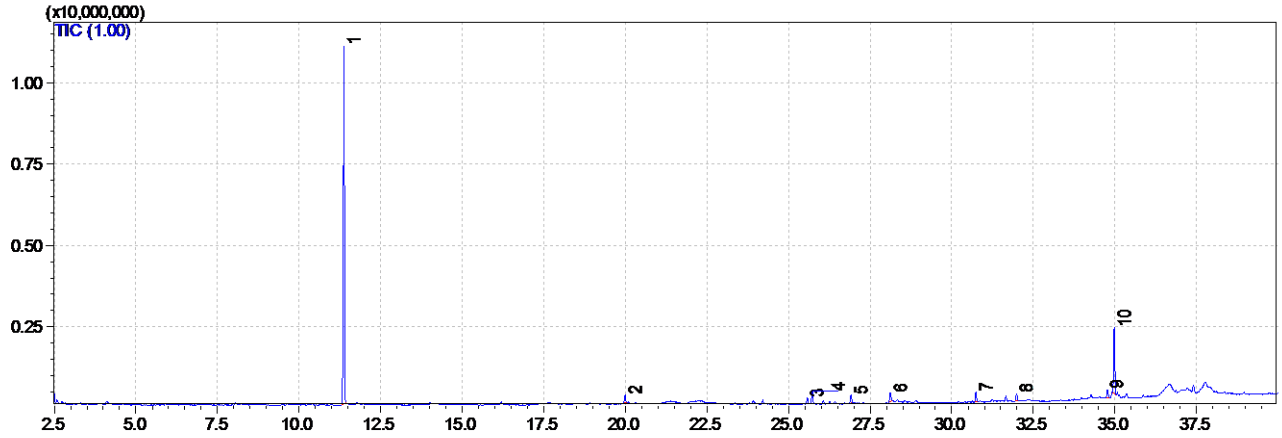
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	2.71	283161	0.47	2,3-Butanediol	0.01
2	11.38	29850234	49.33	1,2,3-trichloromethane	0.88
8	34.98	9166979	15.15	9-Octadecenamide	0.27
9	35.07	8649759	14.29	17-Octadecenal	0.25
10	39.41	3135307	5.18	Pentacosane	0.09

Figure 2.19 GC-MS chromatogram of DCM extract of switch grass



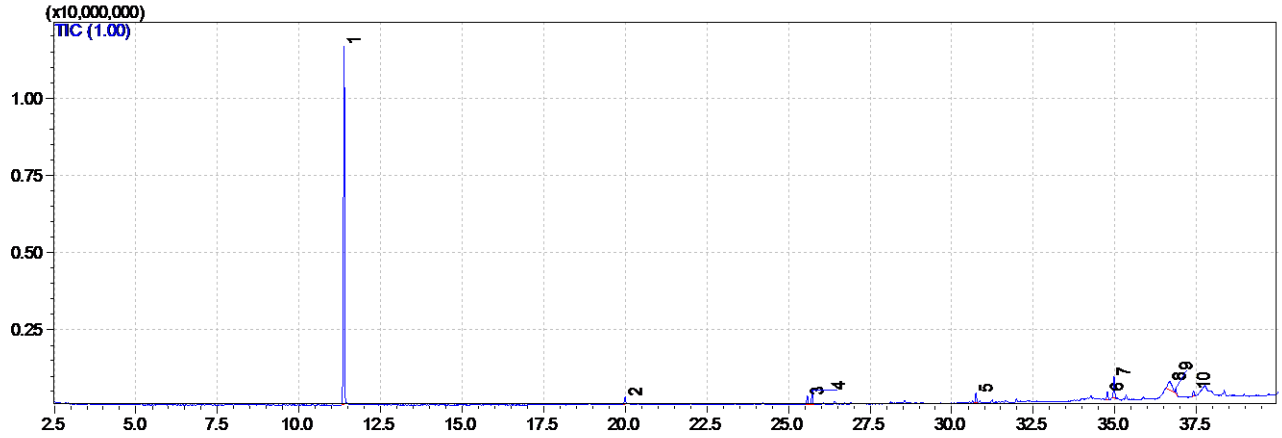
Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	11.38	33409326	62.45	1,2,3-trichloromethane	0.88
2	25.58	731580	1.37	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.02
3	25.72	1089591	2.04	2-Pentadecanone	0.03
4	30.74	712912	1.33	Phytol	0.02
5	34.77	693222	1.30	4,8,12,16-Tetramethylheptadecan-4-olide	0.02
6	34.97	3259903	6.09	9-Octadecenamide	0.09
7	35.34	594732	1.11	Heneicosane	0.02
8	36.49	10420161	19.48	Octadecanal	0.27
9	36.87	1295656	2.42	Pentacosane	0.03
10	38.35	1288418	2.41	Hentriacontane	0.03

Figure 2.20 GC-MS chromatogram of methanolic extract of switch grass



Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	11.38	25838821	70.99	1,3,5-trichloromethane	0.88
2	20.00	589217	1.62	Dodecanoic acid	0.02
3	25.57	446597	1.23	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.02
4	25.72	957949	2.63	2-Pentadecanone	0.03
6	28.12	736734	2.02	n-Hexadecanoic acid	0.03
7	30.74	804207	2.21	Phytol	0.03
8	31.99	624607	1.72	Dodecanamide	0.02
9	34.77	680660	1.87	4,8,12,16-Tetramethylheptadecan-4-olide	0.02
10	34.98	5096777	14.00	9-Octadecenamide	0.17

Figure 2.21 GC-MS chromatogram of ethanolic extract of switch grass



Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	11.38	28661453	75.78	1,3,5-trichloromethane	0.88
2	19.99	632234	1.67	Dodecanoic acid	0.02
3	25.57	754157	1.99	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.02
4	25.73	1100899	2.91	2-Pentadecanone	0.03
5	30.74	841309	2.22	Phytol	0.03
6	34.77	720188	1.90	4,8,12,16-Tetramethylheptadecan-4-olide	0.02
7	34.97	1839906	4.86	9-Octadecenamide	0.06
9	36.87	479035	1.27	Heneicosane	0.01
10	37.41	495573	1.31	Pentadecanal	0.02

Table 2.3 Relative amount of target compounds from different lignocellulosic biomass by ethanol extraction

Lignocellulosic biomass	Relative amount to standard		
	Vanillin	**Apocynin**	**Phytol**
Alkali Lignin	0.56	0.14	--
Protobind-1000	0.54	0.13	0.06
Protobind-2400	0.51	0.27	--
Protobind-5000	--	0.23	--
Sorghum stalk	--	--	0.02
Corn stover	--	--	--
Switch grass	--	--	0.03

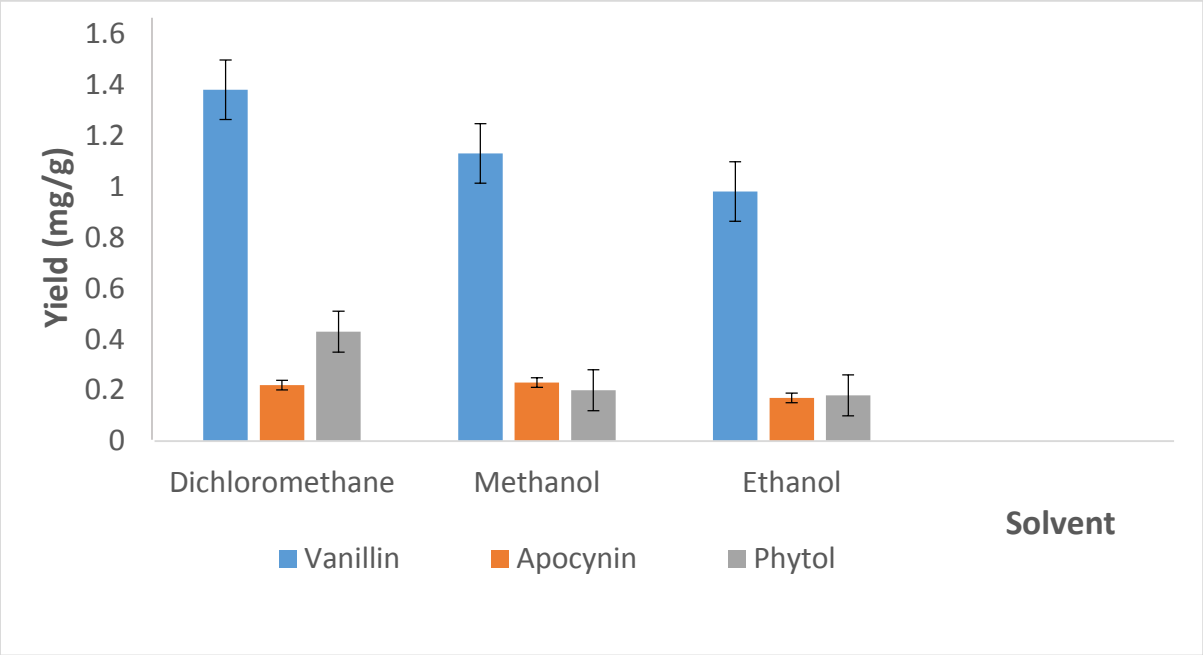


Figure 2.22 Different solvent used for extraction of Protobind-1000.

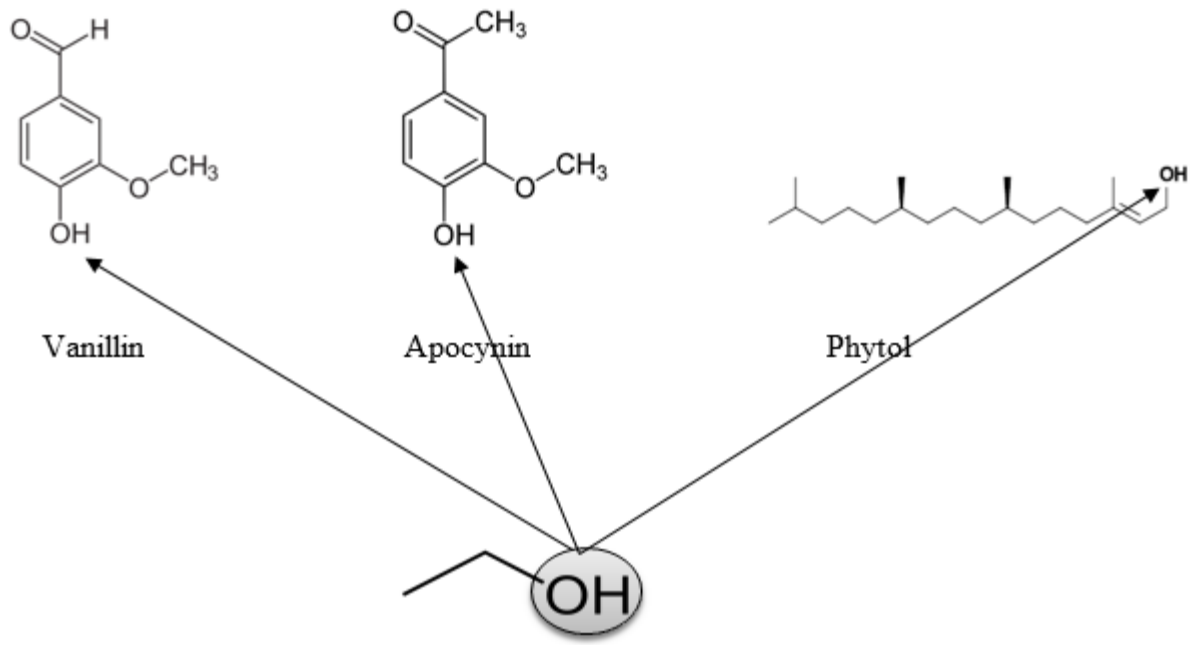


Figure 2.23 Mechanism of ethanol-based extraction

Chapter 3 - Different extraction methods for the target compounds

Introduction

In the previous chapter, we identified optimum solvent, ethanol, for the extraction of three target compounds (vanillin, apocynin and phytol) from lignocellulosic biomass. The next objective of the research was to investigate different extraction techniques and determine the optimum extraction process for the efficient extraction of the three target compounds. Common methods of extraction includes: pressurized liquid extraction (PLE), matrix solid-phase dispersion (MSPD), supercritical-fluid extraction (SFE), microwave-assisted extraction (MAE), solid-phase extraction (SPE), bead beating, solid-phase micro-extraction (SPME), bath sonication, and ultra-sonication (also known as probe sonication). Bead beating, ultra-sonication and combination of bead beating and ultra-sonication methods of extraction were investigated due to their cost-effective properties and the efficiency of extraction between the three methods were compared.

Bead beater use steel beads inside a closed container to grind or homogenize the sample. The bead are vigorously shaken to break up tissue and disrupt cells. In this research, the bead beater helped us to throughly mix the solvent and biomass and also to disrupt the cell walls of the biomass. An advantage of bead beating is that it reduces contamination due to the absence of probes and no sampling port.

Ultra-sonication disrupt tissues and cells through cavitation and ultrasonic waves. The probe of the ultra sonicator vibrates rapidly, causing bubbles in the surrounding solution to rapidly form and collapse. Because of the heat generation, sonicators may not work well with temperatura-sensitive samples. In addition, they are great for cell disruption and particle size reduction.

Materials and methods

Preparations of standards

1,2,3-trichlorobenzene (TCB) was used as the internal standard. Standard samples of vanillin, apocynin, and phytol containing TCB as the internal standard were prepared as follows:

a. Stock solutions of vanillin, apocynin, phytol, and TCB at concentration of 10mg/mL in ethanol were prepared.

b. A mixed alcoholic standard solution containing 0.5 mg/mL TCB (internal standard) and 0.1 mg of vanillin, phytol and apocynin was prepared from stock solution.

Different extraction procedure

Bead beating (Mini Bead Beater-16, Model 607, Bartlesville)**:** One gram of lignin (Protobind-1000), six steel beads (3.2 mm dia. Chrome Steel, Cat No. 11079132c, BioSpec Products) and 4mL ethanol were added into a plastic vial, and attached to bead beater. Bead beating was performed at 3200 RPM for 30s (x 3 times). The supernatant was collected and centrifuged (Centrifuge 5415 R, Eppendorf) at 24x3,75g for 10min. The supernatant from centrifuge was collected and filtered through a 0.45 µm pore size filter. The filtrate (1mL) was transferred to GC vial, and into it was added internal standard (TCB) at concentration of 0.5mg/mL, and analyzed by GC-MS.

Ultra-sonication: One gram of lignin (Protobind-1000) and 4mL ethanol were taken into a plastic and ultra-sonicated using a thin ultra-sonication rod (tip diameter = 1.6mm) for different time duration of 0.5h, 1h, and 1.5h and probe amplitudes of 15, 30, and 45. A mini fridge (MiniFridge II, model 260009, Boekel, Industries Inc.) was used to prevent the overheating of the sample. After sonication, the supernatant was collected and centrifuged at 24x3,75g for 10min.

After the centrifugation, the supernatant was collected and filtered through a syringe filter of 0.45µm pore size. The filtrate (1mL) was transferred to GC vial, and mixed with internal standard (TCB, 0.5 mg/mL) and analyzed by GC-MS.

Ultra-sonication plus bead beating: In this process, the lignin material was first ultrasonicated as explained above, and supernatant was separated from the residue. The supernatant was centrifuged, filtered and analyzed by GC-MS. The residue was dried and weighed, and transferred to a vial for bead beating (30s, 2 x times). The amount of ethanol solvent and the number of Chrome bead used for Bead beating was calculated based on the residue weight. For 1gm of residue, 4mL of ethanol and 6 chrome bead were used. After the bead beating the supernatant was collected and analyzed by GC-MS separately The residue was again extracted by bead beating, and the second supernatant was collected and analyzed by GC-MS. Ultra-sonication + bead beating extraction was performed to determine the concentration of the target compounds present in the second and third extraction, and to evaluate if one time extraction is competent to remove the maximum amount of target compounds from the simple. Different extraction methods are shown in Figure 3.1.

Qualitative and quantitive analysis

Qualitative analysis (i.e. identification and GC-MS peak retention time) of the target compounds was determined using the commercially available standards. The peaks and the retention time of the standards were used to detect the presence of target compounds in the GC-MS chromatogram of the test simple.

Quantification of the target compounds was determined using the internal standard method. This method was used to account for the small variations in the injection volume. In this process,

a mixed standard solution containing the target compounds and the internal standard compound was prepared and analyzed by GC- MS. A known amount internal standard compound (TCB, 0.5 mg.mL) was added in the test sample for GC-MS analysis so that the concentration and peak area of the internal standard can be used in the quantitative calculation of the target compounds. The absolute response of analytes in GC- and GC-MS changes from day to day and instrument to instrument, and also for the same run the response of detector to the standard and the analytes is different. To account for this variation, relative response factor (also known as response factor (RF)) is taken into account. The response factor of each target analyte of known concentration is calculated by using the peak area of the standard solution. The following equation is used to calculate the response factor (RF):

$$RF = \frac{Conc.of\ target\ compound * Peak\ area\ of\ internal\ standard}{Conc.of\ internal\ standard * Peak\ area\ of\ target\ compound}$$

The concentration of the the target compound in the unknown per gram of sample is calculated as follows:

$$Yield = \frac{RF * Conc.of\ internal\ standard * Peak\ area\ of\ COI * Volume}{Peak\ area\ of\ internal\ standard * weight\ of\ biomass}$$

where volume = volume of extract supernatant; weight of biomass = amount of biomass used in the extraction.

Results and discussion

The quantitative results of the target compounds in the extract determined from GC-MS analysis and by using the above equation indicated that bead beating extraction method yielded higher amount of vanillin (0.26 mg/g) compared to the amount of apocynin (0.05mg/g) and phytol (0.04mg/g). This can be explained on the polarity basis of the three target compounds. Vanillin is more polar than the other target compounds. Ethanol is a polar solvent and therefore, extracted more amount of vanillin based on the principle of "like extracts like" (Figure 3.2). The quantitative analysis of the second beat beating extraction indicated very low amount of target compounds. This suggest that first extraction is adequate to extract maximum amount of compounds and proceeding to the second extraction process results in marginal increase in efficiency.

Quantitative analysis (Table 3.1) of target compounds from Ultra-sonication extraction method showed that extraction at 15 amplitude did not yield better results and the concentration of the target compounds are almost similar to the amount extracted by bead beating method. The results also indicated that the amount of target compound is increased with the increase in the amplitude and duration of sonication. It was observed that the optimum duration and the amplitude for maximum yield were at 60 min and 45 amplitude. The yield obtained at this condition of sonication was: vanillin (1.16mg/g), apocynin (0.28mg/g), phytol (0.72mg/g). It was also observed that sonication at 45 amplitude for 90 minutes gave lower yield of the target compounds. This suggests that maximum efficiency of extraction is already reached at 45 amplitude @60 min, and longer duration of more than 60 minutes degrades the targets compounds due to the high input of energy and excessive generation of heat.

Conclusions

Bead beating and ultra-sonication methods were investigated in the determination of efficient extraction of target compounds from the lignocellulosic biomass, namely protobind-1000. Results indicated that ultra-sonication is a better method of extraction than bead beating, and also second time extraction is not necessary, as maximum amount of target compound is extracted in the first extraction process. Ultra-sonication at 45 amplitude for 60 minutes is the optimum parameter to obtain maximum yield of target compounds.

Table 3.1 Yields (mg/g) of target compounds from different extraction methods

	Conditions	Vanillin	Apocynin	Phytol
Bead beating		0.26 ± 0.04^{C}	0.05 ± 0.01^{B}	0.04 ± 0.02^{DE}
Second extraction by bead beating		0.06 ± 0.01^{I}	0.01 ± 0.01^{E}	0.01 ± 0.00^{G}
Ultra-sonication	*15A_30min	0.20 ± 0.01^{F}	0.04 ± 0.01^{CD}	0.03 ± 0.00^{FG}
	15A_60min	0.35 ± 0.02^{DE}	0.06 ± 0.01^{CD}	0.04 ± 0.01^{EF}
	15A_90min	0.37 ± 0.03^{D}	0.06 ± 0.01^{CD}	0.03 ± 0.02^{FG}
	30A_30min	0.31 ± 0.02^{E}	0.06 ± 0.02^{CD}	0.06 ± 0.01^{DE}
	30A_60min	0.37 ± 0.05^{D}	0.07 ± 0.03^{CD}	0.05 ± 0.01^{EF}
	30A_90min	0.31 ± 0.04^{E}	0.07 ± 0.03^{CD}	0.03 ± 0.01^{FG}
	45A_30min	0.37 ± 0.04^{FG}	0.07 ± 0.03^{CD}	0.08 ± 0.01^{CD}
	45A_60min	$\mathbf{1.16 \pm 0.05^{A}}$	$\mathbf{0.28 \pm 0.03^{A}}$	$\mathbf{0.72 \pm 0.03^{A}}$
	45A_90min	0.96 ± 0.05^{B}	0.28 ± 0.02^{A}	0.66 ± 0.04^{B}
Second extraction by bead beating after ultra-sonication	15A_30min	0.07 ± 0.01^{HI}	0.01 ± 0.01^{E}	0.01 ± 0.00^{G}
	15A_60min	0.06 ± 0.02^{I}	0.09 ± 0.02^{C}	0.01 ± 0.00^{G}
	15A_90min	0.05 ± 0.01^{I}	0.01 ± 0.00^{E}	0.01 ± 0.01^{G}
	30A_30min	0.05 ± 0.01^{I}	0.01 ± 0.01^{E}	0.01 ± 0.00^{G}
	30A_60min	0.05 ± 0.02^{I}	0.01 ± 0.01^{E}	0.01 ± 0.00^{G}
	30A_90min	0.05 ± 0.02^{I}	0.01 ± 0.00^{E}	0.01 ± 0.01^{G}
	45A_30min	0.11 ± 0.02^{HG}	0.06 ± 0.02^{D}	0.06 ± 0.01^{DE}
	45A_60min	0.20 ± 0.05^{F}	0.09 ± 0.02^{C}	0.09 ± 0.02^{C}

	Conditions	Vanillin	Apocynin	Phytol
	45A_90min	0.17 ± 0.05^{F}	0.08 ± 0.03^{CD}	0.01 ± 0.01^{G}

Data represent mean value ± standard deviation based on three replications; Values with the same letters in the same column are not significantly different at $p<0.05$.

*15, 30, 45 are the amplitudes we used during the extraction process, A means probe amplitude. E.g. 15A_30min means the extraction time was 30min with the probe amplitude of 15.

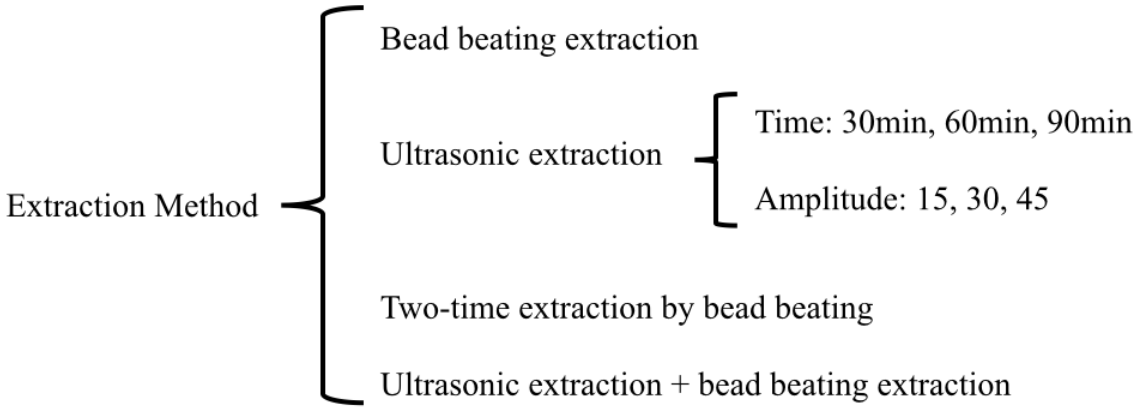


Figure 3.1 Extraction methods used in this research

Chapter 4 - Isolation of compounds of interest (COI) using TLC and column chromatography

Introduction

Thin-layer chromatography

TLC is a simple, cost-effective, and easy-to-operate planar chromatography technique It was originally described by Kirchner et al. (1951) and it has been used in general chemistry laboratories for several decades to routinely separate chemical and biochemical compounds (Sy-Chyi Cheng et al., 2011). Usually TLC is performed on a sheet of glass; it can also be performed on plastic material or aluminum foil (Peter E.W. 2005). The stationary phase of TLC is the layer of absorbent—usually silica gel, and the liquid solvent system is used as the mobile phase. The separation of sample result from the differences in migration of sample components in the direction of the mobile phase. After the separation is complete, individual compounds appear as spots separated vertically. Each factor has a retention factor (Rf) which is equal to the distance migrated by the sample over the the total distance traveled by the solvent.

$$Rf = \frac{distance\ travel\ by\ \ compound}{Distance\ traveled\ by\ solvent}$$

It is necessary to determine a good solvent system to get a good separation of components in the TLC plate. TLC is also a simple method of determining the correct composition of solvent system to separate the COI in preparative scale by column chromatography (Available at http://www.reachdevices.com/SetUpColumn.html).

Column chromatography

Column chromatography is a common and effective method for isolation and purification of compounds from mixtures of organic compounds (Davies & Johnson, 2007). Reports have been published regarding column chromatography technique for isolating organic compounds since the 1990s (Goodrich et al., 1993; Svoronos & Sarlo, 1993). Column chromatography has many advantages, such as the relatively low cost and disposability of the stationary phase used in the process. Different components distribute differently between the stationary phase (usually silica gel, alumina.) and mobile phase (can be liquids or gases); thus, the complex mixture can be separated through the column. Silica gel is widely used as the stationary phase due to its high resolution and economical performance (Shibata et al., 2004). The rate at which the components of a mixture are separated depends on the activity of the adsorbent and polarity of the solvent. If the polarity of the adsorbent is very high and polarity of the solvent is very low, then the separation is very slow but gives a good separation. On the other hand, if the activity of adsorbent is low and polarity of the solvent is high the separation is rapid but gives only a poor separation (Available at http://vlab.amrita.edu/?sub=2&brch=191&sim=341&cnt=1).

Column chromatography is divided into two types: depending on how the solvent flows down the column. If the solvent is allowed to flow down the column by gravity it is called gravity column chromatography. If the solvent flows down the column by positive air pressure, it is called flash column chromatography. Song et al. (2013) reported the isolation of four prazole enantiomers by achiral, gravity-driven silica gel column chromatography, which offers a new method for obtaining a high enantiomeric purity sample. Hadi and Noviany (2009) used gravity column chromatography to isolate hipeahpemol from *Shorea ovalis* blume. Compare to flash chromatography, the particle size of the stationary phase of gravity column chromatography is

bigger. Shibata et al. (2004) improved the separation method for lutein with high purity by using flash column chromatography with silica gel as the stationary phase.

Materials and methods

Chemicals and equipment

Protobind-1000 extract was obtained by ultra-sonication method (with the probe amplitude of 45 and extractive time of 60min) using ethanol as the extracting solvent; Silica gel on TLC Al foils (Lot # BCBN9366V) was obtained from Sigma-Aldrich. Three standards—acetovanillone (apocynin, 98%, Lot: A0333666, Acros Organics), vanillin (Batch # 127K3725, Sigma-Aldrich), and phytol (Cat # 23027, Chem-Impex Int'L Inc.) were purchased and used as received. Silica gel (Silica gel 60 GF254) was purchased from Sigma Aldrich. $CaSO_4.2H_2O$ was purchased from Fisher Scientific, and manganese-activated zinc silicate (zinc fluorescent) was purchased from MP biomedicals.

Thin-layer chromatography plates preparation

TLC plates were made using the method modified from Harborne (1998). A mixture of silica gel, $CaSO_4.2H_2O$, zinc fluorescent (5g: 1g: 0.1g) were mixed in 10mL of H_2O to obtain a thick flowable slurry making TLC plates. All the glass plates (Precleaned microscope slides, 3*1 inch, Sargent-Welch Scientific Company, Skokie, ILL) were cleaned using 1% acetone. The slurry mixture was vigorously stirred for around 90s and then applied as thin coating on the glass plate. The plate coated with slurry was dried in room temperature for 2 hours and then heated for activation in oven at 50°C for 30min.

Sample preparation

0.01 gram of vanillin, apocynin, and phytol were dissolved in 1mL ethanol as standards. Protobind-1000 extract (4 mL) was concentrated to 1 mL by a rotating evaporator (Rotavapor R-210, Buchi Labortechnik AG, Switzerland).

TLC experiment and gravity column chromatography

A 250 mL beaker was used as the TLC chamber. A solvent system (hexane, dichloromethane, ethyl acetate, and chloroform in a ratio of 1:1:1:0.1, v/v) was poured in the beaker covering the bottom of the beaker to a height of about 0.6 cm. The beaker covered with a lid to saturate the chamber with vapor of the mobile phase. Spots of samples were applied to the plate using a capillary tube. The spots were allowed to dry. The plate was placed into the chamber as evenly as possible and leaned it against the side of the beaker. The plate was immediately removed when the solvent reached near the top of the plate. The solvent front was marked. UV light (254 nm) was used to detect the components. The target compounds were detected by comparing with their respective standards spotted on the same plate.

Column chromatography was set up with silica gel (Silica gel for chromatography, 0.060-0.200mm, 40A, CAS # 7631-86-9, Acros Organics) as the stationary phase and the solvent system (hexane, dichloromethane, ethyl acetate, and chloroform in a ratio of 1:1:1:0.1, v/v) as the mobile phase. The column was packed with the stationary phase by following the procedure as follows: The column was placed in a ring stand in a vertical position. A plug of glass wool was pushed down to the bottom of the column. A slurry of silica gel was prepared with the aforementioned solvent system and poured gently into the column. The stopper at the bottom of the column was adjuested slowly to drain out the solvent. The adsorbent was fully covered with the solvent to prevent any crack. The concentrated extra was slowly poured into the column. The column was

run with the eluent. The liquid flow rate of 3-4 drops per second was maintained to obtain efficient separation of the components. A series of fractions were collected with each fraction containing about 20 mL volume of eluent. Each fraction was concentrated to about 1 mL and analyzed by GC-MS.

Results and discussion

Solvent system

Thin layer chromatography was used to detect the target compounds, and to determine the optimum solvent system to isolate and collect the individual compounds of interest in preparative scale by gravitational chromatography. A series of different solvent composition was tested for the effective separation of the target compounds.

Figure 4.1 showed the results of TLC by different solvent systems. It was observed that the target compounds were not separated by the solvent systems A-E. It was found that the three target compounds were eluted close together. Several solvent composition were tested for the good separation of the three target compounds. After several attempt, it was found that the solvent system of composition (hexane: dichloromethane: ethyl acetate (1:1:1 v/v/v/v)) could at least separate phytol from vanillin and apocynin (Figure 4.2). And a solvent system of composition (hexane: dichloromethane: ethyl acetate: chloroform (1:1:1:0.1 v/v/v/v)) could barely separate vanillin and apocynin (Figure 4.3). The chemical behavior of vanillin and apocynin are very similar due to their almost chemical structure, and therefore, it was difficult to separate them by TLC. The separation by TLC was also limited due to the short length of separation times. It was expected that the solvent system (hexane: dichloromethane: ethyl acetate: chloroform (1:1:1:0.1

v/v/v/v) would give better separation with the column chromatography as the elution time is much longer than TLC and has longer length of separation time.

Column chromatography

The chromatography column (Figure 4.4) used in the experiment showed bands of different colors indicates that the constituents of extract were separated in the column.

All the fractions collected during column chromatography were concentrated by rotating evaporator and then injected into GC-MS. Table 4.2 shows the fractions indicating absence or presence of the target compounds.

Table 4.1 shows that the target compounds are present from fraction 5 to 13. Fraction 5-6 indicated the presence of phytol, fraction 7 and 13 showd the presence of vanillin. Fraction 8, 10 and 12 indicated the presence of apocynin. However, fraction 9, and 11 showed the presence of both vanillin and apocynin.

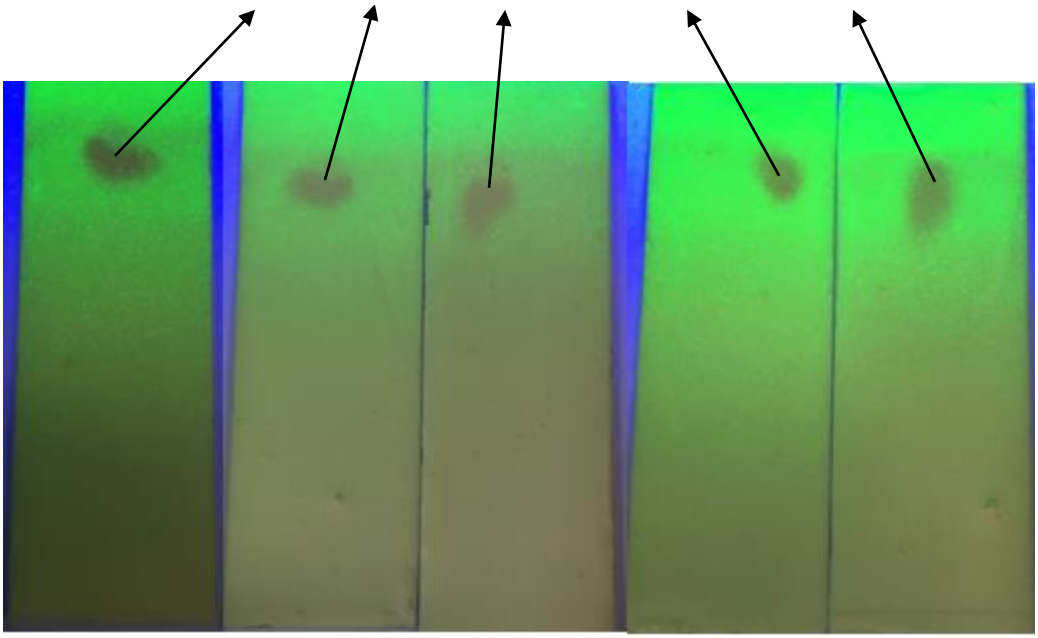
Conclusions

TLC was used to detect and isolate the target compounds and to develop the optimum solvent solvent for preparative scale separation of target compounds by gravitational column chromatography. The solvent system (hexane, dichloromethane, ethyl acetate, chloroform in a ratio of 1:1:1:0.1 v/v) optimized by TLC was efficient in the separation of the targets compounds by column chromatography.

Table 4.1 Analyzation of each fraction by GC-MS

Fraction #	Component
1	No target compounds appeared
2	No target compounds appeared
3	No target compounds appeared
4	No target compounds appeared
5	Only phytol appeared
6	Only phytol appeared
7	Only vanillin appeared
8	Only apocynin appeared
9	Vanillin and apocynin both appeared
10	Only apocynin appeared
11	Vanillin and apocynin both appeared
12	Only apocynin appeared
13	Only vanillin appeared
14	No target compounds appeared
15	No target compounds appeared
16	No target compounds appeared
17	No target compounds appeared
18	No target compounds appeared

Mixture of vanillin, apocynin & phytol



A B C D E

Figure 4.1 Solvent systems A-E

A: Ethyl acetate

B: Acetone: Ethyl acetate (10:90 v/v)

C: Acetone: Ethyl acetate (50:50 v/v)

D: Acetone: Ethanol (90:10 v/v)

E: Acetone: Ethanol (50:50 v/v)

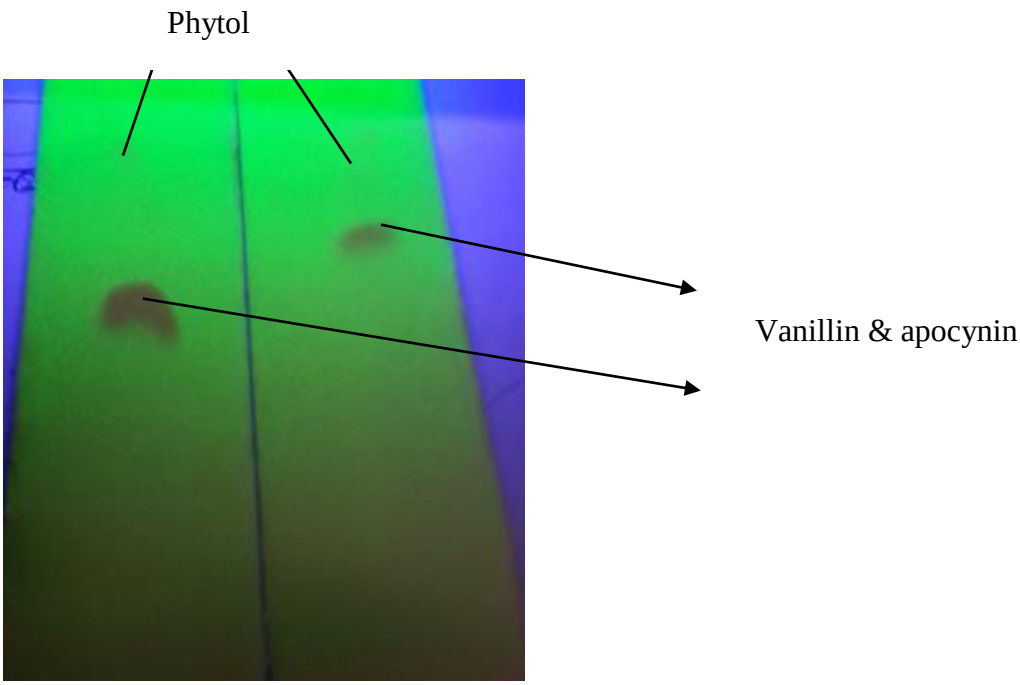


Figure 4.2 Solvent systems F-G

F: Hexane: Dichloromethane: Ethyl acetate (1:1:1 v/v/v)

G: Hexane: Dichloromethane: Ethyl acetate: Ethanol (1:1:1:0.1 v/v/v/v)

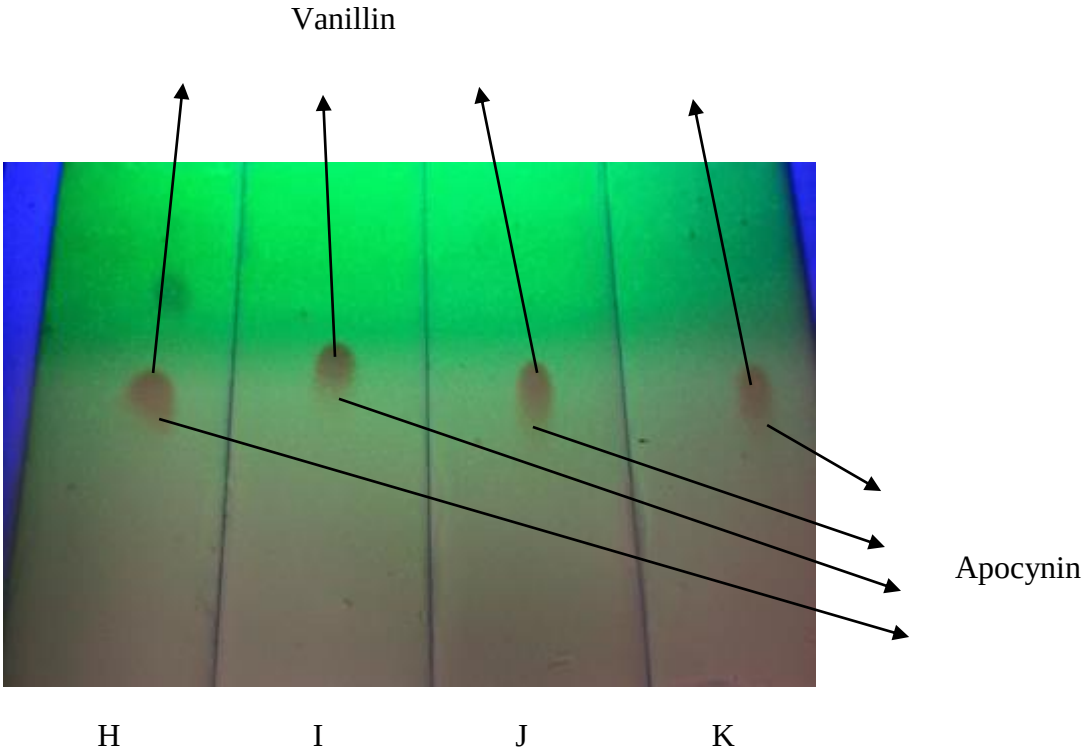


Figure 4.3 Solvent systems H-K

H: Hexane: Dichloromethane: Ethyl acetate: Chloroform (1:1:1:0.025 v/v/v/v)

I: Hexane: Dichloromethane: Ethyl acetate: Chloroform (1:1:1:0.05 v/v/v/v)

J: Hexane: Dichloromethane: Ethyl acetate: Chloroform (1:1:1:0.075 v/v/v/v)

K: Hexane: Dichloromethane: Ethyl acetate: Chloroform (1:1:1:0.1 v/v/v/v)

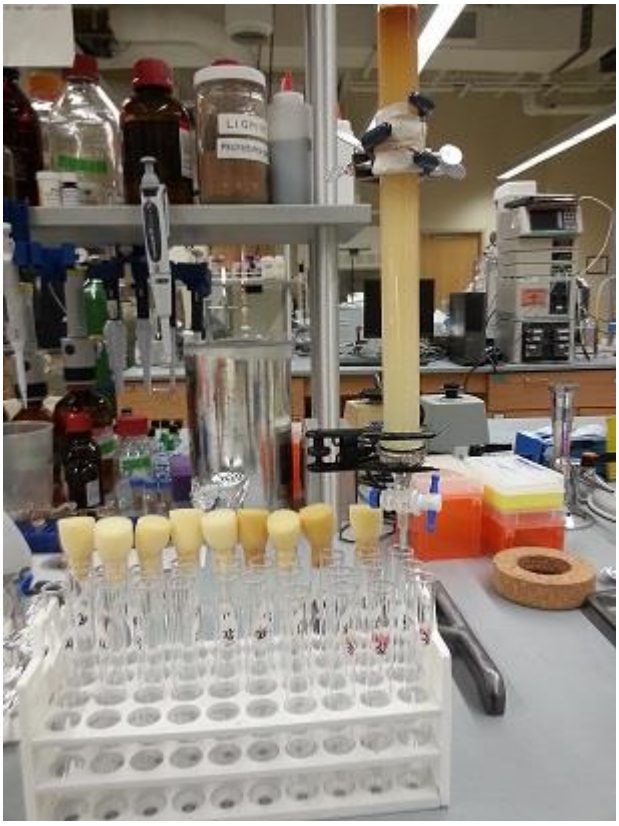


Figure 4.4 Column chromatography set up

Chapter 5 - Biomass-derived lignin

Introduction

Management of large amounts of lignocellulosic biomass in United States has become a major challenge for natural resource sustainability (Ahring et al., 2015). Lignin from lignocellulosic biomass is a good source of macromolecules and miscellaneous monomers. From economic perspective, it would be highly advantageous if lignocellulosic biomass is used as the source of lignin for deriving commercially viable lignin rather than depending on the commercial lignin which is expensive. Commercial lignin is usually derived from wood chips by kraft pulping process (Francis et al., 2008). Kraft pulping is a process in which wood ships are treated with a mixture of sodium sulfide (18-20 wt%) and sodium hydroxide (15-18 wt%) at elevated temperature (170-175°C). This process completely depolymerizes lignin into monomers. Lignin obtained from kraft pulping contains high amount of sulfonate ($-SO_3^-$) group, and therefore, they are also known as lignosulfonates. Due to the use of harsh chemicals and extreme temperatures, lignin derived from kraft pulping is not a suitable raw material for extraction of valuable chemicals.

Materials and methods

Soda/anthraquinone pulping conditions

Sorghum stalk (Texas) was used as the source of lignin and was pretreated by soda/anthraquinone (SAQ) pulping method that was described by Francis et al. (2008). SAQ pulping in ethanol/water (50/50 v/v) was performed with 0.10% anthraquinone, 18% NaOH, with the pulping temperature of 165°C and the pulping time of 90min (Pressure Reactor, PAU). The mixture was centrifuged and the liquid part was neutralized by HCl aqueous and dried in room temperature. The solid part after drying were used as biomass-derived lignin.

Extraction methods

Both the bead beating and ultra-sonication methods with ethanol as the extracting solvents were used for biomass-derived lignin extraction. The same protocols as mentioned in Chapter 3 were used for the bead beating and ultra-soniation extraction procedure.

Bead beating: (Mini Bead Beater-16, Model 607, Bartlesville)**:** One gram of laboratory-made lignin, six steel beads (3.2 mm dia. Chrome Steel, Cat No. 11079132c, BioSpec Products) and 4mL ethanol were added into a plastic vial and attached to bead beater. Bead beating was performed at 3200 RPM for 30s (x 3 times). The supernatant was collected and centrifuged (Centrifuge 5415 R, Eppendorf) at 24x3,75g for 10min. The supernatant from centrifuge was collected and filtered through a 0.45µm pore size filter. The filtrate (1mL) was transferred to GC vial, and into it was added internal standard (TCB) at concentration of 0.5mg/mL, and analyzed . by GC-MS.

Ultra-sonication: One gram of laboratory-made lignin and 4mL ethanol were taken into a plastic and ultra-sonicated using a thin ultra-sonication rod (tip diameter = 1.6mm) for different time interval of 0.5h, 1h, and 1.5h and probe amplitudes of 15, 30, and 45, respectively. A mini fridge (MiniFridge II, model 260009, Boekel, Industries Inc.) was used to prevent the overheating of the sample. After sonication, The supernatant was collected and centrifuged at 24x3,75g for 10min. After the centifugation, the supernatant was collected and filtered through a syringe filter of 0.45µm pore size. The filtrate (1mL) was transferred to GC vial, and mixed with internal standard (TCB, 0.5 mg/mL) and analyzed by GC-MS.

Results and discussion

Composition of biomass-derived lignin

The target compounds (vanillin, apocynin, and phytol) present in the extract of biomass-derived lignin was qualitatively and quantitatively analyzed by GC-MS. The GC-MS chromatograms (Figures 5.1 and 5.2) detected the presence of target compounds in the biomass-derived lignin extract. The results also indicates that ultra-sonication is more efficent extraction method than bead beating. The yield of target compounds (mainly vanillin and apocynin) from biomass-derived lignin is comparable to that of the probind lignin.

The laboratory-made biomass-derived lignin contains chemicals similar to that found in the commercially available lignin (Protobind-1000), which indicates it can be a promising replacement of commercial lignin. The biomass-derived lignin contains vanillin and apocynin; however, phytol was not shown in biomass-derived lignin. Biomass-derived lignin contains less compounds, which can make the separation of target compounds easier. The quantitative results indicates that the laboratory-made biomass-derived lignin can be substituted for commercial lignin for the extaction of commercially viable chemicals. Biomass derived lignin is more economical and is also of better quality for the extraction of other valuable compounds, including lignin oligomer, aromatic and other miscellaneous compounds. The results analysis shows that the optimum bead beating and ultra-sonication protocols developed for the commercial lignin protobind-1000 extraction were equally applicable to the biomass-derived lignin extraction.

Conclusions

Biomass-derived lignin was made in the lab as a replacement of the commercial lignin. The results show that the lab-made lignin has similar composition with commercial lignin and encouraging yields of the target compounds, which indicates that it is a promising replacement of

the commercial lignin. Future work will focus on how to optimize the pretreated method of obtaining lignin and other lignocellulosic materials should be tested for lignin production.

Table 5.1 Comparison of the yield (mg/g) of target compounds between commercial lignin and biomass-derived lignin by ultra-sonication extraction

Lignin type	Yield (mg/g)		
	Vanillin	Apocynin	Phytol
Commercial lignin	1.16 ± 0.05^{A}	0.28 ± 0.03^{A}	0.72 ± 0.03^{A}
Biomass-derived lignin	0.48 ± 0.02^{B}	0.51 ± 0.03^{B}	--

Data represent mean value ± standard deviation based on three replications; Values with the same letters in the same column are not significantly different at $p<0.05$.

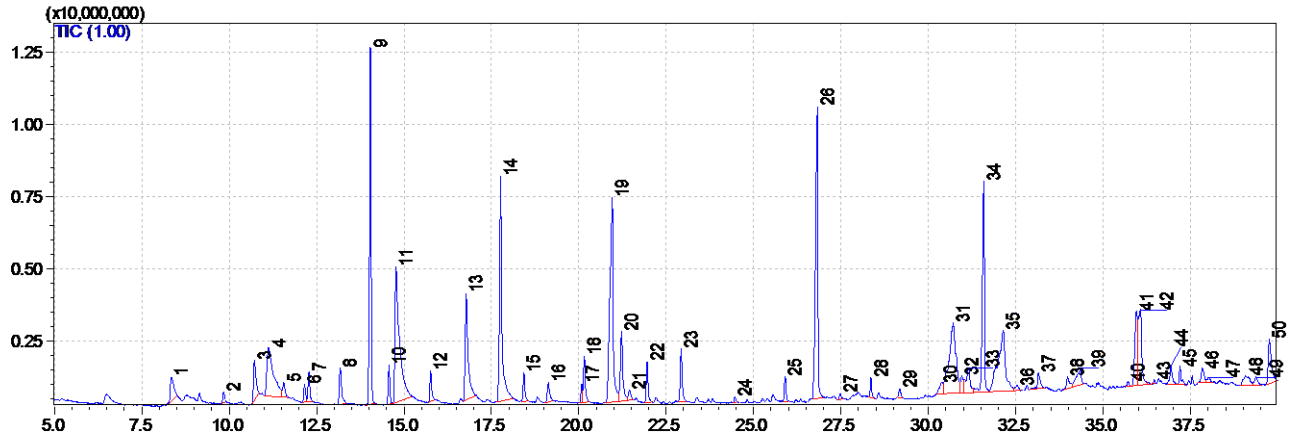
Table 5.2 Comparison of the yield (mg/g) of target compounds from biomass-derived lignin by different extraction methods

Extraction method	Yield (mg/g)	
	Vanillin	Apocynin
Bead beating	0.12 ± 0.02^{A}	0.34 ± 0.03^{A}
*Ultra-sonication	0.48 ± 0.02^{B}	0.51 ± 0.03^{B}

Data represent mean value ± standard deviation based on three replications; Values with the same letters in the same column are not significantly different at $p<0.05$.

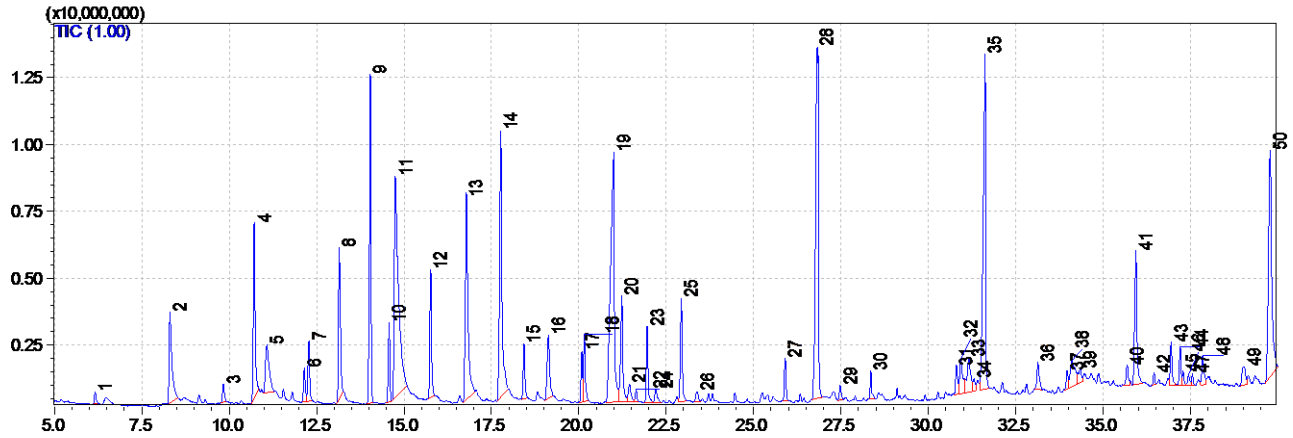
*The optimum extraction condition discussed in Objective 2: Probe amplitude of 45 and extraction time of 60min.

Figure 5.1 GC-MS chromatogram of ethanolic extract of biomass-derived lignin by bead beating extraction method



Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	8.35	5625507	1.08	Phenol	0.12
2	9.84	1304601	0.25	2-Cyclopenten-1-one	0.03
3	10.72	6835957	1.31	Phenol	0.14
4	11.13	22169091	4.25	Cyclopropyl carbinol	0.47
5	11.57	2538156	0.49	2-Cyclopenten-1-one	0.05
6	12.16	2243082	0.43	Bicyclo[2.2.2]octan-1-ol	0.05
8	13.18	5731392	1.10	Phenol	0.12
9	14.04	41488149	7.96	1,3,5-trichloromethane	0.88
10	14.58	4335057	0.83	2-Cyclohexen-1-one	0.09
11	14.78	42571432	8.16	Benzofuran	0.90
12	15.77	3660767	0.70	Phenol	0.08
13	16.78	20825712	3.99	2-Methoxy-4-vinylphenol	0.44
14	17.77	37562504	7.20	Phenol	0.80
16	19.13	3216737	0.62	Vanillin	0.07
18	20.16	5889983	1.13	Phenol	0.12
19	20.96	46657024	8.95	Acetophenone	0.99
20	21.23	11989836	2.30	Apocynin	0.25
21	21.45	1792750	0.34	1,5-Dihydroxy-1,2,3,4-tetrahydronaphthalene	0.04
22	21.96	5129244	0.98	Benzene	0.11
25	25.90	2769063	0.53	Phenol	0.06
26	26.82	45582435	8.74	Ethanone	0.97
28	28.36	2045564	0.39	2-Pentadecanone	0.04
32	30.96	3526105	0.68	5,10-Diethoxy-2,3,7,8-tetrahydro-1H	0.07
33	31.15	6894621	1.32	Anthrone	0.15
34	31.59	30881890	5.92	9,10-Anthracenedione	0.66

Figure 5.2 GC-MS chromatogram of ethanolic extract of biomass-derived lignin by ultra-sonication extraction method



Peak#	Ret.Time	Area	Area %	Name	Yield (mg/g)
1	6.16	1480397	0.19	2-Cyclopenten-1-one	0.03
2	8.31	18844463	2.43	Phenol	0.41
3	9.83	2525884	0.33	2-Cyclopenten-1-one	0.06
4	10.72	24178162	3.11	Phenol	0.53
5	11.08	13194286	1.70	Cyclopropyl carbinol	0.29
6	12.15	4425627	0.57	Bicyclo[2.2.2]octan-1-ol	0.10
8	13.15	21633658	2.79	Phenol	0.47
9	14.04	40384820	5.20	1,2,3-trichloromethane	0.88
10	14.58	9297047	1.20	2-Cyclohexen-1-one	0.20
11	14.75	69307689	8.92	Benzofuran	1.51
12	15.77	16055693	2.07	Phenol	0.35
13	16.78	39397855	5.07	2-Methoxy-4-vinylphenol	0.86
14	17.77	47337547	6.09	Phenol	1.03
16	19.13	12105832	1.56	Vanillin	0.26
18	20.16	8587355	1.11	Phenol	0.19
19	21.00	70498918	9.08	Acetophenone	1.54
20	21.23	17760574	2.29	Apocynin	0.39
21	21.45	3696561	0.48	1-Hydroxy-7-hydroxymethylindane	0.08
23	21.96	9984644	1.29	Benzene	0.22
24	22.20	2653779	0.34	2-Propanone	0.06
28	26.81	79262087	10.20	Ethanone	1.73
31	30.80	4485577	0.58	10-Methyl-9(10H)-anthracenone	0.10
32	30.93	7625068	0.98	n-Hexadecanoic acid	0.17
33	31.16	9771864	1.26	Anthrone	0.21
35	31.62	62450637	8.04	9,10-Anthracenedione	1.36
38	34.11	9129338	1.18	cis-9-Hexadecenal	0.20

Chapter 6 - Conclusions and future work

Conclusions

In this research, different byproducts from bioprocessing industry and raw biomass samples were tested for specialty value-added chemicals. Protobind-1000 was considered as a valuable lignocellulosic raw materials that contains various types of bioactive compounds. Vanillin, apocynin and phytol were regarded as three compounds of economic value.

Different extraction methods such as bead beating, ultra-sonitation, and different organic solvents such as methanol, ethanol and dichloromethane were evaluated to an optimum extraction method for the specific compounds. Eventually, ethanol was regarded as a good solvent for our target compounds due to its availability and environmental friendly properties. Ultra-sonication under the condition of probe amplitude of 45 and extraction time of 60min gave us a relatively higher yields of all the target compounds.

TLC and column chromatography were used for the detection and isolation of vanillin, apocynin, and phytol. It was found that a solvent system of hexane, dichloromethane, ethyl acetate, chloroform in 1:1:1:0.1 is an optimum eluent for the efficient separation of target compounds of interest by column chromatography. GC-MS was extensively used for the qualitative and quantitative analysis of different constituents of extract.

Lignocellulosic biomass was successfully converted into biomass-derived lignin by using SAQ pulping technique. The results indicate that biomass-derived and commercial lignin have almost the same chemical composition and comparable amount of target compounds of interest.

Future prospects

Lignin from differenty biomass sources has remarkable variations with respect to molecular weight and polydispersity, along with different functional groups, due to species specificity, location, cultivar, local soil conditions, water level, processing conditions and pre-treatment methods followed during biomass deconstruction. The complication of lignin structure poses challenges to the lignin-based extraction research and biorefinery process industries. The evaluation of different lignin and their analogs will make substantial contribution toward the extraction of value-added chemicals from these resources.

Depolymerising the lignin to its derivatives can be achieved through various effective depolymerization methods (Wang et al. 2013). The depolymerization can be further studied to obtain the desired materials. The fragmented lignin part could be a valuable source for specialty and other value-added chemicals and materials.

Future work will also focus on increasing the extraction and isolation efficiency of the target compounds. In this research, we investigated the isolation of vanillin, apocynin, and phytol from commercial lignin. The isolation of other valuable compounds from biomass-derived lignin should also be investigated. Biomass-derived lignin will be studied for its application as polymer resin filler, adhesives and raw material for making carbon fiber.

Experimental data for the COI extraction in a laboratory-scale are presented and discussed in this research. In the future, scalability of the extraction process will be attempted to obtain significant amount of the COI. Different types of extractors, extraction columns, and extraction equilibrium will be considered during the scale-up design.

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