Extraction and Enrichment of Flaxseed Lignan (*Linum usitatissimum* L.)

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ABSTRACT

Plant lignans are a class of natural plant diphenolic compounds that are made by the condensation of two phenylpropanoid moieties. Oilseed and cereal crops including flaxseed and sesame seed are major sources of plant lignan with flaxseed containing the highest amount of lignan among all plants tested. Secoisolariciresinol (SECO), a compound that can elicit responses in mammals that mimic estrogen, is the primary lignan produced by flaxseed. The lignan SECO can be transformed into enterodiol (END) and enterolactone (ENL) by intestinal bacteria and these molecules can be absorbed and affect mammalian physiology. Flaxseed SECO is in the form of a heteropolymer that contains SDG crosslinked with HMGA by ester bonds. The objective of this study is to establish procedures to recover the SDG polymer, SDG and SECO with acceptable yield and quality. Experiments were conducted to optimize three operations: first, optimization of aqueous ethanol extraction of SDG polymer from whole flaxseed; second, hydrolysis of SDG polymer ester bonds to release SDG; and finally, enzymatic hydrolysis of SDG ether bonds to produce free SECO. Optimization of ammonium hydroxide hydrolysis and enzymatic hydrolysis were investigated. Whole flaxseed (CDC Sorrel) was used as the raw material; SDG polymer extraction included two extractions with 70% ethanol (v/v) at room temperature; SDG polymer was hydrolysed using 29% (w/w) ammonium hydroxide solution at a ratio of 4:1 (w/w) at 80 °C for 2 h; SDG was refined over resin; finally, SECO was released using 30 U/mL cellulase from *Trichoderma viride* in sodium acetate buffer solution in ratio of 5:7 (v/v) at 60 °C for 48 h. Yields of SDG polymer, SDG and SECO were 2.7(of defatted meal mass), 51.9 (of isolated SDG polymer mass), and 66.6% (of SDG mass), respectively. The content of SDG in the polymer, SDG and SECO were 65.8 (g SDG/g polymer), 89.5 (g SDG/g), and 86.4 (g SECO/g), respectively. These results indicate a possible industrial process for recovery of flaxseed lignan from defatted flaxseed meal. The approach may reduce total production cost and the price of enriched flaxseed lignan. This would be beneficial for intensive applications of flaxseed lignan as a medicine, dietary supplement or other health ingredient.

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LIST OF ABBREVIATIONS

ABS	Aqueous Biphasic Systems
CDC	University of Saskatchewan Crop Development Center
DFHF	Defatted Flaxseed Hull Flour
DHA	Docosahexaenoic Acid
END	Enterodiol
ENL	Enterolactone
EPA	Eicosapentaenoic Acid
GC-MS	Gas Chromatography-Mass Spectrometry
HMGA	3-Hydroxy-3-Methyl Glutaric Acid
HPLC	High Performance Liquid Chromatography
ID-GC-MS	Isotope Dilution-Gas Chromatography-Mass Spectrometry
IR	Infrared Spectroscopy
ISO	Isolariciresinol
LARI	Lariciresinol
MAR	Macroporous Adsorption Resin
MAT	Matairesinol
MS	Mass Spectrometry
NCI	National Cancer Institute of the United States
NMR	Nuclear Magnetic Resonance
PAC	Phosphomolybdic Acid Colorimetric Method
PINO	Pinoresinol
PLPW	Pressurized Low Polarity Water Extraction
PLR	Pinoresinol-Lariciresinol Reductase
SD	Standard Deviation
SDG	Secoisolariciresinol Diglucoside
SECO	Secoisolariciresinol
SFE	Supercritical CO_2 Fluid Extraction
TLC	Thin-Layer Chromatography
UV	Ultraviolet

1. INTRODUCTION

1.1 Overview

Flax (*Linum usitatissimum*), an annual herbaceous plant, is traditionally cultivated as a source of oil and fiber for industrial and food applications (Xu and Huang, 1998). It is widely grown in India, Canada, China, United States, Kazakhstan, Ethiopia and Russia (FAOSTAT, 2020). Its seed, flaxseed, contains nutrients such as α-linolenic acid, dietary fiber and lignan (Yu et al., 2018). It is found to benefit health by inhibiting the development and progression of atherosclerosis, cancer, inflammation, and osteoporosis (Kalac, 1975).

Plant lignans are compounds produced by the phenylpropanoid pathway (Fuentealba et al., 2015; Yang, 2005), which are composed of two propyl-benzene moieties linked by a bond between the 8 and the 8' position (Moss, 2000). Oilseed and cereal crops including flaxseed and sesame seed are reported as major plant lignan sources. In the 1990s, flaxseed was found to have the highest known content of the lignan secoisolariciresinol (SECO) among all plant crops studied (Mazur, 1998). SECO has similar structure to natural and synthetic estrogens and is considered to be a phytoestrogen. Bakke and Klosterman (1956) reported that SECO occurs in flaxseed in the form of the diglucoside, SDG. The response of cells and organisms to SDG suggest that the compound is estrogenic and binds to estrogen receptors, induces specific estrogen-responsive gene products, and stimulates estrogen receptor-positive breast cancer cell growth (Bakke and Klosterman, 1956; Kurzer and Xu, 1997). In addition, flax lignan is a natural antioxidant that can be used to treat eczema and quench active oxygen on the skin (Renault and Catroux, 2004). As one of the most important bioactive components in flaxseed, flaxseed lignan adds value to flaxseed processing where flaxseed and its fractions are utilized for their health benefits.

Previous studies of the detection, extraction, and enrichment of SDG focus on laboratory-scale experiments. Industrial production of SDG is not well developed as it faces many problems including the need for high cost of equipment that requires expensive maintenance combined with low yield and product quality. In current market, there is no commercial SDG polymer product; the price of 40% SDG is 0.2 CAD/g; the price of 97% SDG is 18-29 CAD/g; and the price of 95% SECO is 40.4 CAD/mg (Sigma-Aldrich Canada Ltd.; Oakville, ON, Canada). As SDG is valued for its utility as a dietary supplement and a medicine, establishing a standard method for industrial-scale production of high quality lignan can help to lower the cost and price is important for its

intensive application. The current study was conducted to develop a food-safe protocol to produce SDG and its derived products and provided possible approaches to achieve SDG industrial production.

1.2 Hypothesis

Flaxseed lignan, including SECO, SDG and a polymer composed of SDG and HMGA, can be recovered in an integrated process that optimizes yield and quality.

1.3 Objectives

Previous studies of 0.1-10 kilogram scale extraction and enrichment of flaxseed lignan mostly describe direct hydrolysis of SDG polymer after extraction without the isolation of SDG polymer as an intermediate product. In these processes the final product is generally SDG while SECO is not recovered. However, SDG polymer, SDG and SECO might have different physicochemical properties, bioactivity and potentially commercial value. Establishing an industrial process that is able to produce all three products might both increase the product value and reduce production costs.

The specific objectives of this study are as follows:

1) to establish a simple method with few procedures to extract SDG polymer from defatted flaxseed meal suitable for increased scale production;
2) to establish an environmentally friendly method to release SDG from SDG polymer; and
3) to establish an environmentally friendly method to hydrolyze SDG to SECO.

2. LITERATURE REVIEW

2.1 Flaxseed Overview

Flaxseed (*Linum usitatissimum*) is an important commercial oilseed crop with long history of use in industrial, food and feed applications (Zhou and Li, 2010). It is a member of the genus *Linum* in the family Linaceae, and its species name, *usitatissimum*, is Latin for "most useful" (Vaisey-Genser and Morris, 2003). Flax originated in central Asia and the Mediterranean region. It has been cultivated for more than 8,000 years as an industrial oil and a fiber crop (Vaisey-Genser and Morris, 2003; Zhou and Li, 2010). Flax is an annual herbaceous plant with erect stem, linear or lanceolate leaves, and lignified base. The structure of flax phloem fiber is long and thin, shiny, elastic and strong. It is an ideal raw material for textile and papermaking. Flax flower is umbrella shape with 5 petals that can be white, pale blue, or purple-blue. It grows on the top of the plant or the axils of the upper leaves and flowers from June to August. Some related species are used as ornamental plants (Xu and Huang, 1998). Mature flaxseed is oblong and flat in shape with pointed tip, may be yellow, olive, brown or dark brown based on genetics. A typical seed is approximately 2.5 × 5.0 × 1.5 mm in width, length and height, respectively (Freeman, 1995; Oomah et al., 1996; Wanasundara and Shahidi, 1997) though considerable variation is observed. Flaxseed consists of three parts: epidermis, endosperm and cotyledon. The thick epidermis is made of four cell layers: an outer mucilage layer that makes up about 8% of the seed weight, followed by a layer of surrounding cells, fibers, and pigments (Bhatty and Cherdkiatgumchai, 1990; Oomah et al., 1996). The endosperm is directly below the epidermis, and it has both high protein and oil contents. The innermost seed structures are the two cotyledon and germ. Figure 2.1 shows side view and cross-section of flaxseed (Shim et al., 2014). Oil is mainly stored in the endosperm while fiber and gum are mainly in the hull. Flaxseed endosperm adheres to the epidermis when seed is milled making it difficult to separate the flaxseed kernel from its hull. This property is not conducive to efficient flaxseed processing and utilization as quality and yield of flaxseed oil and gum may be decreased by the presence of contaminants (Oomah and Mazza, 1997; Oomah and Mazza, 1998; Oomah et al., 1996; Wiesenborn et al., 2003).

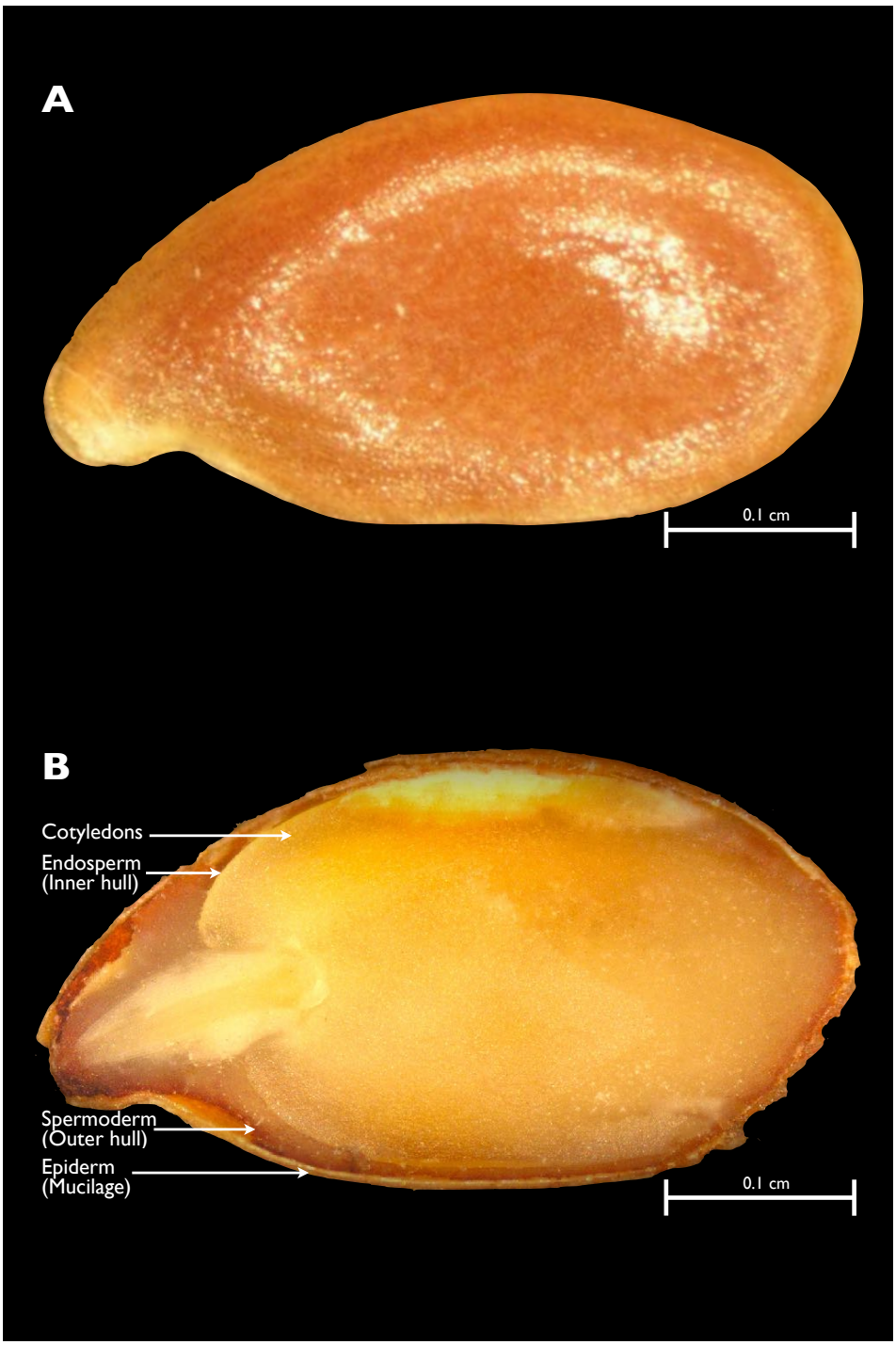


Figure 2.1. Hand-cut sections of flaxseed (*L. usitatissimum* L., var. CDC Bethune) mounted in distilled water showing anatomical structures: (A) the side of flaxseed; (B) hand-cut section of flaxseed (Shim et al., 2014).

As an important oil and textile crop, flaxseed is now widely grown including, in the Siberian steppe, central Asia, west Asia and the Mediterranean region (Li, 2007). In the most recent 20

years, India, Canada, China, the United States, Kazakhstan, Ethiopia and Russia produce the majority of flaxseed (Chen and Xu, 2004; FAOSTAT, 2020). According to the latest annual flaxseed production records (FAOSTAT), since 1998, flaxseed production in China, the United States and India remain stable; data in Canada fluctuates the most, with production in 2005, 2006, 2009 and 2015 counting for 2.5 times the output in 2011; Kazakhstan and Russia had relatively low production during 1998 to 2008 and substantially increased output in the last decade; Kazakhstan surpassed Canada in 2018 to become the world's major flaxseed producer (FAOSTAT, 2020).

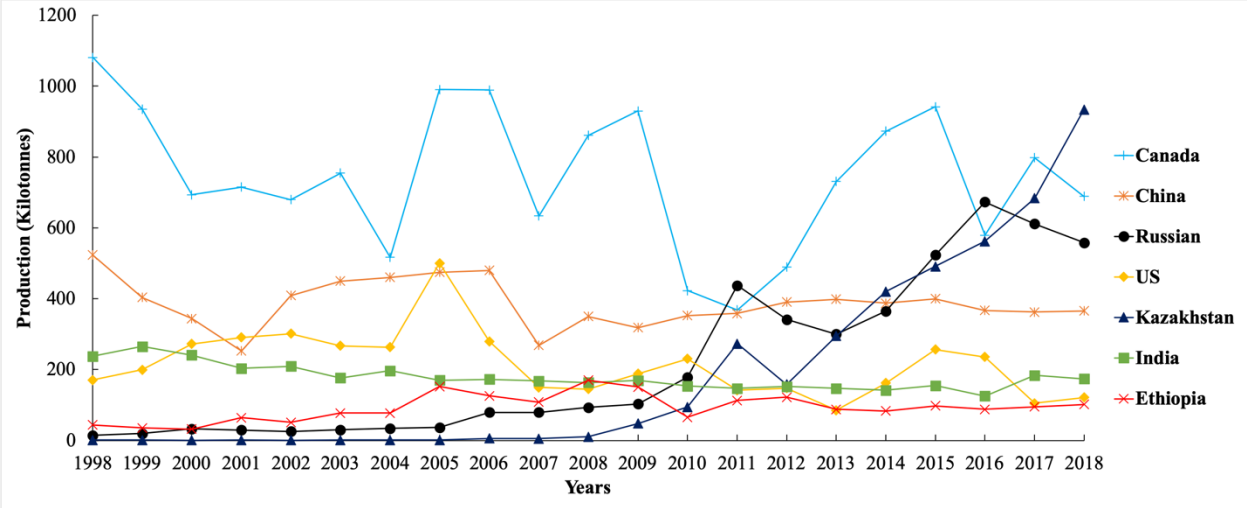


Figure 2.2. Annual flaxseed production in Canada, China, Russian, the United States, Kazakhstan, India and Ethiopia in 1998-2018; data collected from the Food and Agriculture Organization of the United Nations (FAOSTAT, 2020).

2.1.1 Flaxseed Composition

Flaxseed has a high content of oil and protein. Flaxseed oil mainly consists of α-linolenic acid, linoleic acid and oleic acid; α-linolenic acid constitutes about 52% of total fatty acids (Rubilar et al., 2010) though cultivars are known that produce a wide range of concentrations of this constituent. Flaxseed protein contains all essential amino acids, among which arginine, glutamic acid and aspartic acid account for 40% of the total content (Dev and Quensel, 1986; Madhusudhan and Singh, 1983; Yang, 2016). Flaxseed protein has high nutritional value. Studies have shown that flaxseed protein can support heart health and improve human immunity (Oomah and Mazza, 1993; Yang, 2016). Flaxseed meal can have substantial commercial value as it imparts water-

binding capacity, emulsifying ability, and foamability to foods (Oomah and Mazza, 1993). There are more than 3,600 flaxseed cultivars held in world collections (Vaisey-Genser and Morris, 2003). The chemical composition of flaxseed is 7-9% moisture, 34-45% oil, 26-34% protein, 3-6% ash, and 20-25% fiber; but composition can vary with the origin, cultivar, climate and growing conditions. Protein content is often negatively correlated with flaxseed oil content (Bhatty and Cherdkiatgumchai, 1990; Coskuner and Karababa, 2007; Oomah and Mazza, 1997). In addition, both flaxseed gum and lignan contribute to its bioactivity. Table 2.1 shows content of major and minor components of 79 flaxseed cultivars grown in China and Canada (Yu et al., 2018).

Table 2.1. Composition of whole flaxseed (Yu et al., 2018).

Major Component	Content (%)	Mineral	Content (mg/100g)
Moisture	6.75-9.09	Mn	15.9-38.0
Crude Fat	33.93-44.71	Fe	2.5-10.8
α-Linolenic Acid	33.42-59.74	Cu	0.3-1.2
Total Carbohydrate	30.21-36.93	Zn	1.1-4.1
Ash	3.31-5.95	Ca	92.2-262.6
Crude Protein	25.95-34.27	P	517.4-1016.1

2.1.2 Flaxseed Nutritional Components and Utilization

Nutritional components of flaxseed consist of oil, protein, minerals, lignan, flaxseed gum and vitamins. Scientists in Canada, Europe and the United States have conducted substantial research into the benefits of flaxseed nutritional components including flaxseed lignan, α-linolenic acid and flaxseed gum. In the last decade, flaxseed lignan and α-linolenic acid have received attention as potential nutrients for reducing the incidence of cancer and cardiovascular disease. The National Cancer Institute of the United States (NCI) has listed flaxseed as one of six plants that mitigate the risk of cancer. Flaxseed and its processed products have been widely used in the daily life of a growing number of Western consumers (Petzinger et al., 2014; Sainvitu et al., 2012; Sugasini and Lokesh, 2012; Thakur et al., 2009; Zhang et al., 2009).

2.1.2.1 Flaxseed Oil

Flaxseed oil has attracted wide attention as a nutritional edible oil in recent years. It has been reported that long term consumption of flaxseed oil improves blood clotting, while it ameliorates the development of hyperlipidemia, colon cancer, atherosclerosis, diabetes and cardiac arrhythmia.

These studies indicate potential beneficial effects of flaxseed and flaxseed products in prevention and treatment of chronic diseases including cardiovascular and cerebrovascular diseases (Pradhan et al., 2010; Özkal, 2009).

Li and Ding (2005) analyzed the chemical composition of flaxseed oil using Gas Chromatography-Mass Spectrometry (GC-MS) and reported 13 fatty acids, including four saturated and nine unsaturated fatty acids. The unsaturated fatty acids account for 87.1% of the total lipids, among which α-linolenic acid (49.05%), oleic acid (22.34%) and linoleic acid (13.73%) were the most prominent (Li and Ding, 2005). In another study unsaturated fatty acids constituted 82.38% of total fatty acids with α-linolenic acid (74.01%) having the highest content (Wang, 2012).

Both α-linolenic acid and linoleic acid are essential fatty acids for mammals including humans. Human hepatic metabolism can produce other ω-3 fatty acids including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) from α-linolenic acid (Payne, 2000). Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) promote brain and retinal development, regulate immunity, blood cholesterol, and ameliorate cardiovascular and cerebrovascular diseases. For adult humans a daily intake of 0.65g DHA and EPA or 2.22g α-linolenic acid is recommended (Zhang and Liu, 2000). Compared with linoleic acid, which is an ω-6 unsaturated fatty acid, foods with substantial contents of α-linolenic acid are limited. For example, α-linolenic acid content in soybean oil and peanut oil is 7.8% and 1.5%, respectively (Payne, 2000). Lack of ω-3 unsaturated fatty acids might lead to unbalanced fatty acid metabolism and eventually impact health. Sea fish oils have long been a source of ω-3 fatty acid supplements. However, long-term use of fish oils can lead to excessive consumption of vitamins A and D, unbalanced intake of vitamin E, and cholesterol; moreover, sea fish oils are expensive as marine resources are increasingly being depleted. In contrast, flaxseed oil is a rich and convenient source of α-linolenic acid that is associated with less pollution, safe consumption, and a comparably lower cost. Since α-linolenic acid is the main source of ω-3 unsaturated fatty acids, flaxseed and its processed products, with high content of α-linolenic acid, can be considered as substitutes for sea fish oils (Di, 2002; Oomah and Mazza, 1993; Payne, 2000; Wang, 1986).

2.1.2.2 Flaxseed Gum

Flaxseed gum is a water-soluble dietary fiber present in flaxseed hull. It is a major co-product in flaxseed meal and can be used as natural food gum. Flaxseed gum constitutes 2~10% of whole flaxseed weight depending on cultivar and conditions during plant growth (Lu et al., 2007). Flaxseed gum is composed of protein, small amount of minerals, and polysaccharides made up of arabinose, galactose, rhamnose, xylose, fucose and glucose (Marpalle et al., 2014; Martinchik et al., 2012; Whistler and BeMiller, 1993). Flaxseed gum is an ideal source of soluble dietary fiber. Dietary flaxseed gum can mitigate the effects diabetes, help to prevent obesity, lower the risk of cardiovascular diseases, colon cancer, cholelithiasis, decrease blood cholesterol, and protect against exposure to heavy metals and pesticides (Chen, 2004; Elif Bilek and Turhan, 2009; Oomah and Mazza, 2001; Williams et al., 2007). In addition, as a hydrophilic colloid, flaxseed gum has good solubility, water-binding capacity, emulsification properties, gelation properties, and rheological properties. Therefore, flaxseed gum can be used as dietary supplement and food additive that can thicken, stabilize, emulsify and create edible foams (Chen et al., 2006; Wang et al., 2011; Whistler and BeMiller, 1993).

2.1.2.3 Flaxseed Lignan

Lignan is a group of phenolic compounds, of which flaxseed lignan induces estrogen like effects due to the ability of lignan metabolites to interact with estrogen receptors. Flaxseed lignan can be transformed by human intestinal microorganisms and in the human body to become bioactive compounds. Studies have shown that lignan is found in many plant materials, of which flaxseed has the highest content (Johnsson et al., 2000; Johnsson et al., 2002; Yang and Tian, 2011). Bakke and Klosterman reported the extraction of lignan from flaxseed in 1956 (Bakke and Klosterman, 1956). Since then, many researchers have conducted comprehensive analyses on the nutritional and medical potential flaxseed lignan and lignan products of microbial and mammalian metabolism. The structure, biosynthesis, metabolism and bioactivities of flaxseed lignan are summarized in section 2.2, and the detection, extraction and enrichment are described in section 2.3.

2.2 Lignan

Lignan constitutes a widely distributed group of plant natural products with more than 200 structures identified (Macrae and Towers, 1984). Lignan polyphenols are synthesized from phenylalanine which is metabolized to coniferyl alcohol which is then dimerized. Most lignans are dimers, though trimers and tetramers exist (Fuentealba et al., 2015; Yang, 2005). Recent studies have primarily focused on lignan antioxidant activity and structural similarity to human estrogen.

Lignan is found in many common plants and grains including oilseeds, beans, vegetables, fruits, berries and cereals. Plant lignan content can be affected by many factors such as genetics, and environment (climate, and cultivation conditions). Among common edible plants, oilseeds, nuts and berries have relatively higher lignan contents. Table 2.2 shows lignan content in selected foods and flaxseed is reported as containing highest amount of lignan. Flaxseed lignan accounts for 1~4% of whole flaxseed weight, which is 75~800 times that of other plants (Johnsson et al., 2000; Thompson et al., 1991; Xu and Sun, 2007). Therefore, flaxseed may be considered as a major plant lignan source.

Table 2.2. Phytoestrogen (SECO) content of selected food (Mazur, 1998).

Plant Food Trivial Name (*Botanical Name*)	SECO Content (μg/100g Dry Weight)
Grains and Cereals	
Rye (*Secale cereale*)	47.1
Wheat (*Triticum dicoccum*)	8.1
Barley (*Hordeum* spp)	58.0
Oats (*Avena sativa*)	13.4
Maize (*Zae mays*)	8.0
Rice (*Oryza sativa*)	16.0
Oilseeds and Nuts	
Flaxseed (*Cuscuta epilinum*)	369,900
Sunflower Seed (*Helianthus*)	610
Clover Seed (*Trifolium* spp)	13.2
Peanut (*Arachis hypogaea*)	298.0
Walnut (*Juglans nigra*)	163.0
Hazelnut (*Corylus avellane pontica*)	119.0
Berries and Currants	
Bramble (*Rubus fructicosus*)	3,718
Strawberry (*Fragaria x ananassa*)	1,500

Cranberry (*Vaccinium macrocarpum*)	1,054
Red Raspberry (*Rubus ideaus*)	139.0
Blackcurrant (*Ribes nigrum*)	388.0
Redcurrant (*Ribes rubrum*)	165.3
Fruits	
Plum (*Prunus domestica*)	5.0
Banana (*Musa sapientum*)	10.0
Avocado (*Persea americana*)	76.7
Tomato (*Lycopersicum esculentum*)	51.6
Lemon (*Citrus limon*)	61.3
Orange (*Citrus sinensis*)	76.8
Vegetables	
Cabbage (*Brassica oleracea*)	33.0
Broccoli (*Brassica oleracea italica*)	414.0
Onion (*Allium cepa*)	83.0
Garlic (*Allium sativum*)	379.0

2.2.1 Major Lignan in Flaxseed

Flaxseed hull contains the lignans matairesinol (MAT), pinoresinol (PINO), isolariciresinol (ISO), lariciresinol (LARI) and SECO. Figure 2.3 shows structure of flaxseed lignans. These lignans are derived from same basic structure, a molecule comprising two propyl-benzene moieties linked by a bond between the 8 and the 8' carbons (Moss, 2000). SECO and MAT constitute the majority of flaxseed lignan (Hosseinian, 2006; Shim, et al., 2016). According to previous studies, SECO content is 3,699 mg/kg for whole flaxseed, while MAT content is 10.87 mg/kg. MAT accounts less than 1% of flaxseed SECO content, while the concentration of other lignans is much lower than MAT (Blitz et al., 2007; Eliasson et al., 2003; Mazur, 1998; Mazur et al., 1996; Meagher and Beecher, 2000). Therefore, flaxseed lignan typically only refers to the SECO content.

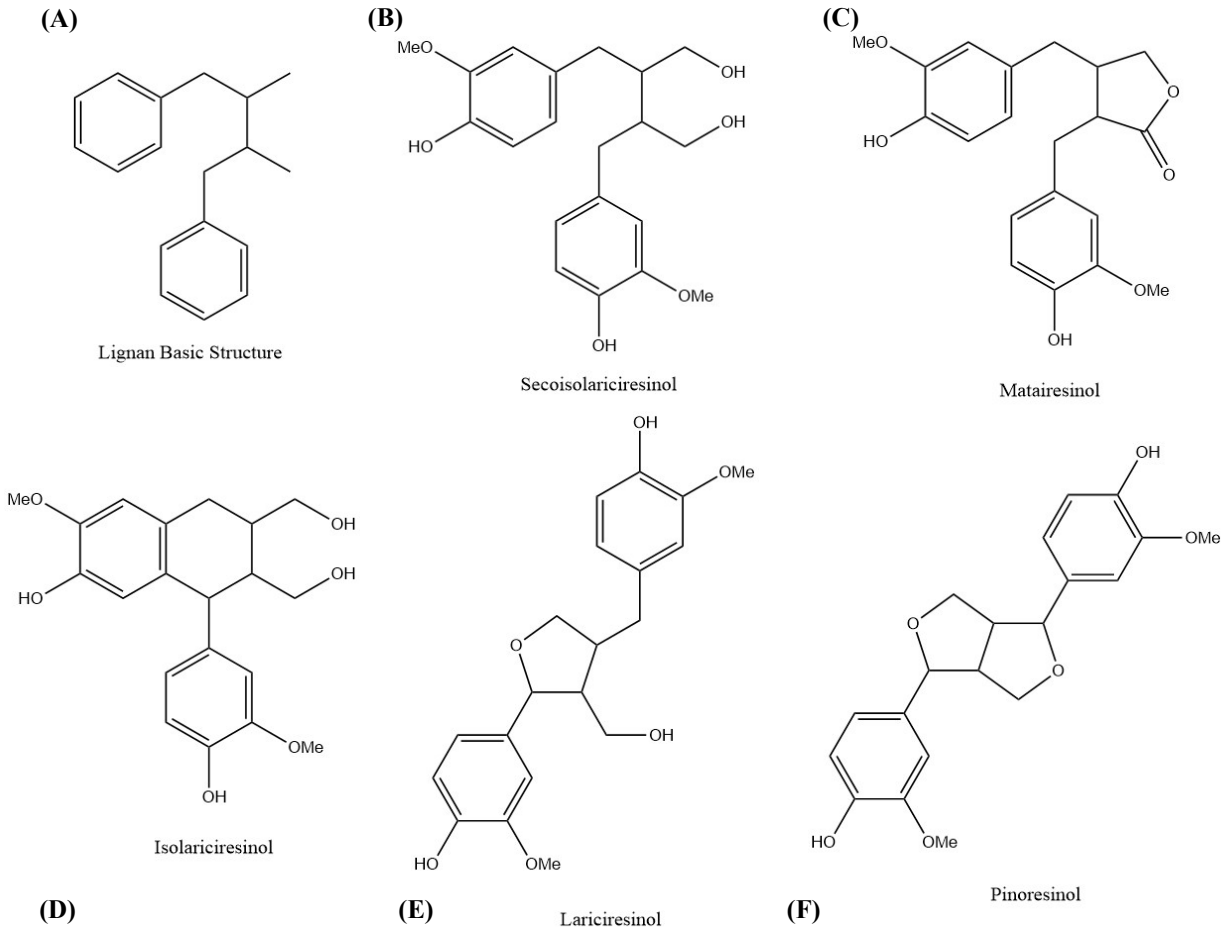
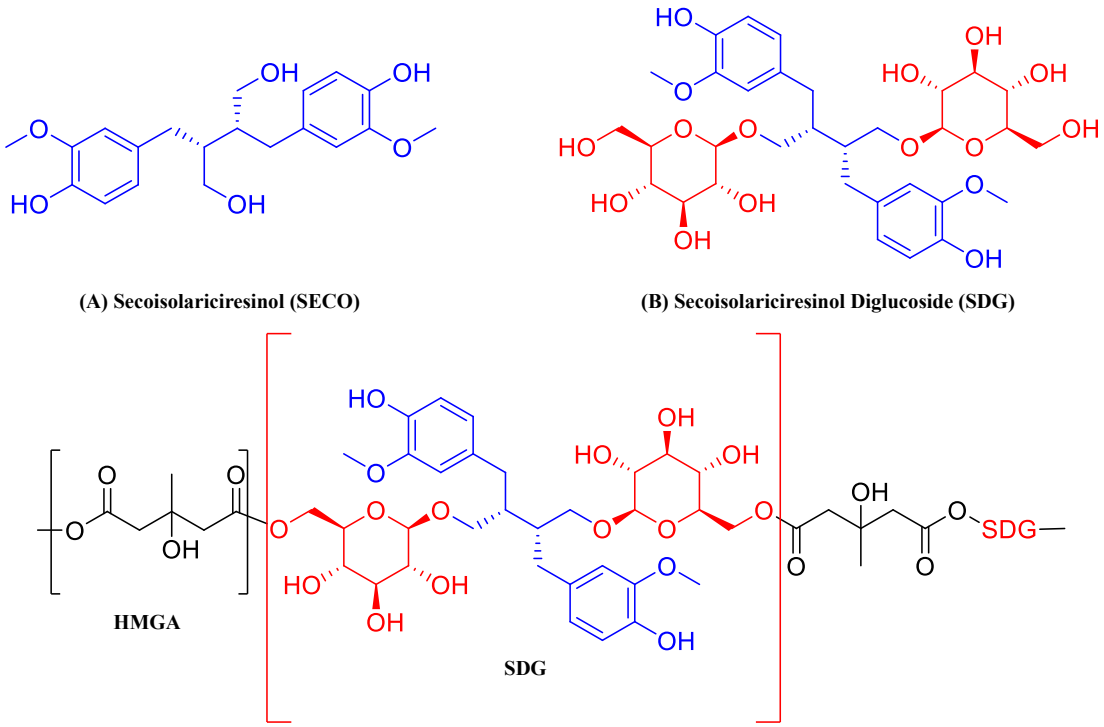


Figure 2.3. Structure of flaxseed lignans: (A) lignan backbone structure; (B) SECO; (C) MAT; (D) ISO; (E) LARI; (F) PINO.

2.2.2 SECO in Flaxseed

Secoisolariciresinol is found in flaxseed in the form of an SDG co-polymer. The SDG polymer is crosslinked through ester bonds with HMGA. Figure 2.4 shows structure of SECO, SDG and a polymer composed of SDG and HMGA. The SECO molecule is composed of two C_6C_3 units. For nomenclature purposes, Moss (2000) numbered the phenyl moiety from 1 to 6, starting from the carbon attached to the propyl group, with the propyl group is numbered from 7 to 9, starting from phenyl moiety. With the second C_6C_3 unit the numbers are designated in the same manner, but each number is followed with a single quote or prime (Moss, 2000). SECO has two hydroxyl groups attached on C-9 and C-9' position while these groups are bound to glucose in SDG. In the polymer, SDG molecules are ester-linked with HMGA through glucose residues (Ford et al., 2001; Kamal-Eldin et al., 2001). The SDG polymer structure varies depending on ratio of SDG to HMGA,

and it is not literarily determined (Yang, 2005). In the SDG biosynthetic pathway two coniferyl alcohol molecules are condensed by an oxidase in the presence of a dirigent protein to produce PINO. In turn, PINO is reduced by PINO-LARI reductase (PLR) into LARI, which is further reduced to SECO in the presence of PLR (Ford et al., 2001; Hu, 2005). SDG is seen as the major constituent in commercial flaxseed lignan products that is also the precursor to bioactive lignans.



(A) Secoisolariciresinol (SECO)

(B) Secoisolariciresinol Diglucoside (SDG)

(C) A Polymer Composed of Secoisolariciresinol Diglucoside (SDG) and 3-Hydroxyl-3-Methyl-Glutaric Acid (HMGA)

Figure 2.4. Structure of (A) SECO, (B) SDG, and (C) a polymer composed of SDG and HMGA.

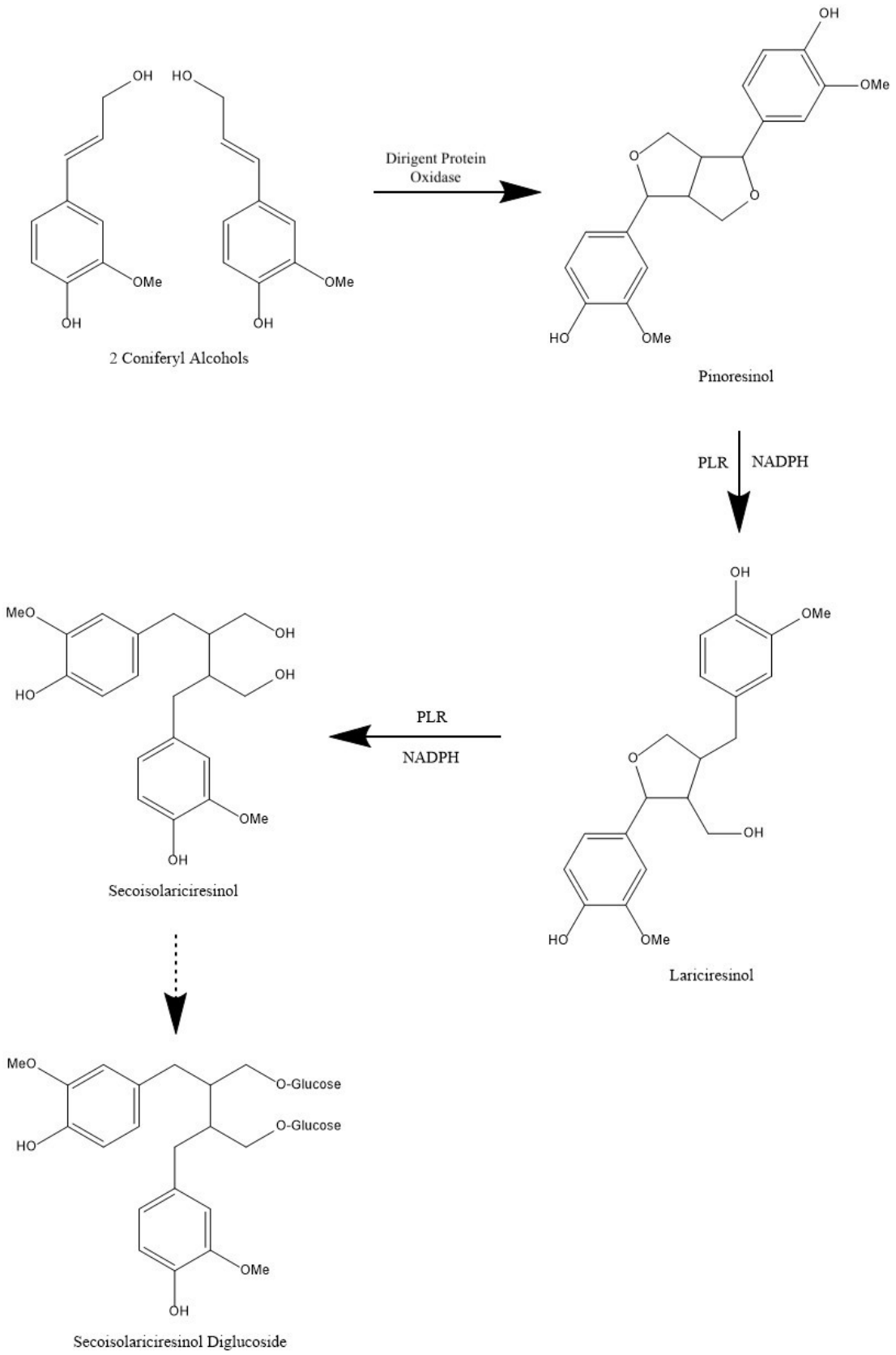


Figure 2.5. Biosynthetic pathway of SDG.

2.2.3 Metabolism of Flaxseed Lignan

As early as the 1980s, mammalian lignans, enterodiol (END) and enterolactone (ENL), with structures similar to lignans found in plants, were reported in human urine (Setchell et al., 1983). Figure 2.6 shows structure of END and ENL. Both END and ENL have the basic lignan carbon bond structure (Figure 2.3). Differences between mammalian lignans and plant lignans were related to aromatic ring substituents on the mammalian lignans. In mammalian lignans hydroxyl residues are present but no other groups. In addition to phenolic hydroxyl groups plant lignans can have other residues. Mammalian lignans like END and ENL are formed from plant lignans through the action of gut bacterial enzymes. Once modified these mammalian lignans are absorbed from the intestines. Absorbed lignans are then converted to sulfate and glucuronide conjugates by intestinal enterocyte and liver enzymes. These conjugates may undergo enterohepatic recirculation. Both END and ENL exhibit estrogenic activity *in vivo* and *in vitro* as their structure is similar to 17β-estradiol, a steroid estrogen (Axelson and Setchell, 1980; Wang et al., 2010).

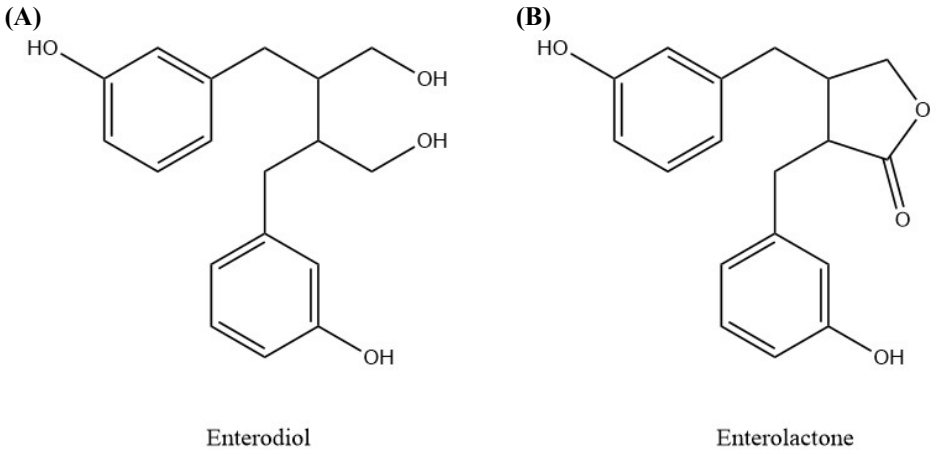


Figure 2.6. Structure of (A) END and (B) ENL.

Plant lignans including SDG and MAT are transformed into enterodiol (END) and enterolactone (ENL) in the presence of intestinal bacteria (Axelson and Setchell, 1980; Penalvo et al., 2005; Wang et al., 2000; Xie et al., 2003). Monogastric animals such as humans mainly absorb plant lignan after microbial metabolism in the colon and cecum (Alhassane and Xu, 2010; Quartieri et al., 2016). Figure 2.7 shows metabolism of SDG in mammals. Plant lignans are eventually transformed and activated to form END and ENL by several metabolic steps including deglycosylation, demethylation, dihydroxylation, and oxidation (Clavel et al., 2006a; Clavel et al., 2005; Clavel et al,. 2006b).

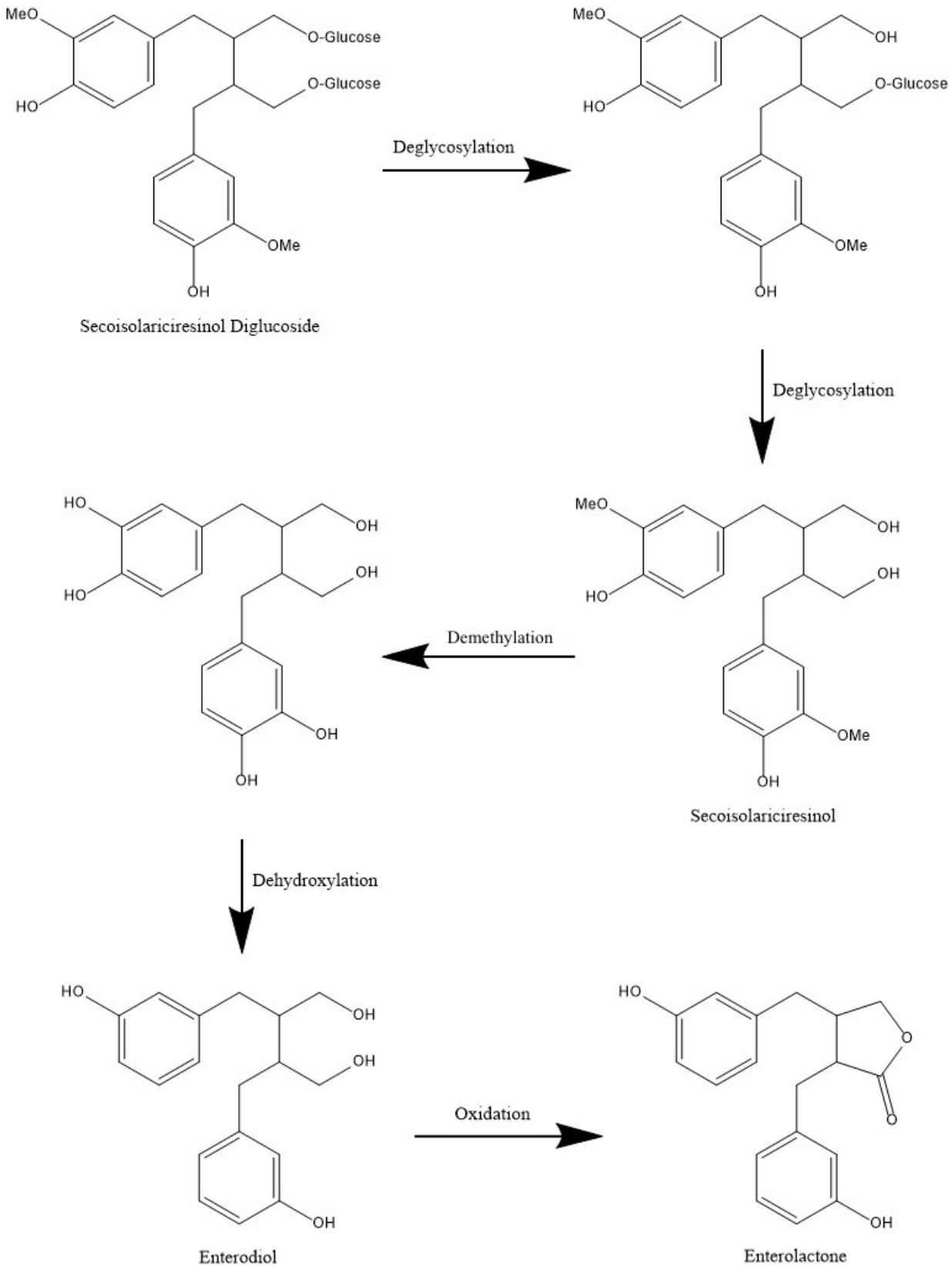


Figure 2.7. Metabolism of SDG in mammals.

2.2.4 Bioactivities of Flaxseed Lignan

Axelson's group first reported that flaxseed is an important source of mammalian lignan precursor (Axelson et al., 1982). According to previous reports, flaxseed lignan is both an antioxidant and an anti-inflammatory agent; it also can be used as a dietary prophylactic that mitigates the onset and severity of diabetes, cardiovascular disease, kidney disease, and especially some estrogen-dependent diseases such as breast cancer, premenstrual syndrome and osteoporosis (Adlercreutz, 2002; Alhassane and Xu, 2010; Clark et al., 2000; Dai et al., 2002; Haliga et al., 2013; Li et al., 2012; Masuda et al., 2010; Murkies et al., 1998; Ososki and Kennelly, 2003; Pan et al., 2009; Prasad, 2013; Prasad, 2001; Rodriguez-Leyva et al., 2013; Setchell et al., 2014; Tian et al., 2017; Yang et al., 2014; Zanwar et al., 2011; Zhang, 2013). Therefore, flaxseed lignan can be investigated as a bioactive for inclusion in medicine and health care products.

2.3 Flaxseed Lignan Detection, Extraction and Enrichment

The accurate determination of food lignan content is a difficult task as most lignans are present at low concentrations in plants and many are unstable. Flaxseed is the richest known source of lignan where most of its lignan is SECO. A number of researchers have reported methods for detection and quantification flaxseed lignan. These analytical methods can be generally described as being either direct or indirect. Indirect methods have been used to determine the mammalian lignan, enterodiol (END) and enterolactone (ENL), content of urine, while direct methods mainly focus on extraction of lignan from products and analysis of the extracts using Gas Chromatography (GC) or High-Performance Liquid Chromatography (HPLC). Indirect methods rely on the activity of intestinal bacteria and experimental conditions, which might not always be consistent. These methods are not widely applied in flaxseed lignan analysis (Thompson et al., 1991). Common methods for flaxseed lignan detection, extraction and enrichment are discussed below.

2.3.1 Common Methods for Sample Preparation

Prior to analysis SDG is generally extracted from either flaxseed meal after oil extraction or an enriched hull fraction after dehulling. A hull fraction with low protein and fat content is preferred for SDG extraction and enrichment as it requires less processing to remove impurities. According to a previous study, SDG was prepared by extraction from defatted flaxseed hull flour (DFHF). In the study whole flaxseed was first soaked in 70 °C water to remove gum. After drying the seed

was ground into large particles. Subsequently kernel and hull fractions were separated by sieve (Madhusudhan et al., 2000; Wiesenborn et al., 2003). Later, the separated hull fraction is dried (either freeze dried or oven dried) and defatted by stirring with hexane (1:10; w/v) for 1~2 h followed by centrifugation (3,000 rpm) for 10 min. After centrifugation, the sediment is washed with 5 volumes of hexane and then centrifuged at 3,000 rpm for another 10 min. The sediment is dried at 60 °C with airflow and is ground to a fine powder then screened through a 60-mesh sieve to generate the DFHF sample. The average powder particle size is 250 μm (Zhang and Xu, 2006c). SDG is then extracted from DFHF using polar organic solvents. For example, DFHF was extracted with 50% aqueous ethanol (v/v) at 60 °C for 3 h followed by centrifugation at 3,000 rpm for 15 min. The supernatant liquid was then evaporated at 45 °C to remove the solvent and mixed with 0.25 M sodium hydroxide (NaOH) solution at 40 °C for 3 h. Later, the pH was adjusted to 4.0 by using 2.0 M hydrochloric acid (HCl) solution and the product centrifuged for another 15 min. The final supernatant liquid was filtered through a 0.45 μm film to afford a sample for SDG analysis (Zhang and Xu, 2006a).

2.3.2 Flaxseed Lignan Detection

2.3.2.1 Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) has been used in lignan detection since the 1960s. It is a simple and inexpensive technique that is particularly suitable for screening large numbers of samples. Typically, TLC is a qualitative analysis as quantitative TLC requires densitometric detection. The most used phase is silica gel and intermediate polarity solvents like dichloromethane-ethanol (93:7; v/v) serve as eluents. Compounds on thin layer plates like SDG can be observed after spraying with 10% sulfuric acid in ethanol followed by 2-min heating at 150 °C. This approach converts lignans to visible spots. Quantitative analysis of SDG content by TLC is not widely applied in modern lignan analysis to monitor processing (Willför et al., 2006).

2.3.2.2 Phosphomolybdic Acid Colorimetric (PAC) Method

Heating lignans in the presence of phosphomolybdic acid at 100~120 °C produces an observable colour reaction. Zhang and Xu (2006c) reported the development of a stable color when heating samples in a boiling water bath for 15 min. For this approach, SECO (>95%) was first dissolved

in methanol to make a 11.65 μg/mL standard. The standard solution was then mixed with three volumes of 5% phosphomolybdic acid-ethanol solution after which solvent was removed by heating the mixture in an 80 °C water bath and colour was developed by transferring the heated residue to a boiling water bath for 15 min. After cooling the samples to room temperature, 10 mL of distilled water was added to completely dissolve the residue. Light absorbance at 700 nm was observed with a UV-Vis spectrophotometer. A linear standard curve of can be plotted of SECO concentration versus absorbance at 700 nm. This method can be used to quantify extracted SDG samples treated in the same way as SECO standards (Zhang and Xu, 2006c). The phosphomolybdic acid-ethanol reagent can be used for rapid and convenient determination of SDG yield and quality during SDG extraction and enrichment. Unfortunately, as the SDG content determined this way is based on a standard curve that use SECO as a standard, the results can be easily affected by quality of original SECO standard and errors during experimental operations.

2.3.2.3 Ultraviolet Spectrometry (UV) and Mass Spectrometry (MS)

Researchers have reported both UV and MS method for SDG detection. For these analyses sample preparation is similar to that of phosphomolybdic acid colourimetry (PAC) methods. In these procedures mild enzyme hydrolysis is used to prevent by-product formation during isolation. HPLC coupled with UV or MS detection is often applied to characterize plant phenolic components including SDG and flavonoids. In Johnsson's study (2000), HPLC analyses were performed with UV/DAD detection at wavelengths between 210 and 400 nm. Chromatograms were recorded at 280 nm. A linear HPLC calibration curve for standard SDG was previously obtained. The detected SDG peaks were identified and quantified by comparison with those of SDG standard. (Johnsson et al., 2000; Obermeyer et al., 1995). These two methods are rapid and robust and the cost for each analysis is minimal. Most importantly a benefit of enzyme hydrolysis is that it avoids the formation of undesired products that are generated by acid hydrolysis and derivatization of SDG. Conversely, the weakness of these methods is that SDG quantitation may be affected by numerous experimental factors.

2.3.2.4 Isotope Dilution-Gas Chromatography-Mass Spectrometry (ID-GC-MS)

A combined method of ID-GC-MS was developed for SDG quantification in flaxseed (Mazur et al., 1996). This is a very sensitive technique that can be very accurate. The method is suited for

quantitative determination of SDG and other plant phytoestrogens. Due to the high sensitivity, standard preparation for this method can be more complicated compared with analyses that do not use cold labelled internal standards. The unit operations of ID-GS-MS include sample preparation, enzymatic hydrolysis, ether extraction, acid hydrolysis, chromatographic analysis and GC/MS detection. Enzymatically active *Helix pomatia* intestinal extract, the enzyme used for hydrolysis is first enriched with 1% charcoal in 9 mL of 0.66 M acetate buffer at pH 4.1 (Adlercreutz et al., 1991; Kraushofer and Sontag, 2002; Mazur et al., 1996; Schmidt et al., 2006). An outline of the procedures involved with ID-GC-MS are shown in Figure 2.8 (Mazur et al., 1996). However, this method has some disadvantages. For example, adding internal standard is required to estimate sample losses during workup. In addition, the cost per analysis is relatively high. Therefore, this method is not commonly used in many laboratory and industrial settings. Nevertheless, extraction and detection steps can be modified and combined with simpler methods for low-cost monitoring of industrial processes.

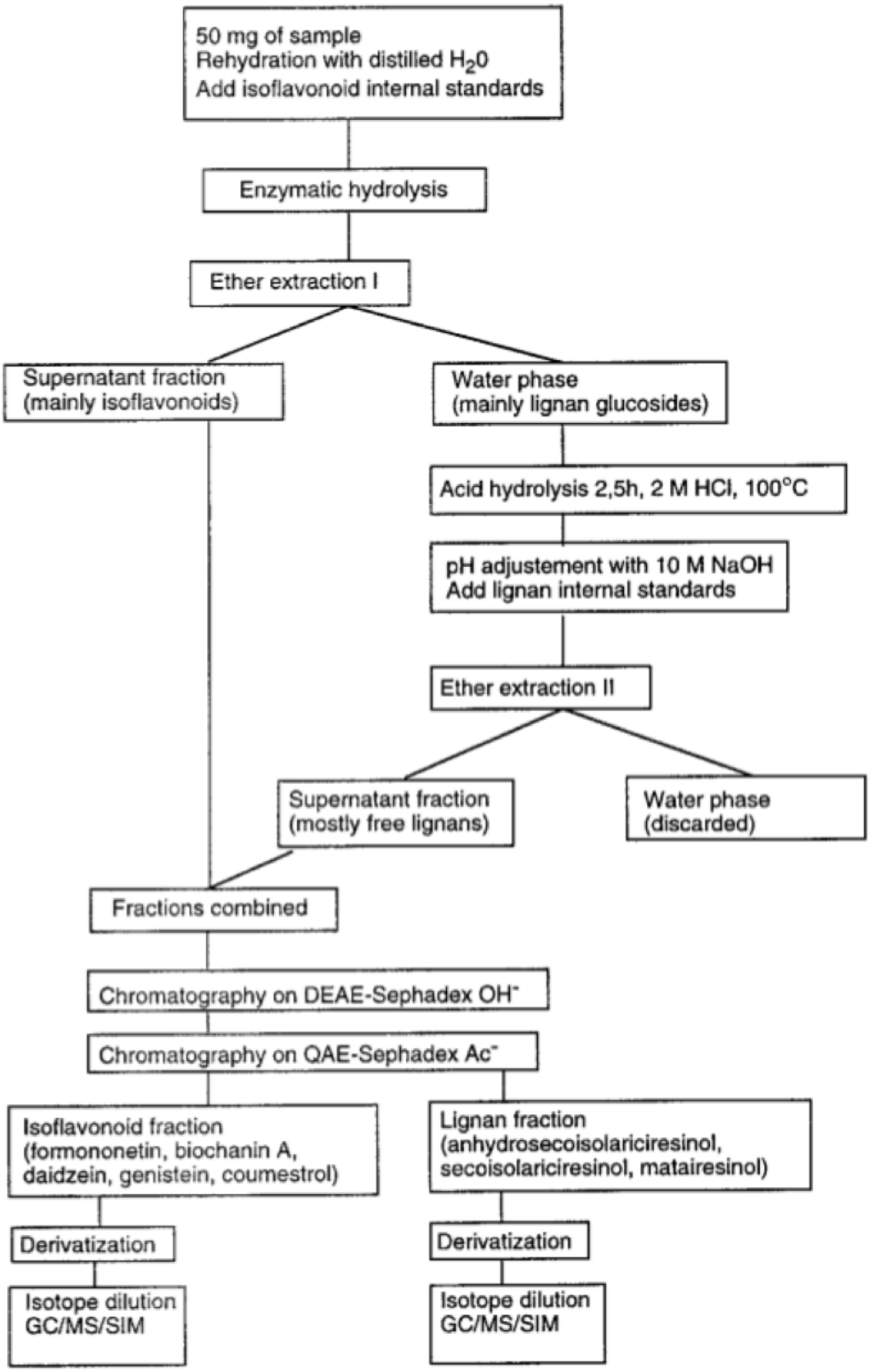


Figure 2.8. Outline of steps involved in isotope dilution-gas chromatography-mass spectrometry (ID-GC-MS) method (Mazur et al., 1996).

2.3.2.5 High Performance Liquid Chromatography (HPLC)

Huang and Yan (2008) reported a rapid, simple, and accurate HPLC method for quantifying extracted flaxseed SDG that uses highly enriched SDG (>99.86%) as a reference. The method uses a stationary reversed phase column (a Hypersil ODS C_{18} 4.6 mm × 250 mm, 5 μm column) and a gradient generated from acetonitrile and water (Table 2.3) as the mobile phase. The mobile phase flow velocity is 1.5 mL/min, column temperature 25 °C, and injected sample volume 10 μL. Eluting compounds were determined by their absorbance at 220 nm. Under these conditions SDG eluted with an ideal peak shape at 10.8 min (Huang and Yan, 2008). Such a rapid, simple, and inexpensive HPLC method can directly determine SDG content with high accuracy for quality control of flaxseed SDG extracts during industrial production.

Table 2.3. Gradient elution of mobile phase of HPLC analysis on SDG (Huang and Yan, 2008).

Time (min)	0.12% Phosphoric Acid	Acetonitrile
0	94	6
10	71	29
13	71	29
15	30	70
20	94	6

2.3.3 Flaxseed Lignan Extraction

2.3.3.1 Organic Solvent Extraction

Polar organic solvents are often reported for SDG extraction. The most commonly used solvents are methanol and ethanol, though a few researchers have used dioxane: ethanol (1:1, v/v), acetone and isopropanol as solvents followed by alkaline hydrolysis to release SDG from the polymer. Conditions for extraction with the highest yield is based on the solvent used. Improved extraction conditions can be identified by single factor tests, orthogonal methods, and response surface analysis (Kitts et al., 1999; Prasad, 2000; Prasad, 2002). Organic solvent extraction can be both convenient and rapid, however, impurities and unexpected derivatives can be introduced if alkaline hydrolysis is used as a step. Conversion of SDG to other products can affect extraction yield and lead to inefficient enrichment. In addition, microwave-assisted and ultrasonic-assisted methods are commonly used to improve the efficiency of organic solvent extraction (Chen and Gao, 2007; Sun

et al., 2009; Yuan et al., 2013). Microwave irradiation can be applied for improved extraction of a variety of compounds from soil, seeds, foods and feeds prior to chromatography (Ganzler et al., 1986). For SDG extraction, 70% ethanol (v/v) was mixed with DFHF with several different weight-to-volume ratios and stirred for 10, 30, or 60 min, respectively. The mixtures were then exposed to 700 W microwave irradiation for 2, 5 and 8 min, respectively. During irradiation, iced-water cooling, and solvent backflow are alternated with intervals of 30 s until the full irradiation exposure was reached. Extracted SDG solutions were combined and concentrated for further analysis. The optimal condition for microwave-assisted extraction included 700 W microwave irradiation for 5 min (Chen and Gao, 2007). Microwave-assisted extraction is a simple, rapid and efficient method. Wider application of microwave extraction has been limited by risks and hazards related to microwave leakage. Ultrasonic-assisted extraction is also used for improved organic solvent extraction. Ultrasonic waves induce cavitation, turbulence, and mass transfer at phase interfaces to improve mass transfer. Ultrasound is widely applied in component extraction, membrane separation, crystallization, adsorption and desorption, and aqueous two-phase extraction (Rutkowska et al., 2017). Ultrasonic-assisted extractions can decrease solvent consumption and shorten extraction times. Also, such methods help to increase extraction yields at lower temperatures. This can be important where higher temperatures might alter or destroy analytes. Reported optimized conditions for SDG extraction included the use of a 400 W ultrasonic generator (working frequency of 20~25 kHz) with sample-to-solvent ratio of 1:17 (w/v) for 21 min (Sun et al., 2009). Such methods might improve industrial extraction by increasing recovery and speed of extraction while reducing costs. However, due to the high power, many impurities might also be extracted with the lignans. Fractions of lower quality require more processing including additional enrichment steps.

2.3.3.2 Pressurized Low Polarity Water (PLPW) Extraction

Cacace and Mazza (2006) studied extraction of SDG from flaxseed with pressurized low polarity water (PLPW). In this method, subcritical water, which has similar polarity with alcohols, is used for SDG extraction; with whole flaxseed used as the raw material. The extraction equipment consists of an HPLC syringe pump, operated at 0~10 mL/min, 3.0 m preheating coil, extraction cell, temperature-controlled oven, 1.0 m cooling coil, back pressure regulator with a cartridge of 5.2 MPa (750 psi), and collection vessel (Figure 2.9). In order to maintain porosity, the same bed

depth was used for all extractions of equal masses of seed such that bulk density was constant. During extraction, deionized and degassed subcritical water was pumped into the system at a constant flow rate bringing the pressure to 5.2 MPa. Water extracts were concentrated by evaporation under nitrogen before analysis. The optimal condition determined testing orthogonal variables included temperature, water flow rate, and water-to-seed ratio, of 160 °C, 0.5 mL/min and 30~40 mL/g, respectively. This approach provided a high yield of SDG, but the complicated process limits its application in industrial production. More recently, PLPW at 40°C over 28 h (Zhang et al., 2007) achieved higher SDG yield than Cacace and Mazza's method. Most importantly, it provides a possible way to further modify PLPW extraction for industrial production of SDG.

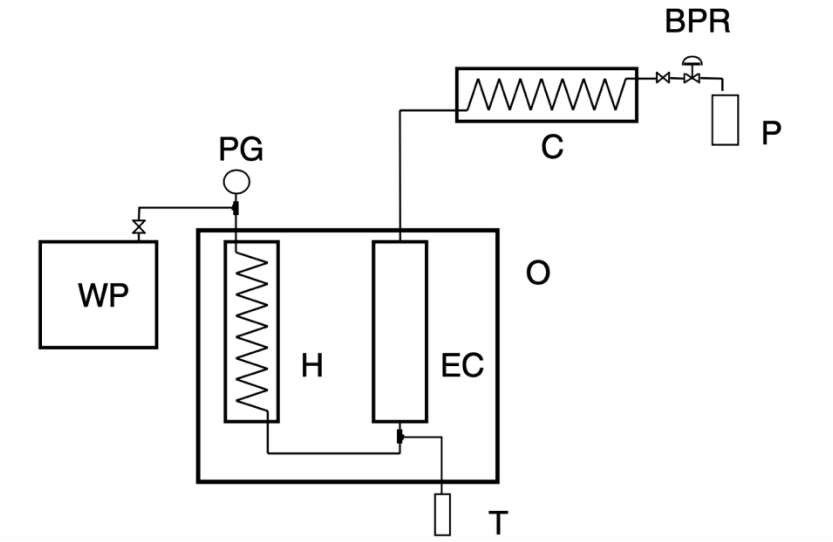


Figure 2.9. Diagram of pressurized low polarity water extractor, WP, water pump; PG, pressure gauge; H, heating coil; T, thermometer; EC, extraction chamber; O, oven; C, cooling coil; BPR, back pressure regulator; P, product collector (Cacace and Mazza, 2006).

2.3.3.3 Supercritical CO_2 Fluid Extraction (SFE)

Carbon dioxide (CO_2) is the most used supercritical fluid extraction (SFE) solvent. Supercritical CO_2 is a fluid state of carbon dioxide where it is held at or above its critical temperature, 31.1°C, and pressure, 7.3 MPa (Budisa and Schulze-Makuch, 2014). As a solvent, supercritical CO_2 is non-toxic, stable, and non-flammable so that to be considered an excellent solvent for food processing (Liao and Huang, 2004). SFE requires specialized equipment to generate the high pressure required for extraction (Zhang et al., 2014).

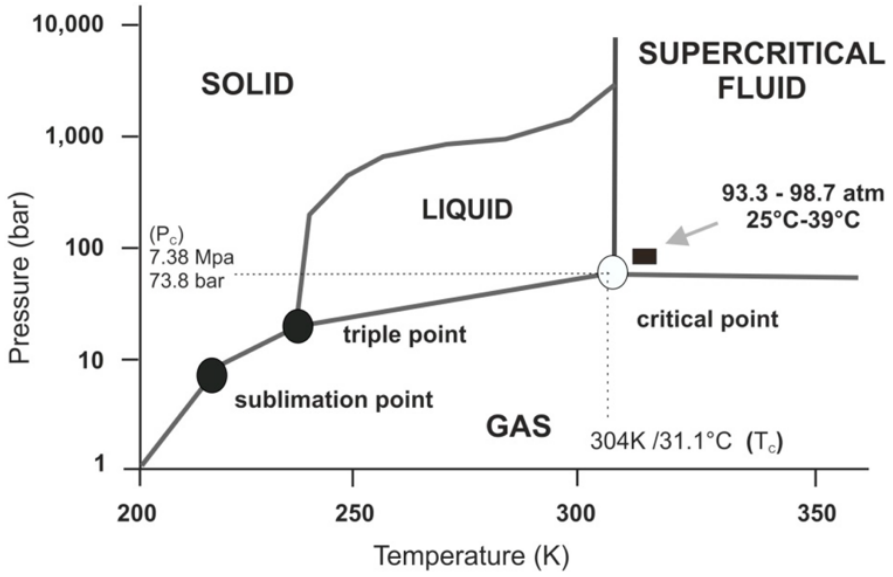


Figure 2.10. Supercritical CO_2 (Budisa and Schulze-Makuch, 2014).

Yang (2005) used CO_2 SFE to extract SDG from DFHF. Extraction efficiency was improved by including 70% ethanol solution (v/v) as an entrainer and tetrahydrofuran: water: acetic acid (5:5:1, v/v/v) solution as a regulator. During SFE extractions, CO_2 completely recovered any remaining flax oil, and addition of the entrainer enabled SDG extraction. Optimal condition for SFE included 70% ethanol to extract SDG at 60 °C and 30 MPa for 30 min (Yang, 2005). When compared with traditional organic solvent extraction, SFE can lower processing costs, shorten processing times, increase extraction yields and avoids the use of toxic solvents. However, due to the high capital cost of high-pressure equipment, this method is not widely applied to SDG production.

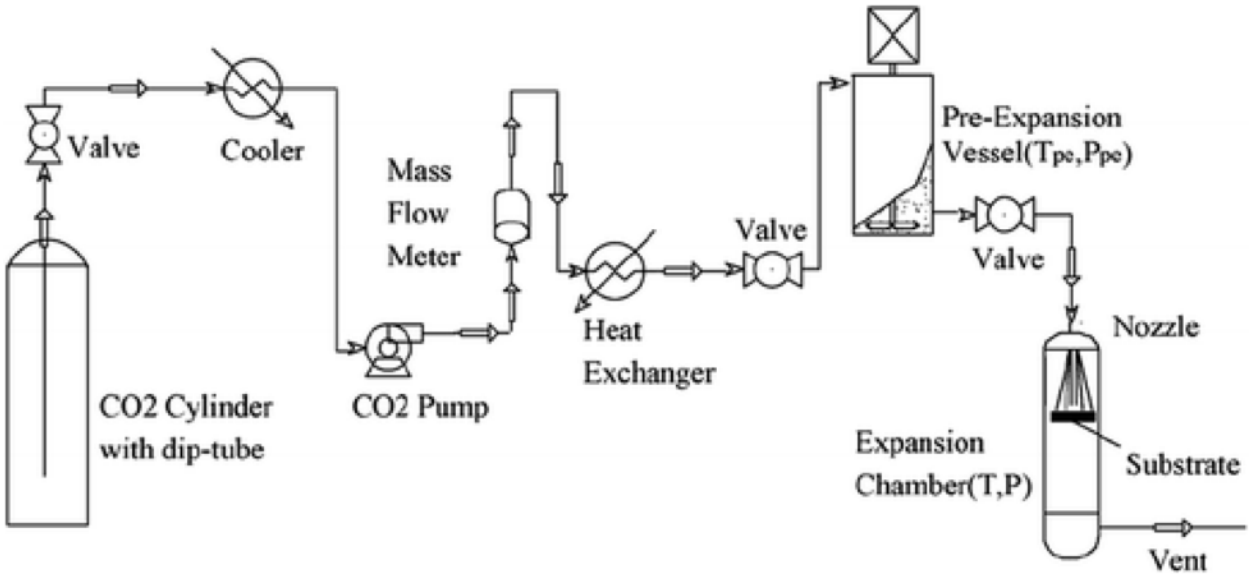


Figure 2.11. Diagram of typical supercritical CO_2 fluid extraction apparatus (Zhang et al., 2014).

2.3.4 Flaxseed Lignan Enrichment

2.3.4.1 Chromatography

Many chromatographic methods have been applied for SDG enrichment. The commonly used methods including packed and preparative reversed phase column chromatography (e.g. C_{18}), silica gel column chromatography, and high-speed countercurrent chromatography (Degenhardt et al., 2002; Muir et al., 1999; Pihlava et al., 2004; Willför et al., 2006). Each method uses different mobile phase eluents. A key aspect of chromatographic enrichment is reproducibility of the elution time so that fractions with high SDG contents can be reliable collected and then concentrated to enrich SDG. However, methods mentioned above require different organic solvents for elution, and many are expensive, toxic, and non-recyclable. As SDG is used in food and medicines, trace solvents remaining in enriched SDG could lead to safety concerns.

2.3.4.2 Macroporous Adsorption Resin (MAR)

Macroporous adsorption resins are polymer materials that are used for efficient adsorption, and convenient desorption of compounds as part of a purification protocol. Such resins have been utilized for SDG recovery (Guo and Wang, 2006). Some researchers have discussed the use of MAR in SDG enrichment (Li et al., 2008; Li et al., 2007; Zhang and Xu, 2006). MARs are typically soaked in an organic solvent (e.g. acetone for 24 h) for complete swelling. Then the swelled MARs are eluted sequentially using a series of solvents. Pre-treatment ensures that impurities in MARs are removed and no longer interfere with processing. For SDG extracts loaded onto MAR AB-8 resin eluting with 10~30% ethanol (v/v) at 1.5×10^{-3} L/min afforded the best enrichment (Li et al., 2008). The application of MAR extraction methods can be cost effective as they are reusable and when used properly, they are safe for contact with food. However, researchers report low SDG quality compared with other methods. Processes that use MAR are normally used for rough separation and enrichment of SDG. Fractions recovered using MAR typically require further enrichment.

2.3.4.3 Sephadex LH-20 Column Chromatography

Sephadex LH-20 is a hydroxypropyl glucan gel that affords excellent flavonoid separation. Due to the presence of crosslinked hydroxypropyl groups, the eluent for Sephadex LH-20 can be water, organic solvents or mixtures of water with organic solvents. Sephadex LH-20 has amphiphilic

properties, good physical and chemical properties as well as good reproducibility. The use of Sephadex LH-20 gel column chromatography for SDG enrichment was reported (Zhang and Xu, 2007). The common eluents include water, methanol and ethanol. For SDG enrichment, eluting samples with water achieved better separation than ethanol as SDG contains hydrophilic glucoside groups. Sephadex LH-20 has different swelling behavior with different eluents. When using water as the eluent, glucan gel swells less than in ethanol; the gel column has less interspace and SDG is retained longer on the column. This might be the reason that using water as an eluent gives better SDG separation efficiency. The extracted SDG quality was determined by HPLC-PAD-MS. PAD UV/Vis adsorption was measured between 200~600 nm and MS spectra were recorded for mass ratios of m/z 300~800. Sephadex LH-20 is reusable and food safe; the relatively high cost of the resin is offset by its durability. Most importantly, it gives very high SDG yield and quality, which are 97.2 and 96.6%, respectively. This method can be modified for large-scale production of high-quality SDG (Zhang and Xu, 2007).

2.3.5 Challenges in Modern Flaxseed Lignan Production

Previous studies on SDG extraction and enrichment mainly focus on laboratory-scale experiments but SDG is a precursor for commercial flaxseed lignan products. Methods established for analytical purposes cannot typically be applied in industrial flaxseed lignan production as there are many restrictions on solvents and equipment. Industrial production of SDG faces many problems such as low extraction yield of SDG polymer from flaxseed and low quality of hydrolyzed SDG. The efficiency of SDG production is lower in commercial than analytical extractions and the total cost of analytical approaches is comparitively higher. Without improvements to analytical approaches the price of high-quality SDG as a raw material for dietary supplements and medicine would be relatively high. Therefore, establishing a standard protocol to produce SDG and its derived products in industrial scale is critical to increase the commercial value of flaxseed lignan.

2.4 Summary

Flaxseed composition and its significance as an important oilseed crop was described. The nutritional components in flaxseed including flaxseed lignan and their utilization were introduced. The structure, biosynthesis pathway, metabolism in mammals, and bioactivities of flaxseed lignan

are described. Methods for flaxseed lignan detection, extraction and enrichment are elucidated. Challenges in current flaxseed lignan production are outlined. The following studies address this shortfall of knowledge. Here we will present a food-safe protocol for producing SDG polymer, SDG and SECO in a series of unit operations. SDG polymer, SDG and SECO products will be analyzed to determine their structure, yield, and quality.

3. MATERIALS AND METHODS

3.1 Materials

Flaxseed (CDC Sorrel; harvested in 2016 from the University of Saskatchewan Crop Development Center) or was provided by Bioriginal Food & Science Crop (Saskatoon, SK, Canada). Seed was cleaned and freed of broken seed, dust and other foreign materials. Recycled ethanol (70%; v/v) was provided by Prairie Tide Diversified Inc. (Saskatoon, SK, Canada). Ammonium hydroxide (29%; w/w) was purchased from VWR Chemicals BDH (Radnor, PA, USA). Resin (Amberlite FPX-66) was provided by Prairie Tide Diversified Inc. (Saskatoon, SK, Canada). Three types of cellulase (from *Trichoderma viride*; from *Trichoderma sp*.; from *Aspergillus niger*) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). All other reagents were of analytical grade and used as received.

3.1.1 Content of SDG in Whole Flaxseed

Flaxseed (2 g) was ground in a coffee grinder (Black & Decker; Towson, MD, USA). Then a sample (500 mg) was stirred in 10 mL dichloromethane (>99.8%; Sigma-Aldrich Canada Ltd.; Oakville, ON, Canada) at room temperature for 2 h. Subsequently, the mixture was filtered, and the aqueous phase was collected then concentrated until dry. Deuterated water (10 mL) with 5% KOH (w/v) was added and the mixture stirred at room temperature for 2 h. An additional solution of 500 mg 1.844% pyrazine in D_2O (w/w) was then added as an internal standard. Samples were transferred to nuclear magnetic resonance (NMR) tubes (CA89041-318; VWR Chemicals BDH; Radnor, PA, USA) for analysis. SDG content was calculated based on resonance area ratio between internal standard and target resonances. Resonance area of the internal standard was normalized. As the molecular mass of pyrazine is 80.09 g/mole and the molecular mass of SDG is 687 g/mole the SDG content in whole flaxseed was calculated as:

$$\text{SDG Content (\%)} = \frac{M_{IS} \times C_{IS} \times 4 \times A_{SDG} \times 687}{80.09 \times 6 \times M_{GF} \times A_{IS}} \times 100 \qquad (3.1)$$

Where, M_{IS} is mass of internal standard (mg), C_{IS} is concentration of internal standard (%), A_{SDG} is resonance area of SDG peaks at ~6.6 PPM, M_{GF} is mass of ground flaxseed (mg), and A_{IS} is the area of the internal standard at ~8.6 PPM.

Experiments were performed in triplicate; SDG content in whole flaxseed was expressed as mean ± standard deviation (SD).

3.2 Sample Preparation

Whole flaxseed was pressed using an oil expeller (IBG Monforts Oekotec CA-59-6-3; Germany) at room temperature with a motor speed setting of 1.5 (screw speed 30 rpm). Pressed oil was collected separately for further treatment. Whole flaxseed meal after oil pressing was collected as the raw material for flaxseed lignan extraction. Partially defatted whole flaxseed meal was stored at 4°C before ethanol extraction.

Yield of defatted flaxseed meal was calculated as:

$$\text{Yield (\%)} = \frac{M_{FM}}{M_{WF}} \times 100 \tag{3.2}$$

Where, M_{FM} is mass of defatted flaxseed meal (kg), M_{WF} is mass of whole flaxseed (kg).

Three batches of oil pressing were performed, and yield of defatted flaxseed meal was expressed as mean ± SD.

3.3 Ethanol Extraction of SDG Polymer

For pilot scale ethanol extraction of flaxseed lignan defatted whole flaxseed meal was first soaked in 70% ethanol in ratio of 1:3 (w/w) for 24 h. For this treatment, 18 kg of 70% ethanol was used to soak 6 kg of flaxseed meal. Then, after removing the first portion of ethanol that contains extracted SDG polymer additional 70% ethanol was added at a ratio of 1:1 (w additional 70% ethanol/w-soaked flaxseed) for a second extraction. In this study, wet flaxseed meal remaining after the first extraction absorbed 5 kg ethanol and the total weight of meal plus ethanol was 11 kg. Therefore, another 11 kg 70% ethanol was added to soak the 11 kg wet flaxseed meal for 24 h after which the ethanol was drained from the meal. The two portions of ethanol were combined and concentrated by pilot scale rotary evaporation. The SDG polymer was obtained after concentration and further dried by vacuum oven drying.

Yield of SDG polymer based on defatted flaxseed meal was calculated as:

$$\text{Yield (\%)} = \frac{M_{SDGP}}{M_{FM}} \times 100 \tag{3.3}$$

Where, M_{SDGP} is mass of SDG polymer (g), M_{FM} is mass of defatted flaxseed meal (g).

Yield of SDG polymer from whole flaxseed was calculated as:

$$\text{Yield (\%)} = \frac{M_{SDGP}}{M_{WF}} \times 100 \tag{3.4}$$

Where, M_{SDGP} is mass of SDG polymer (g), M_{WF} is mass of whole flaxseed (g).

Quality of SDG polymer was determined by NMR analysis. SDG polymer (100 mg) was hydrolyzed in 1 mL D_2O with 5% KOH (w/v) for 2 h at room temperature. After hydrolysis, 50 mg 1.844% pyrazine in D_2O (w/v) was added to the sample to act as the internal standard. Then, sample was pipetted into NMR tubes for analysis. The concentration of SDG polymer was calculated based on resonance area ratio between internal standard and target resonances. The resonance area of the internal standard was normalized. Molecular mass of pyrazine is 80.09 g/mole; molecular mass of SDG is 687 g/mole.

The concentration of SDG polymer in the extracted product was expressed as:

$$\text{Concentration of SDG Polymer (\%)} = \frac{M_{IS} \times C_{IS} \times 4 \times A_{SDG} \times 687}{80 \times 6 \times M_{SDGP}} \times 100 \qquad (3.5)$$

Where, M_{IS} is mass of internal standard (mg), C_{IS} is concentration of internal standard (%), A_{SDG} is resonance area of SDG peaks at ~6.6 PPM, M_{SDGP} is mass of SDG polymer (mg), and A_{IS} is the area of the internal standard at ~8.6 PPM.

Four batches of extraction in pilot scale were performed, and SDG polymer yield and concentration were expressed as mean ± SD.

3.4 Ammonium Hydroxide Hydrolysis of SDG Polymer

Ammonium hydroxide (29%; w/w) was used to hydrolyze SDG polymer to SDG and HMGA. Hydrolysis efficiency might be improved by varying reaction temperature, reaction time and ammonium hydroxide concentration. After extraction crude SDG was enriched by adsorption on polystyrene divinylbenzidene (PSDVB) resin (Amberlite FPX-66) followed by selective elution.

Amberlite FPX-66 is a macroporous absorbent resin of a crosslinked aromatic polymer with a surface area of 700 m^2/g, particle size of 0.60~0.75 mm, and pore envelope of 200~250 Å. Previous studies comparing Amberlite FPX-66 to similar commercial polymers indicate that it had the highest adsorption capacity and desorption ratio, which is 99.8 and 121.1%, respectively. Amberlite FPX-66 has a relatively large particle size, which allows higher mass exchange rates and allow more material to be transferred to and from the resin than possible with smaller particles, thereby desorption ratio and recovery are improved (Buran et al., 2014; Kammerer et al., 2005; Sandhu et al., 2017; Soto et al., 2011). Most importantly, many Amberlite resins including Amberlite FPX-66 are compliant with the US Food and Drug Administration Food Additive Regulation and are safe for food contact applications (Uzdevenes et al., 2018). It is also reported

as the most suitable resin of those tested for the recovery of flavonoids, including anthocyanins, from blueberry water extract (Buran et al., 2014). In this study, resin (Amberlite FPX-66) was used to extract and enrich SDG from the hydrolyzed mixture.

3.4.1 Ammonium Hydroxide Hydrolysis

3.4.1.1 Effect of Temperature

For each experiment, 100 mg ammonium hydroxide solution was used to hydrolyze 100 mg SDG polymer (41.1%; w/w) at the specified temperature for 1 h. One drop (~50 mg) of 1.844% pyrazine (w/w) was added as an internal standard; the weight of internal standard added was recorded for NMR analysis and calculation. Polymer hydrolysis was calculated based on resonance area ratio between internal standard and target resonances (6.4~6.6 ppm). Hydrolysis was conducted at room temperature (20 °C), 50, 60, 70, and 80 °C. The amount of free SDG was determined after hydrolysis.

Free SDG (%) in samples was calculated by using the resonance of SDG aromatic protons observed using ^{1}H-NMR (6.3-6.7 ppm; 6 protons per mole) and the mass of SDG polymer used for ammonium hydroxide hydrolysis into Equation 3.5.

Hydrolysis of SDG was calculated as:

$$\text{Unbound SDG (\%)} = \frac{\text{SDG Content (\%)}}{\text{Content}_{\text{SDGP}}\ (\%)} \times 100 \qquad (3.6)$$

Where, unbound SDG Content (%) was calculated by using Equation 3.5, and $\text{Content}_{\text{SDGP}}$ (%) was calculated SDG polymer content from section 3.3.

Experiments were performed in triplicate; the temperature effect was expressed as SDG conversion (%) versus temperature change (°C). Each point with error bar represented mean ± SD.

3.4.1.2 Effect of Ammonium Hydroxide Concentration

Results from 3.4.1.1 showed that higher temperatures liberated more SDG from the polymer. Therefore, 80 °C was selected for further studies of ammonium hydroxide concentration on SDG polymer hydrolysis. In addition, the ratio of material to solution of 1:1 (w/w) in previous experiments was not ideal as the ammonium hydroxide solution used was not sufficient to hydrolyze all bound SDG. Therefore, the ratio of material and total solution was increased to 1:4

(w/w) for further analysis. Moreover, reaction time was extended to 2 h in order to observe significant differences.

Specifically, 100 mg SDG polymer (41.1%; w/w) was hydrolyzed by 400 mg ammonium hydroxide solution at 80 °C for 2 h. The concentration of ammonium hydroxide solutions used were 3.62, 7.25, 14.5, and 29% (w/w), respectively. Ammonium hydroxide concentration was adjusted by adding different volumes of distilled water, 350 mL, 200 mL, 100 mL and 0 mL, respectively.

Concentration of ammonium hydroxide was determined from Equation 3.7:

$$\text{Concentration (\%)} = \frac{M_{NH_3 \cdot H_2O} \times 0.29}{400} \times 100 \quad (3.7)$$

Where, $M_{NH_3 \cdot H_2O}$ is the mass of original ammonium hydroxide (29%; w/w).

One drop of 1.844% pyrazine (w/w) was added into cooled sample as an internal standard. NMR analyses were performed to determine the free SDG content after hydrolysis. Results were calculated by applying resonance area of detected SDG and mass of SDG polymer added to Equation 3.5 and Equation 3.6.

Each experiment was performed in triplicate; concentration effects were expressed as free SDG accumulation (%) versus ammonium hydroxide concentration (%). Data are presented with error bars represented by mean ± SD.

3.4.1.3 Effect of Reaction Time

Results from 3.4.1.1 and 3.4.1.2 showed that higher temperatures and higher ammonium hydroxide concentrations improved SDG yield. Therefore, in order to determine the effect of reaction time, 100 mg SDG polymer (41.1%; w/w) was hydrolyzed by 400 mg 29% (w/w) ammonia solution at 80 °C for 16 h. Samples were collected at 1, 2, 4, 8 and 16 h, respectively. One drop (~50 mg) of 1.844% pyrazine (w/w) was added into each cooled sample as an internal standard. Exact weight of internal standard was recorded for each sample. NMR analysis was performed to determine the SDG conversion. Data was analysed according to Equations 3.5 and 3.6.

Experiments were performed in triplicate; reaction progress was expressed as free SDG accumulation (%) versus time (h). Data is expressed as mean ± SD.

3.4.2 Pilot-Scale Ammonium Hydroxide Hydrolysis

Considering results from section 3.4.1., pilot-scale ammonia hydrolysis involved reacting SDG polymer with 29% (w/w) ammonium hydroxide solution at ratio of 1:4 (w/w) at 80 °C for 2 h. After the reaction, ammonia was removed by oven drying and trapped by reacting with CO_2 then the material was cooled to room temperature, adjusted to pH 7 and used for further enrichment studies.

3.4.3 Enrichment of SDG by PSDVB Resin

The amberlite FPX-66 resin was pretreated using acid and base as described by Buran et al. (Buran et al., 2014). The pretreated resin was stirred with reacted mixture at 22 °C for 2 h at a ratio of 20:1 (w/v). Then, all materials were loaded onto a 10 L glass chromatography column and washed by 10 times volume of distilled water. The free SDG absorbed on the resin was eluted by washing with six column volumes of 30% ethanol. The aqueous ethanol eluate containing the free SDG was concentrated by evaporation and SDG was collected as the product.

The SDG concentration was calculated as:

$$\text{Free SDG (\%)} = \frac{M_{IS} \times C_{IS} \times 4 \times A_{SDG} \times 687}{80 \times 6 \times M_{SDG} \times A_{IS}} \times 100 \quad (3.8)$$

Where, M_{IS} is mass of internal standard (mg), C_{IS} is concentration of internal standard (%), A_{SDG} is resonance area of SDG peaks at ~6.6 PPM, M_{SDG} is mass of SDG (mg), and A_{IS} is the area of the internal standard at ~8.6 PPM.

Four batches were performed for each experiment; SDG yield and content were expressed as mean ± SD.

The free SDG product was analyzed by NMR, mass spectrometry (MS) and infrared spectroscopy (IR) to confirm its structure.

3.5 Enzymatic Hydrolysis of SDG

Cellulase was used to hydrolyze SDG to release SECO and glucose. Hydrolysis efficiency can be affected by enzyme species, enzyme concentration, reaction temperature and time. Product was extracted and enriched by several methods including liquid-liquid partition and resin enrichment.

3.5.1 Condition Optimization on Enzymatic Hydrolysis of SDG

3.5.1.1 Sample Preparation

Sodium acetate buffer (0.1 M) was prepared from 5.772 g of anhydrous sodium acetate (82 g/mole), 1.778 g acetic acid (60.05 g/mole) and 800 mL distilled water and adjusted to pH 5 (pH = 5.01). The buffer was used to dissolve cellulase and the sample.

A solution of SDG was prepared by dissolving 1.200 g of commercial SDG (40%; Ruibo, Xi'an, China) in 60 mL sodium acetate buffer (20 mg/mL).

Cellulase solution was prepared by dissolving 0.150 g of cellulase in 50 mL of sodium acetate buffer (3 mg/mL).

3.5.1.2 Effect of Cellulase Species

For each experiment 5 mL of 20 U/mL cellulase solution (100U) was added to 7 mL 20 mg/mL SDG solution and mixed well. Reaction mixtures were placed in the stirred reactor (1000 rpm) at 60 °C for 96 h. Samples (0.5 mL) were taken periodically, cooled to room temperature then combined with 0.5 mL $CDCl_3$. The organic solvent phase of each sample was transferred to an NMR tube for ^{1}H-NMR analysis. SECO released from SDG by cellulase was directly expressed as resonance area ratio between $CDCl_3$ and target resonances (resonance area of $CDCl_3$ is normalized).

Each experiment was performed in triplicate; effect of enzyme species was expressed by comparison of resonance areas of NMR spectra.

3.5.1.3 Effect of Cellulase Concentration

SDG solution (7 mL 20 mg/mL) was prepared and 5 mL of cellulase (from *Trichoderma viride*) solution was added (10 U/mL, 20 U/mL, 30 U/mL, 40 U/mL and 50 U/mL). Samples were placed in a stirred (1000 rpm) reactor at 60 °C for 96 h. Samples of the reaction mixture (0.1 mL) were cooled then added to 1.4 mL methanol then the mixture was filtered (0.2 μm PTFE filter). The content of SDG and SECO in solution was determined using high performance liquid chromatography (HPLC).

A standard solution was prepared by dissolving 7.3 mg enriched SDG (89.5%) and 3.6 mg enriched SECO (84.3%) into 10 mL sodium acetate buffer. The molecular weights of SDG and SECO are 686.7 g/mole and 362.4 g/mole, respectively. The mole ratio of SDG to SECO is 1.1:1.

Retention times of SDG and SECO are 1.064 min and 2.811 min, respectively. Integrated resonance areas of SDG were divided by a mole ratio of 1.1 for normalization; integrated resonance area of SECO remains the same as its mole ratio to SDG is 1. Conversion from SDG to SECO was expressed as SDG content (%) and SECO content (%); sum of SDG content (%) and SECO content (%) is 1.

Content of SDG as reactant in enzymatic hydrolysis was calculated as:

$$\text{SDG Content (\%)} = \frac{\frac{A_{SDG}}{1.1}}{\frac{A_{SDG}}{1.1} + A_{SECO}} \times 100 \quad (3.9)$$

Where, A_{SDG} is resonance area of SDG peaks at ~6.6 PPM, A_{SECO} is the integration of resonance area of SECO.

Similarly, content of SECO as product in enzymatic hydrolysis was calculated as:

$$\text{SECO Content (\%)} = \frac{A_{SECO}}{\frac{A_{SDG}}{1.1} + A_{SECO}} \times 100 \quad (3.10)$$

Experiments was performed in triplicate; effects of cellulase concentration were expressed as SDG content (%) versus SECO content (%) versus concentration change (U/mL). SDG content and SECO content were normalized as 1. Each point represented mean ± SD.

3.5.1.4 Effect of Reaction Temperature

For each experiment, 5 mL 30 U/mL cellulase (*Trichoderma viride*) solution was added to 7 mL 20 mg/mL SDG solution. Samples were placed in a stirred (1000 rpm) reactor at a specified temperature for 144 h. Temperature was adjusted to 40, 50, 60 and 70 °C. Samples of the reaction mixture (0.1 mL) were taken periodically and cooled after which 1.4 mL methanol was added to stop the reaction. The diluted samples were then filtered (0.2 μm PTFE filter) and the filtrate was analyzed by HPLC and reaction progress was calculated as described above (3.5.1.3).

Experiments were performed in triplicate; effects of reaction temperature were expressed as SDG content (%) versus SECO content (%) versus temperature (°C). Each point represented mean ± SD.

3.5.1.5 Effect of Reaction Time

For each experiment, 5 mL 30 U/mL cellulase (from *Trichoderma viride*) solution was added to 7mL 20 mg/mL SDG solution. Samples were placed in a stirred (1000 rpm) reactor at 60 °C for

144 h. Samples (0.1 mL) were taken at 0, 4, 24, 48, 72 and 144 h, cooled and 1.4 mL methanol was added to stop the reaction. The diluted samples were filtered using a 0.2 μm PTFE filter then analyzed by HPLC. Reaction progress was reported as described above (3.5.1.3).

Experiments were performed in triplicate; the effect of reaction time was expressed as SDG content (%) versus SECO content (%) versus time (h). Each point is expressed as mean ± SD.

3.5.2 SECO Extraction and Enrichment Conditions

3.5.2.1 Liquid-Liquid Partition

Liquid-liquid partition is achieved by aqueous biphasic systems (ABS). Due to the different polarity of SECO and SDG, SECO is readily dissolved in organic solvent while SDG is more soluble in water. Therefore, organic solvents that are not miscible with water were used to extract SECO from the reacted solution.

In a series of extractions, 5 mL dichloromethane (>99.8%), ethyl acetate (>99.8%) and chloroform (>99.5%) were added into 5 mL of reaction solution in a test tube. These solvents were mixed well and allowed time for separation. Subsequently the organic phase and aqueous phase were separated then concentrated under vacuum. NMR was used to determine the ratio of SECO and SDG in each fraction.

Experiments were performed in triplicate; SECO concentration was expressed as mean ± SD.

3.5.2.2 Enrichment of SECO using Resin

In each experiment, 10 mL of each reaction mixture was loaded onto 200 g of resin (Amberlite FPX-66) with ratio of material to resin 1:20 (v/w). The resin was then eluted with 100 mL distilled water to remove impurities. An SDG enriched fraction was then eluted 60 mL 30% ethanol (v/v) and, subsequently 60 mL anhydrous ethanol was added to recover a SECO enriched fraction. The two fractions were concentrated and dried separately under reduced pressure. NMR was used to determine SECO yield and concentration.

Experiments were performed in triplicate; SECO yield and concentration were expressed as mean ± SD.

3.5.3 Pilot-Scale SDG Enzymatic Hydrolysis and SECO Enrichment

In pilot-scale enzymatic hydrolysis, SDG solutions (20 mg/mL) were prepared by dissolving 50 g commercial SDG (40%) into 2500 mL sodium acetate buffer (pH 5.01). Cellulase solution was prepared by dissolving 5.357 g cellulase (from *Trichoderma viride*) into 1785.7 mL sodium acetate buffer. Two portions of solution were mixed well and stirred at 60 °C for 48 h. The reaction mixture (100 mL) was loaded onto 2 kg resin (Amberlite FPX-66) in a column and washed with water. Subsequently, 600 mL of 30% ethanol (v/v) was used for a first wash and 600 mL of anhydrous ethanol was used for a second wash. Resin was recycled to enrich further fractions of the reaction solution. The SECO fractions in anhydrous ethanol from all extracts were combined and concentrated under reduced pressure. NMR was used to determine SECO yield and concentration.

3.6 Statistical Analysis

Three replicates were used to obtain average values and standard deviations for all tests. Data were presented as mean ± standard deviation (SD) (n = 3). The analyses were conducted using a completely randomized design and Microsoft Excel (Microsoft Corp., Albuquerque, NM) and OriginPro 2020 (OriginLab Corp., Northampton, MA, USA). Statistical significance was accepted at $P < 0.05$.

4. RESULTS AND DISCUSSIONS

4.1 Content of SDG in Whole Flaxseed

Whole flaxseed (CDC Sorrel) was measured as containing 2.1% SDG, which matches results of Eliasson's group in 2003 (Eliasson et al., 2003). This value is helpful to investigate efficiency of ethanol extraction.

Yield of defatted flaxseed meal from whole flaxseed was 68.3 ± 1%. Meal yield might be affected by seed condition, temperature of oil expeller, motor speed and choke size selected for pressing. Oil content in defatted flaxseed meal indicated oil pressing efficiency

4.2 Efficiency of Ethanol Extraction of SDG Polymer

4.2.1 Yield of SDG Polymer

Yield of SDG polymer from defatted flaxseed meal was 4.0%; SDG polymer yield in terms of whole flaxseed was 2.7%. There are no literature reports of SDG polymer yield in previous studies. Flaxseed lignan research mainly focus on SDG, so that SDG polymer after extraction is directly hydrolyzed to SDG without yield and quality determination. SDG polymer yield can be affected by ethanol concentration, efficiency of rotary evaporation, and vacuum oven drying. In addition, significant sample loss was observed during transfers. Figure 4.1 shows picture of flaxseed raw material, flaxseed meal after oil pressing, flaxseed meal after lignan extraction and dried SDG polymer.

Figure 4.1. Picture of (A) whole flaxseed (CDC Sorrel), (B) flaxseed meal after oil pressing, (C) flaxseed meal after lignan extraction and (D) dried SDG polymer.

4.2.2 Content of SDG Polymer

Content of SDG polymer was 65.8 ± 2%. Polymer content was calculated by combining content of detected SDG and HMGA in NMR. Ratio of HMGA to SDG in polymer was 1:1.3 ± 0.1. Figure 4.2 shows ^{1}H-NMR spectrum of SDG polymer and HMGA.

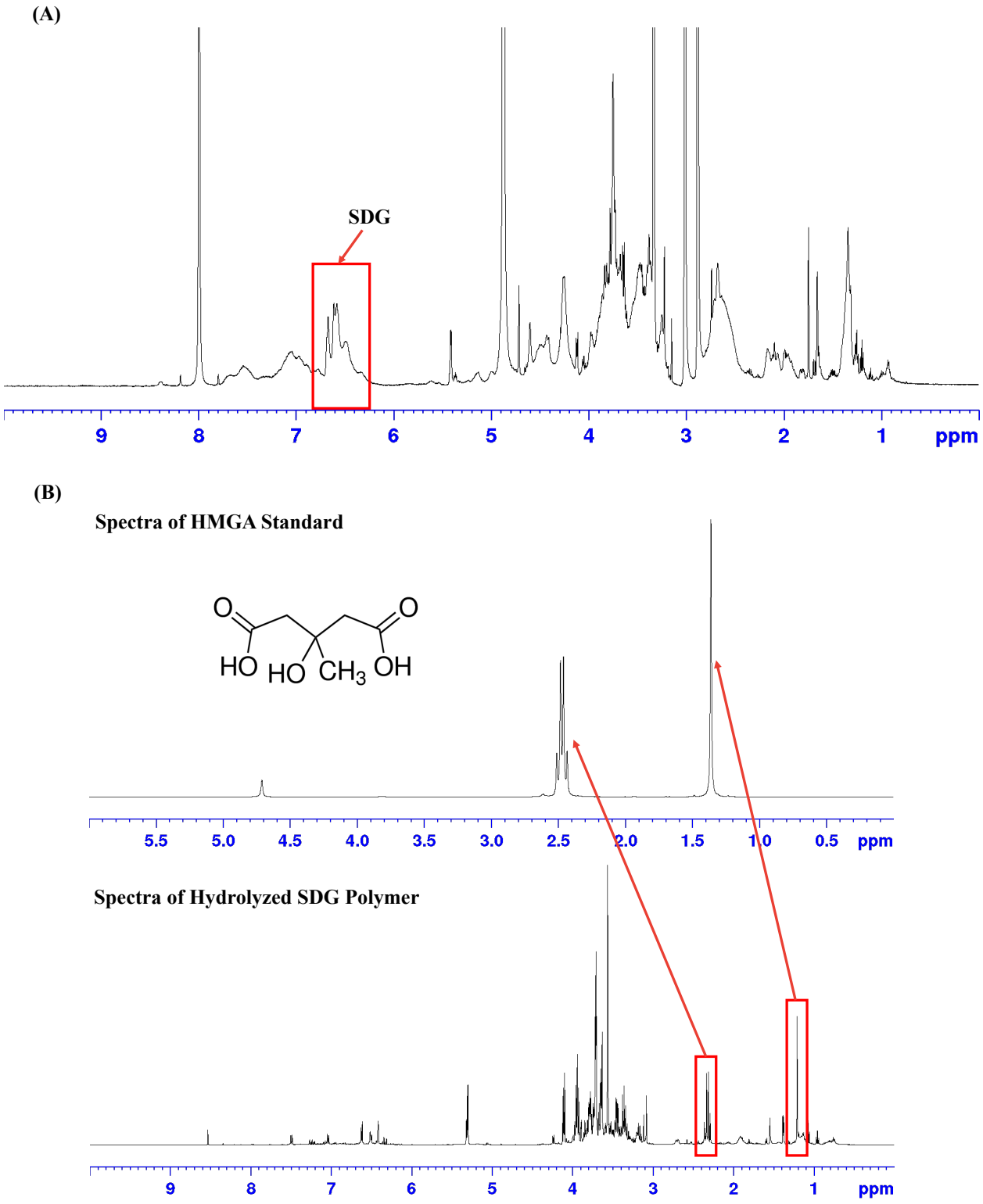


Figure 4.2. [1]H-NMR spectrum of (A) SDG polymer and (B) HMGA.

4.2.3 Structure Investigation of SDG Polymer

Based on the NMR spectrum of hydrolyzed SDG polymer the ratio of SDG to HMGA in the polymer was 1:1.3 ± 0.1. This SDG polymer structure has not been reported in previous studies. The structure of SDG polymer may varies depending on flaxseed cultivar and the proteins and enzymes involved in biosynthesis of flaxseed lignan (Ford et al., 2001; Yang, 2005). Figure 4.3 shows the proposed structure of SDG polymer extracted in this study. Based on the ratio of SDG to HMGA, the investigated chain length of SDG polymer is relatively short. The extracted product may be classified as an SDG oligomer.

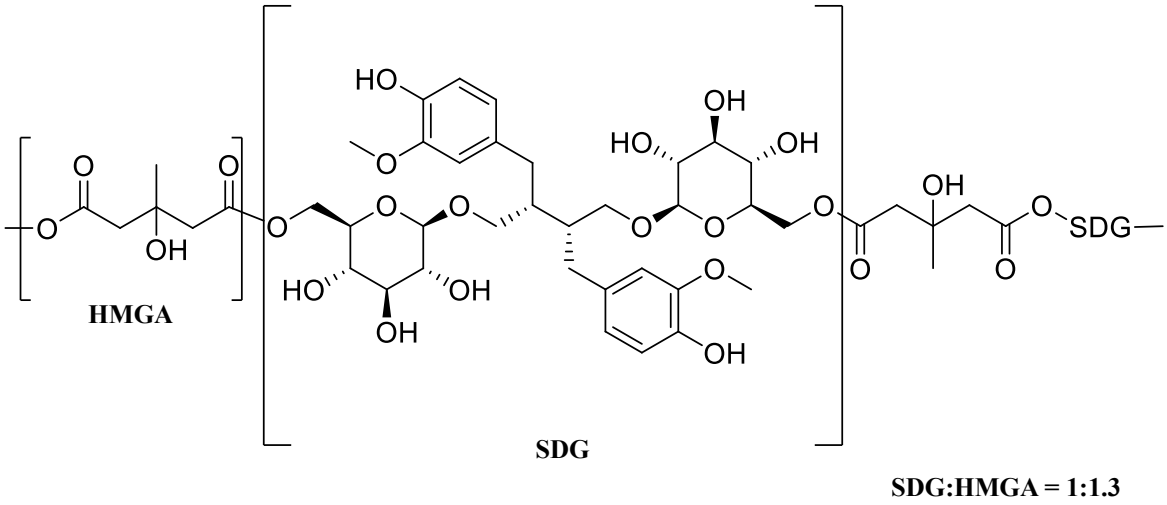


Figure 4.3. Proposed SDG polymer structure.

4.2.4 Design of Ideal Industrial Process of SDG Polymer Extraction

The ideal industrial process was designed based on achieved pilot-scale ethanol extraction, and it is plotted by Aspen Plus V10.0 (Aspen Technology, Inc., Bedford, MA, USA). Figure 4.4 shows ideal industrial process design of SDG polymer extraction. Flaxseed meal after oil pressing is soaked 2 times with 70% ethanol (v/v); SDG polymer from 2 extractions were combined; ethanol is removed by evaporation and is recovered for continuous extraction processing; SDG polymer after evaporation is dried and collected as the product; flaxseed meal after lignan extraction is dried and collected as the co-product.

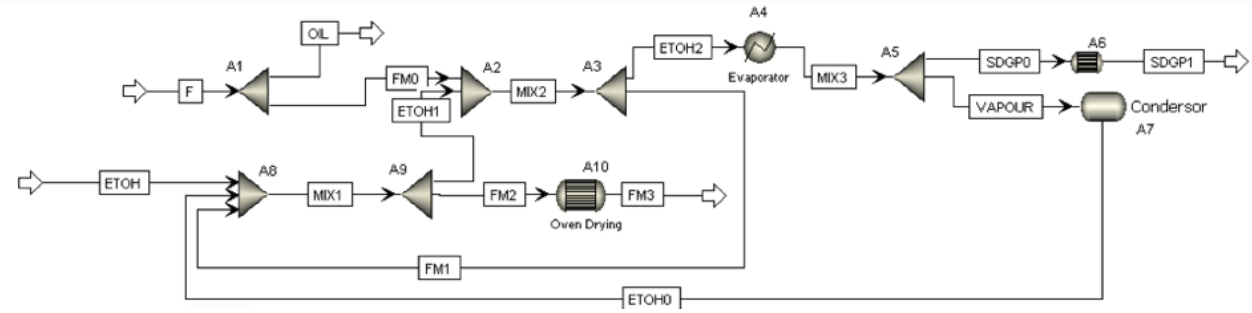


Figure 4.4. Conceptual design of industrial process of SDG polymer extraction.

Where, F is flaxseed raw material, FM0 is flaxseed meal after oil pressing, FM1 is flaxseed meal after first ethanol extraction, FM2 is flaxseed meal after second lignan extraction, FM3 is dried flaxseed meal after lignan extraction as by-product, ETOH is 70% ethanol (v/v), ETOH0 is recycled ethanol (70%; v/v), ETOH1 is ethanol after first lignan extraction, ETOH2 is ethanol containing SDG polymer of 2 extractions, MIX1 is mixture of flaxseed meal after first lignan extraction and ethanol, MIX2 is mixture of flaxseed meal after oil pressing and ethanol after first lignan extraction, MIX3 is mixture of ethanol and SDG polymer, SDGP0 is extracted SDG polymer with high moisture content, SDGP1 is SDG polymer product, A1 is oil pressor, A2 is mixing chamber, A3 is separator, A4 and A5 are evaporator, A6 is dryer, A7 is condenser, A8 is mixing chamber, A9 is separator, and A10 is dryer.

4.3 Ammonium Hydroxide Hydrolysis of SDG Polymer

According to previous studies, after ethanol extraction SDG polymer is typically subjected to alkaline hydrolysis to release SDG (Chen and Gao, 2007; Kitts et al., 1999; Prasad, 2002; Sun et al., 2009). However, subsequent processing of SDG is complicated as alkaline materials such as potassium hydroxide and sodium hydroxide require neutralization before removal. In this study, ammonium hydroxide is used to replace mineral alkalis since it can be removed by simple evaporation after hydrolysis. In addition, evaporated ammonia can be trapped by CO_2 to form ammonium carbonate, ammonium bicarbonate, or ammonium carbamate. The latter compound can be converted to urea by dehydration (Choi et al., 2012; Liu and Lu, 2019; Randall and Wright, 2011). The process of ammonium hydroxide hydrolysis is simple, inexpensive, food-safe and environmentally friendly, which would be an advantage for further SDG enrichment. Figure 4.5 shows the approach of ammonium hydroxide hydrolysis.

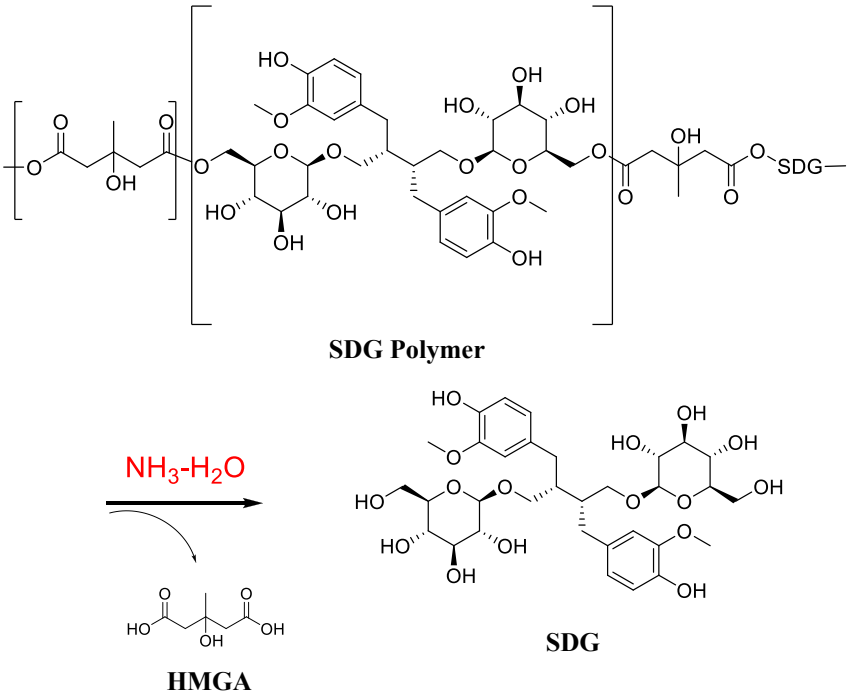


Figure 4.5. Proposed ammonium hydroxide hydrolysis.

4.3.1. Condition Optimization on Ammonium Hydroxide Hydrolysis

4.3.1.1 Effect of Temperature

Effect of temperature on ammonium hydroxide hydrolysis was investigated (Figure 4.6). At room temperature, SDG conversion was lower than 5%, which meant ammonium hydroxide hydrolysis did not occur at this temperature. With increased temperature, a higher content of SDG was released from the SDG polymer. In addition, based on safety and economic considerations, temperatures higher than 80 °C were not selected for ammonia hydrolysis. Therefore, 80 °C was utilized for further treatments.

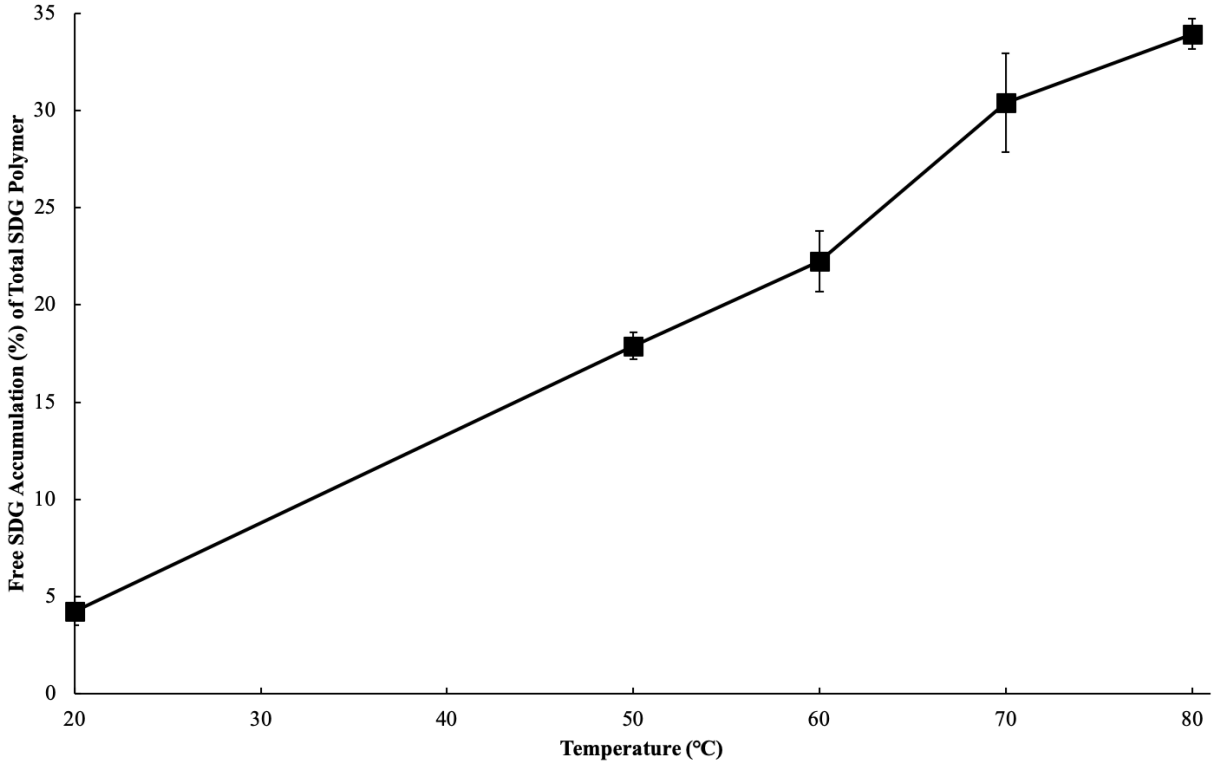


Figure 4.6. Effect of temperature on ammonium hydroxide hydrolysis of SDG polymer.

4.3.1.2 Effect of Ammonium Hydroxide Concentration

Effects of ammonium hydroxide concentration were investigated (Figure 4.7). Ammonium hydroxide solution is available at 29% (w/w) commercially. The amount of 350, 300, 200, and 0 mg distilled water was added to 50, 100, 200, and 400 mg of 29% (w/w) ammonium hydroxide solution, respectively. Ammonium hydroxide concentrations were 3.625, 7.25, 14.5 and 29%, respectively. Effect of ammonium hydroxide concentration was plotted as concentration (%) versus free SDG accumulation (%). Higher ammonium hydroxide concentrations more effectively hydrolyzed SDG polymer to SDG (Figure 4.7). Commercial ammonium hydroxide solution with concentrations higher than 29% (w/w) can be purchased from VWR or Sigma-Aldrich. However, as ammonia can be easily evaporated in high temperature, using higher concentrations might not achieve higher conversion efficiency. In addition, prices of ammonium hydroxide solution with different concentration vary. Therefore, 29% ammonium hydroxide solution was selected for pilot-scale (500g) SDG polymer hydrolysis.

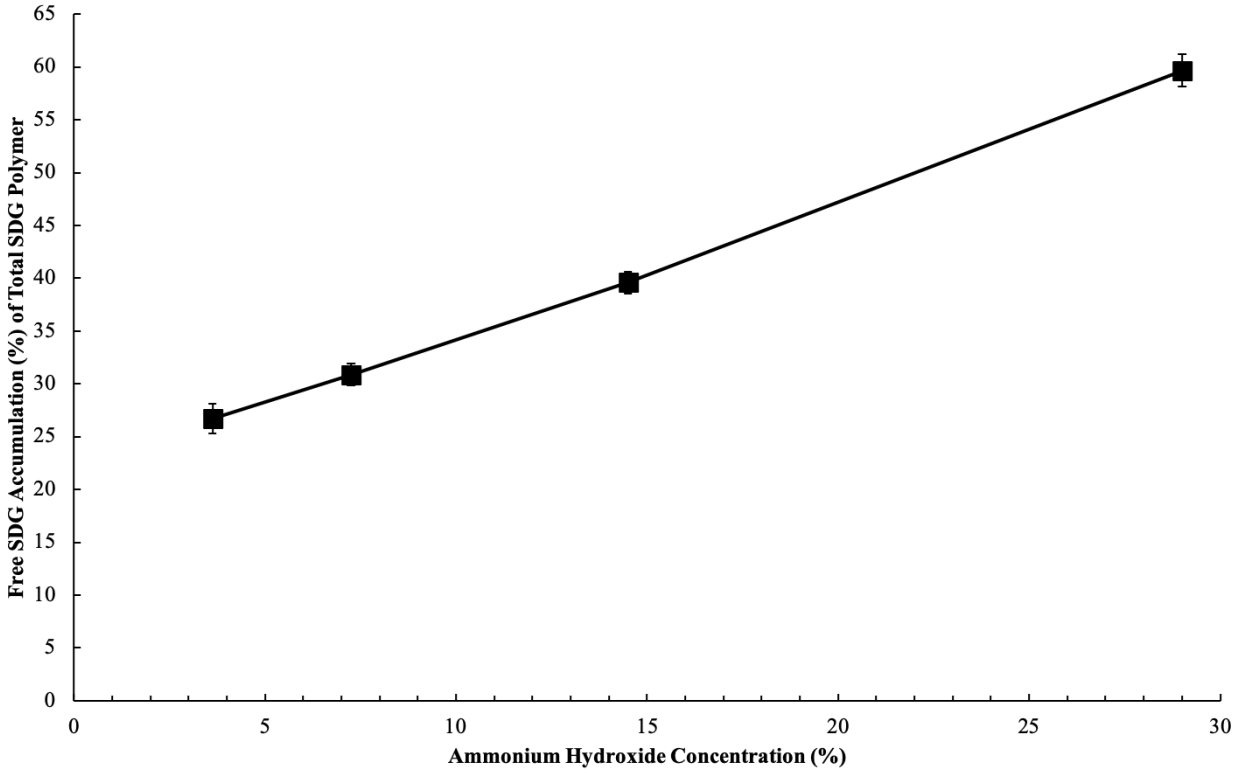


Figure 4.7. Effect of ammonium hydroxide concentration on SDG polymer hydrolysis.

4.3.1.3 Effect of Reaction Time

Effect of reaction time on ammonium hydroxide hydrolysis was investigated (Figure 4.8). The slope between each two date points indicated the reaction rate. The reaction rate at 80 °C decreased with increased reaction time. The conversion efficiency was highest in the first hour and thereafter. In the next 14 h, smaller amounts of SDG were released. Therefore, 2 h was considered as the preferred time for future studies.

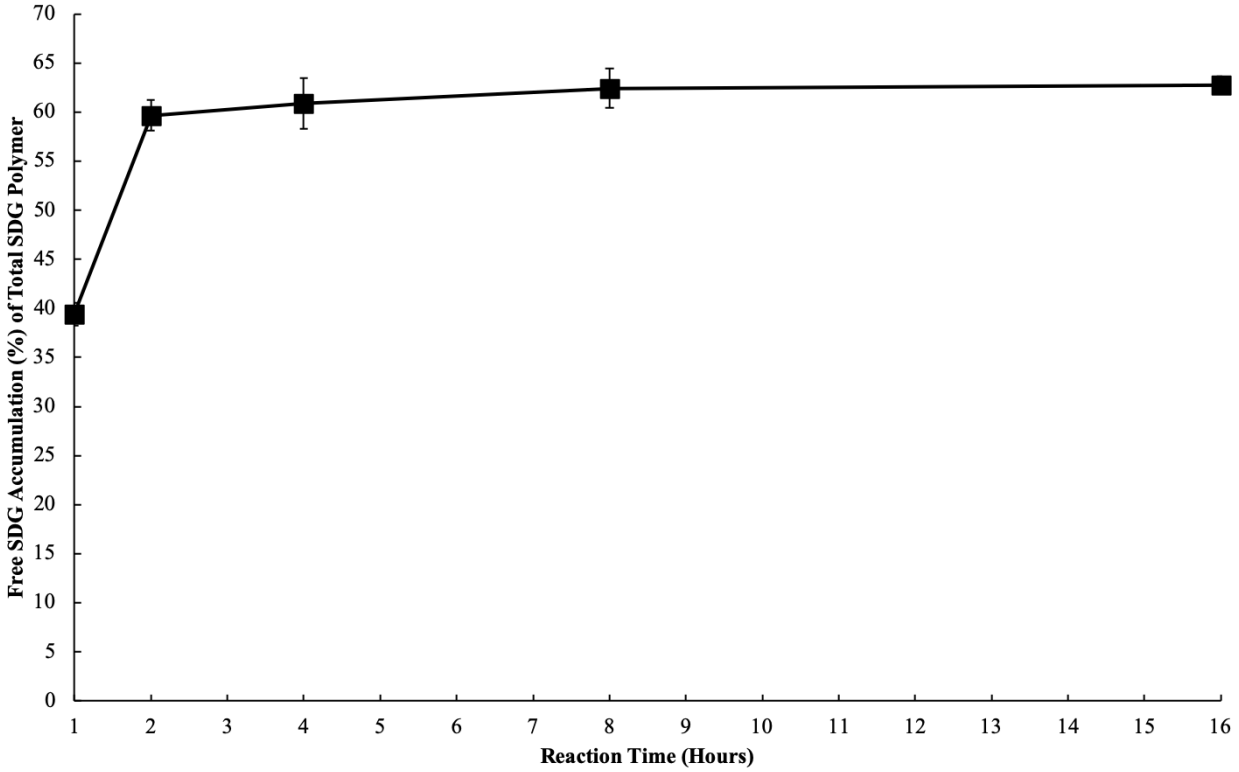


Figure 4.8. Accumulation of SDG during ammonium hydroxide hydrolysis of SDG polymer.

4.3.2 Yield and Content of SDG of Pilot-Scale Ammonium Hydroxide Hydrolysis

Yield of SDG was 51.9 ± 3%; while SDG concentration was 89.5 ± 1%. Ammonium hydroxide hydrolysis reached a maximum of 60% SDG conversion at 80 °C (4.4.1). In pilot-scale experiments, average conversion was 52%. The lower recovery might be due to slight changes in conditions related to the reaction vessel. Larger reaction vessels might have space for ammonia gas evaporation so that SDG polymer did not fully react with the ammonium hydroxide solution. This disadvantage can be controlled in future experiments using suitably sealed processing equipment.

Enriched SDG product was light yellowish powder with molecular weight 686.7 g/mole. The product was subjected to NMR and IR to determine its structure. Results matched literature descriptions (Chimichi et al., 1999; Qiu et al., 1999; Zanwar et al., 2014). Spectra are attached in appendix A.

4.3.3 Design of an Industrial Process for SDG Polymer Hydrolysis

An improved industrial process was designed based on pilot-scale SDG polymer hydrolysis with ammonium hydroxide, and it is plotted by Aspen Plus V10.0 (Aspen Technology, Inc., Bedford, MA, USA). Figure 4.9 shows ideal industrial process design of SDG polymer hydrolysis. SDG polymer produced from flaxseed lignan extraction is reacted with ammonium hydroxide (29%; w/w); ammonia is removed by simple evaporation and is trapped by reacting with CO_2; urea is collected as by-product; hydrolyzed SDG polymer is mixed with resin; water is used to elute impurities; ethanol (30%; v/v) is used to recover SDG from the resin; resin is, thereby, recycled for continuous SDG enrichment processing; ethanol containing SDG is subjected to rotary evaporation; ethanol is removed from SDG product and recycled; SDG is dried and collected as product.

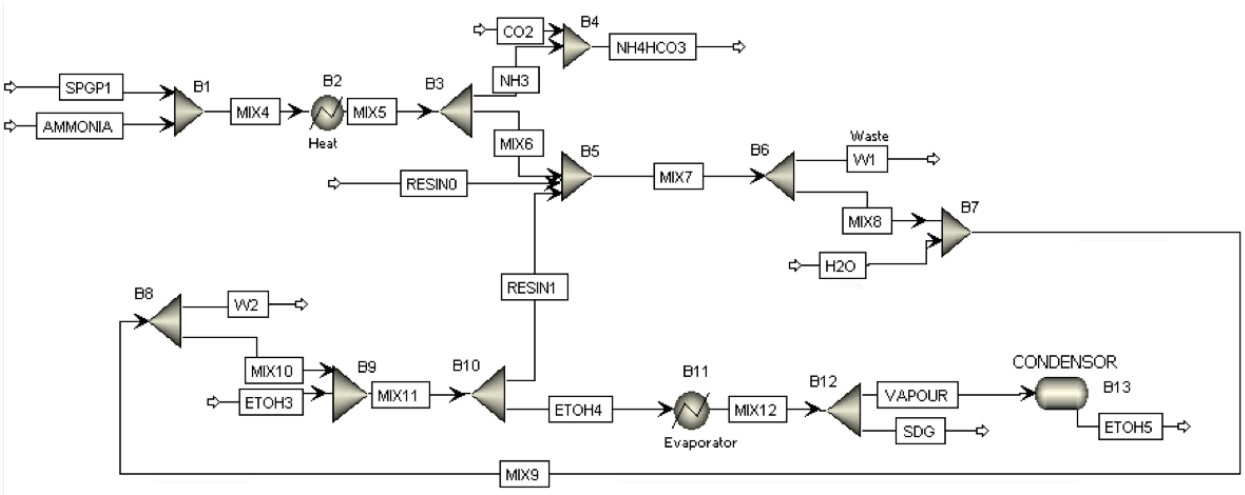


Figure 4.9. Conceptual design of industrial process of SDG polymer hydrolysis.

Where, SDGP1 is dried SDG polymer from flaxseed lignan extraction, SDG is dried SDG product, AMMONIA is ammonium hydroxide (29%; w/w), ETOH3 is 30% ethanol (v/v), ETOH4 is ethanol containing SDG, ETOH5 is recycled ethanol, RESIN0 is pretreated resin, RESIN1 is recycled resin, MIX4 is mixture of SDG polymer and ammonium hydroxide, MIX5 is mixture of SDG, HMGA and ammonium hydroxide, MIX6 is mixture of SDG and HMGA, MIX7 is mixture of resin, SDG and HMGA, MIX8 is mixture of resin, SDG and minor impurities, MIX9 is mixture of resin, SDG and water, MIX10 is mixture of resin and SDG after water wash, MIX11 is mixture of ethanol, resin and SDG, MIX12 is mixture of SDG, water and ethanol vapor, W1 and W2 are waste, B1 is reaction chamber, B2 is heater, B3 is evaporator, B4 is reaction chamber, B5 is mixing

chamber, B6 is separator, B7 is mixing chamber, B8 is separator, B9 is mixing chamber, B10 is separator, B11 is evaporator, B12 is separator, and B13 is condenser.

4.4 Enzymatic Hydrolysis of SDG

SECO is an intermediate metabolite of SDG in mammalian metabolism. It can be dissolved in oil and absorbed and utilized by intestinal bacteria (Axelson and Setchell, 1980; Wang et al., 2010). According to previous studies, SECO is generally produced by acid hydrolysis of SDG (Zang et al., 2017; Zhang et al., 2007). However, unexpected by-products may form during acid hydrolysis (Johnsson et al., 2000; Obermeyer et al., 1995). Bacteria isolated from human faeces also hydrolyze SDG to SECO (Li et al., 2012). This hydrolysis efficiency highly relies on intestinal bacteria and experimental conditions that are outside the scope of this study. In contrast, commercially available cellulase enzyme (EC 3.2.1.4.) is inexpensive and an ideal reagent for SDG hydrolysis. Figure 4.10 shows approaches for SDG enzymatic hydrolysis. In this study, cellulase from three different sources were selected to hydrolyze SDG to SECO and glucose. Effects of enzyme species, enzyme concentration, reaction temperature and reaction time on SDG hydrolysis are investigated.

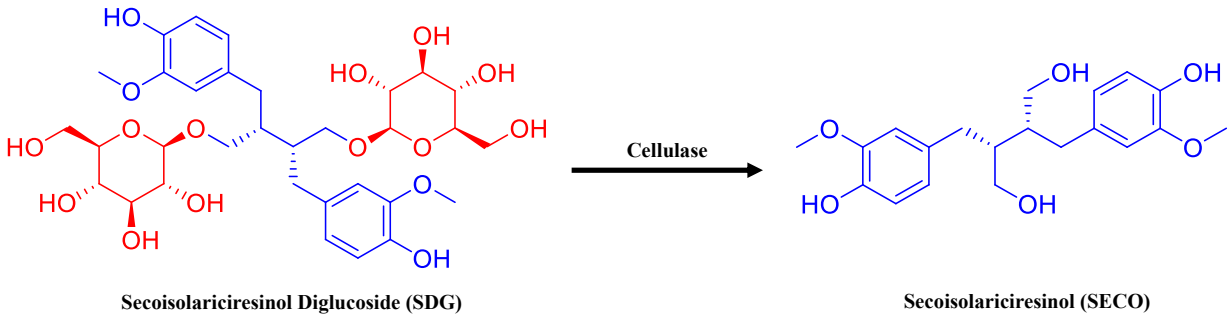


Figure 4.10. Approach of enzymatic hydrolysis of SDG.

4.4.1 Conditions of SDG Enzymatic Hydrolysis

4.4.1.1 Effect of Cellulase Source

Three sources of commercial cellulase were tested for SDG hydrolysis. Prices and sources of cellulase are listed in Table 4.1. Enzyme activity (U) is defined as the enzyme quantity that liberates 1 mg of product per mL of the reaction mixture per min (usually at 25 °C). As cellulases used for experiments are powder, enzyme activity is expressed as U per mg of solid powder. Prices of 1000 U are compared.

Table 4.1. Comparison of prices, sources and enzyme activities among three cellulases.

Enzyme	Sources	Price of 1000U (CAD)	Enzyme Activity (U/mg solid)
Cellulase 1	*Trichoderma viride*	2.34	10
Cellulase 2	*Trichoderma sp.*	36.8	0.8
Cellulase 3	*Aspergillus niger*	6.75	3-10

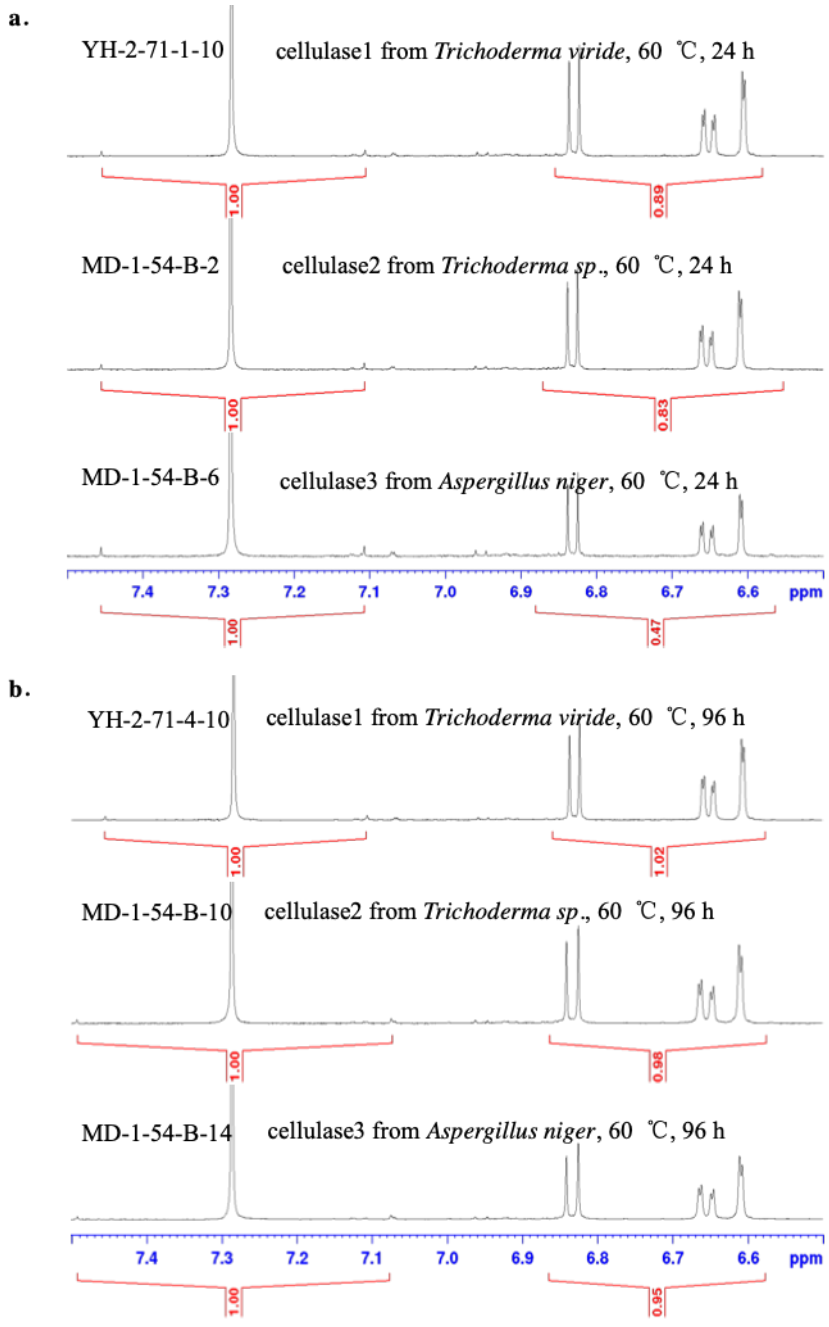


Figure 4.11. Enzymatic conversion of SDG to SECO.

In these experiments, the volume of sample collected and $CDCl_3$ used was consistent. During analysis, $CDCl_3$ resonance area in ^{1}H-NMR spectrum was considered as 1 and SECO resonance area was calculated as shown. Enzyme species effect on SECO conversion was determined by comparing differences of SECO resonances. Figure 4.11 shows SECO converted by cellulase from three sources. Figure 4.11(a) indicated that SECO conversion by cellulase 1 and cellulase 2 were similar at 24 h reaction while SECO conversion by cellulase 3 was about half the yield. Figure 4.11(b) indicated that final SECO conversion by the three cellulases were similar at 96 h reaction. According to Table 4.1, cellulase 1 had lowest price and relatively efficient hydrolysis; cellulase 2 achieved a relatively efficient hydrolysis but had the highest price; cellulase 3 had lowest reaction rate and higher price than cellulase 1. Therefore, cellulase 1 (from *Trichoderma viride*) was selected for further optimization experiments.

4.4.1.2 Effect of Cellulase Concentration

Conversion of SDG and SECO at different enzyme concentrations are shown in Figure 4.12. Figure 4.12(a) and Figure 4.12(b) show SDG (reactant) conversion and SECO (product) caccumulation, respectively. Based on the results, with same amount of SDG, higher enzyme concentration achieves higher initial reaction rates; the lowest enzyme concentration (10 U/mL) produced the slowest reaction rate and required the longest time to reach equilibrium; all other tested enzyme concentrations required similar times to reach reaction equilibrium. Considering reaction time and costs, 30 U/mL cellulase 1 (from *Trichoderma viride*) was selected for further optimization experiments.

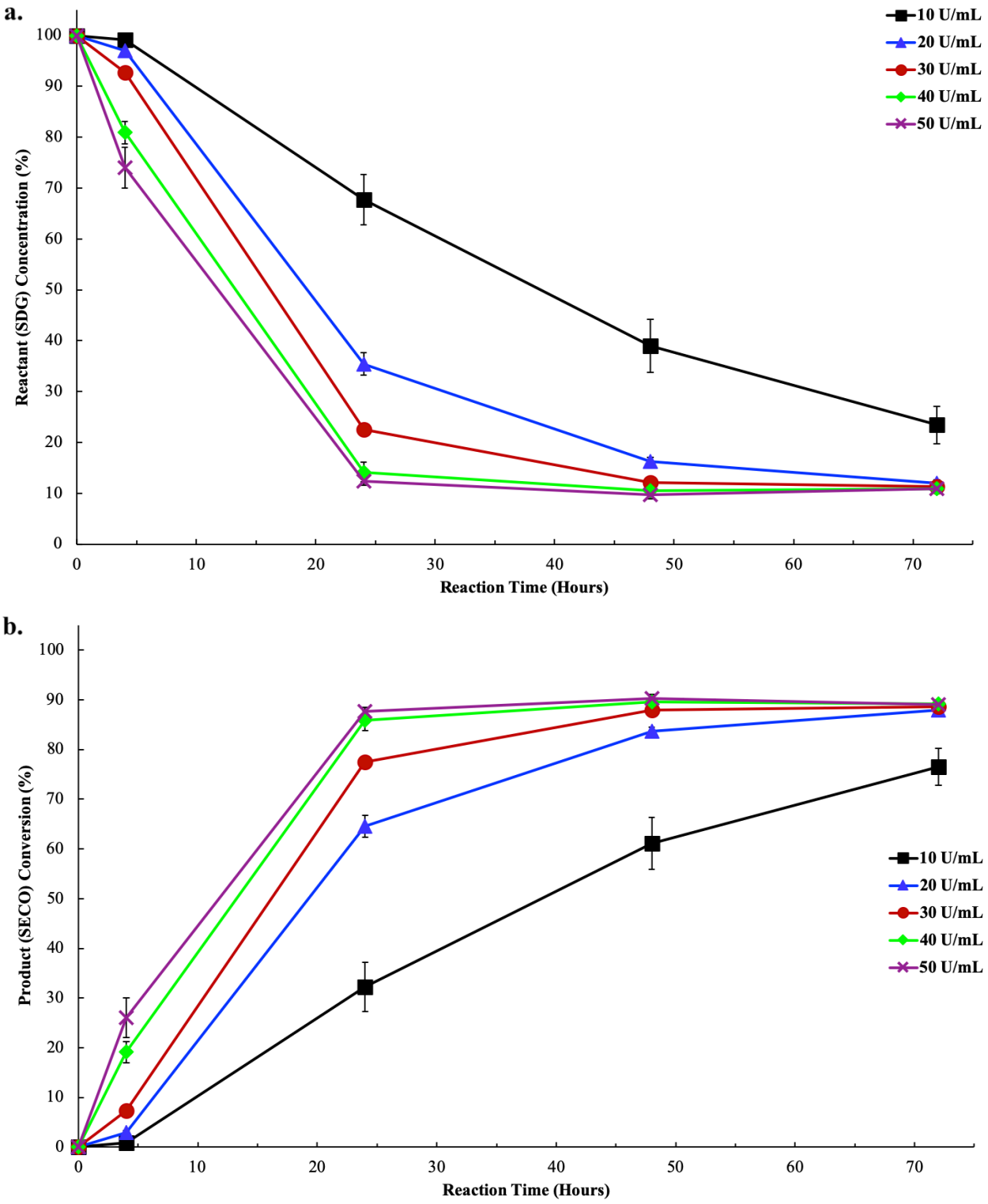


Figure 4.12. Consumption of SDG (a) and accumulation of SECO (b) at different enzyme concentrations.

4.4.1.3 Effect of Reaction Temperature

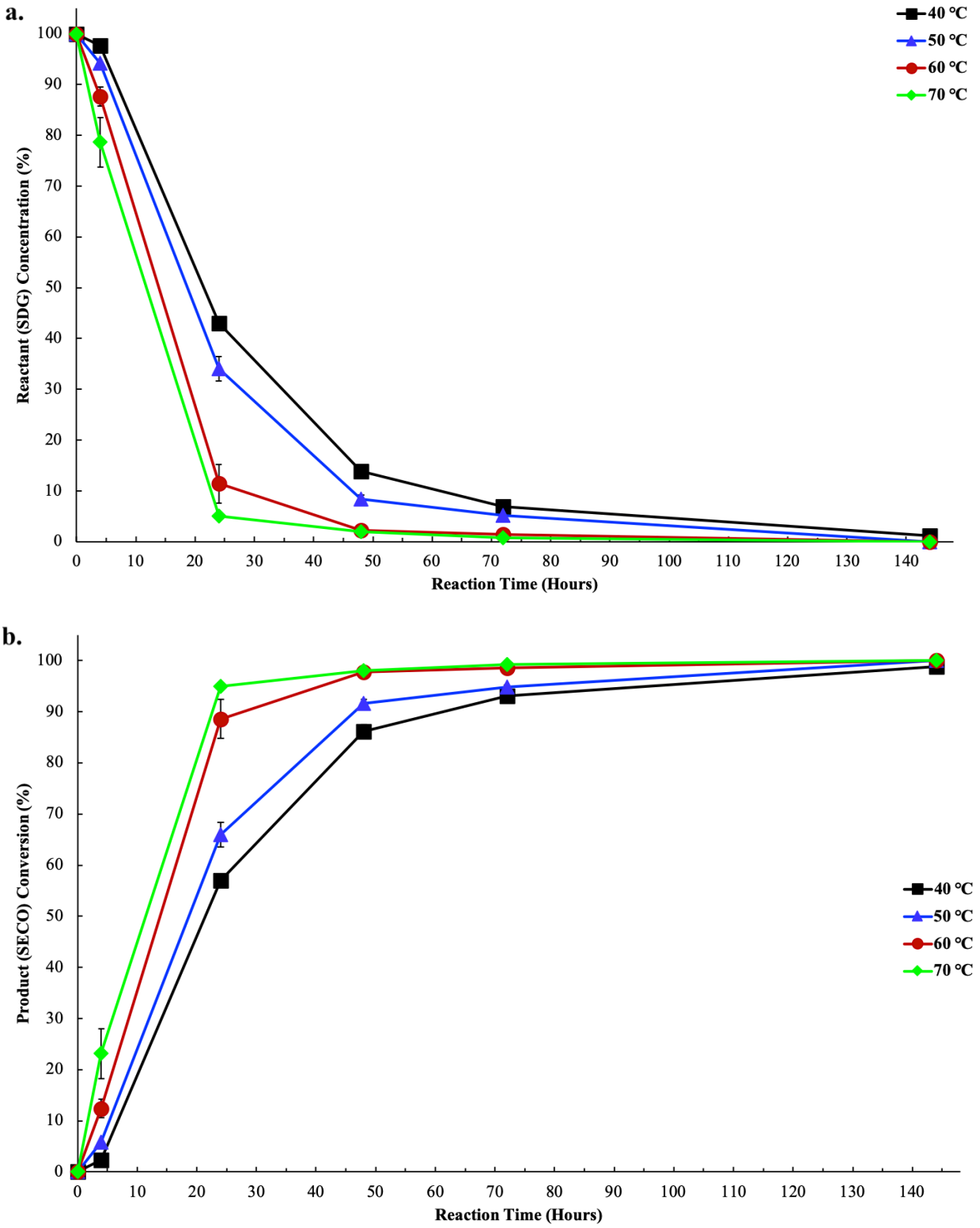


Figure 4.13. Consumption of SDG (a) and accumulation of SECO (b) at different reaction temperatures.

Conversion of SDG and SECO at different temperatures are shown in Figure 4.13. Figure 4.13(a) and Figure 4.13(b) showe SDG (reactant) conversion and SECO (product) accumulation, respectively. Based on the results, with same amount of SDG and enzyme concentration, reaction rate was increasing with increased temperature and reaction took shorter time to reach equilibrium. Reaction rate had significant increase when temperature changed from 50 to 60 °C and slight increase when temperature kept changing to 70 °C. Considering energy consumption and reaction time, 60 °C was selected as the preferred reaction temperature.

4.4.1.4 Effect of Reaction Time

Conversion of SDG and SECO at different reaction time was shown in Figure 4.14. The reaction mostly took place during the first 24 h; after 24 h, the reaction rate kept decreasing with increased reaction time; reaction was about to reach equilibrium after 48 h. Therefore, 48 h was considered as preferred reaction time.

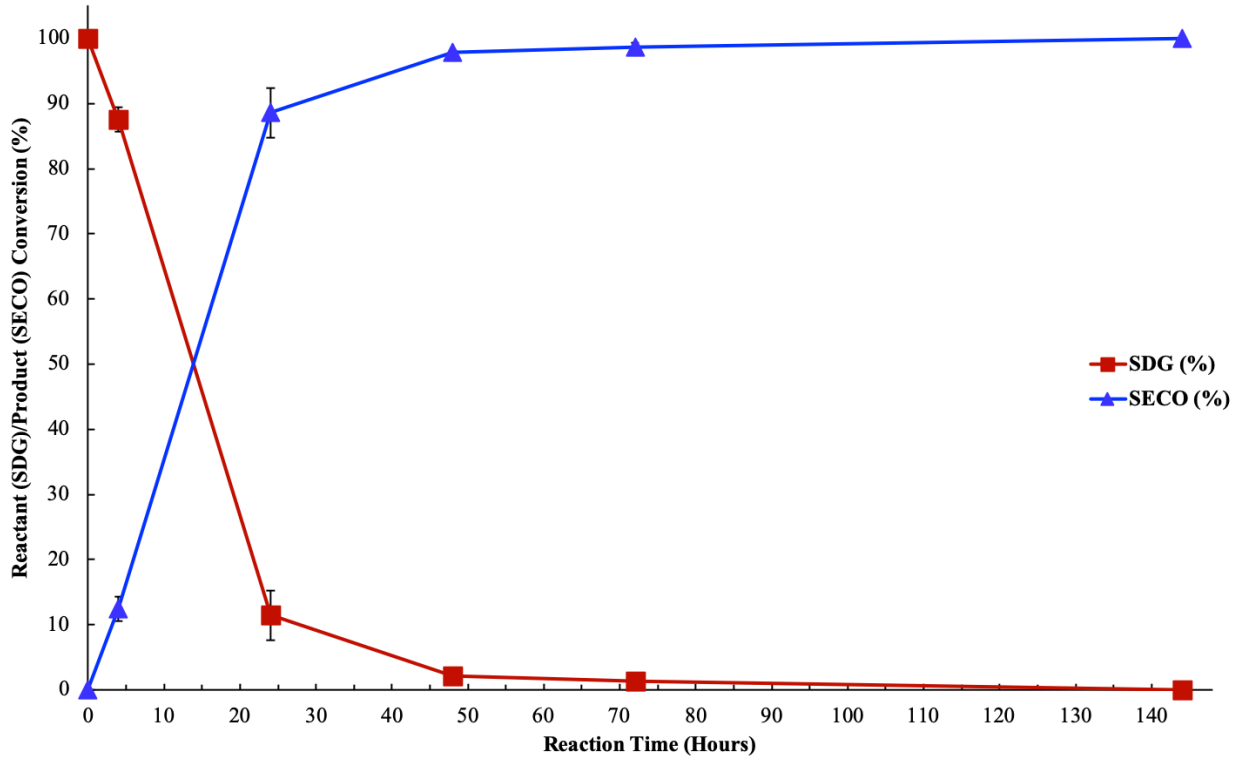


Figure 4.14. Consumption of SDG and accumulation of SECO over time.

4.4.1.5 Kinetics of SDG Cellulase Hydrolysis

According to previous results, optimal condition of SDG cellulase hydrolysis was determined as using 30 U/mL cellulase from *Trichoderma viride* hydrolyzing SDG at 60 °C for 48 h. During the reaction, SDG as substrate was converted into product SECO by cellulase. The kinetic of this reaction can be modelled using Michaelis-Menten equations that describing enzymatic reaction rated, by relating the reaction rate to substrate concentration. The reaction rate can be expressed as:

$$v = \frac{d[P]}{dt} = \frac{V_{max}[S]}{k_m + [S]} \tag{4.1}$$

Where, v is reaction rate (mmol/h), [P] is the concentration of product SECO (mmol/L), [S] is the concentration of substrate SDG (mmol/L), V_{max} is the maximum reaction rate achieved by system (mmol/h), and k_m is the value of the Michaelis constant, which is numerically equal to the substrate concentration at which the reaction rate is half of maximum reaction rate.

In this study, since sample size was small and time interval was relatively large, the V_{max} is difficult to be determined precisely. Therefore, the Lineweaver-Burk plot was used for this data:

$$\frac{1}{v} = \frac{k_m + [S]}{V_{max}[S]} = \frac{k_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \tag{4.2}$$

By applying experimental data into above equation, a plot of 1/[S] vs 1/v was derived as Figure 4.15.

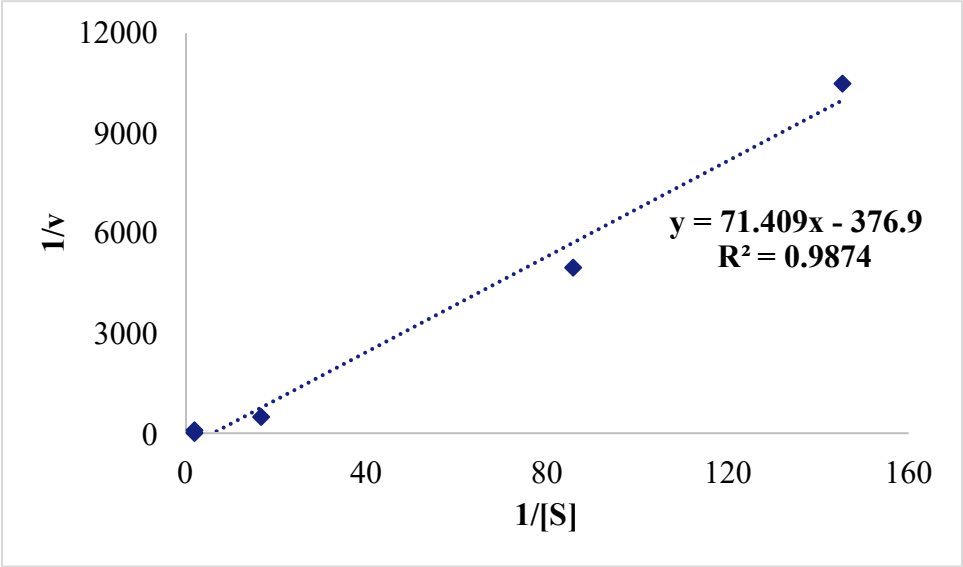


Figure 4.15. Lineweaver-Burk plot of SDG substrate concentration vs reaction rate (1/[S] vs 1/v).

From the Lineweaver-Burk plot, the y-intercept is negative, which means the maximum reaction rate, V_m, is negative. This might be due to the errors in experiment al conditions and calculations. During the experiments, the concentration of substrate (SDG) is determined by integration of resonance area in HPLC spectrum. Inaccurate determination of the resonance area may lead to overestimated substrate concentration (especially for low concentrations). In addition, concentration of SDG and SECO are normalized to 1 to better monitor the process of cellulase hydrolysis, normalization may also shift the plot. Therefore, the reaction rate of cellulase hydrolysis could not be determined using available data. Future work on determining the enzymatic kinetic of cellulase hydrolysis may include repeating the experiments with a large sample size, more collected data points and improved methods to monitor the hydrolysis.

4.4.2 Extraction and Enrichment of SECO

4.4.2.1 Extraction of SECO by Liquid-Liquid Partition

Organic solvents used in liquid-liquid partition that extracted SECO from reacted mixtures included dichloromethane (>99.8%), ethyl acetate (>99.8%) and chloroform (>99.5%). The yield of each extraction was not collected as sample size was too small. A fraction extracted by dichloromethane (>99.8%) was 82.2% SECO; the ethyl acetate (>99.8%) extract was 79.1 ± 1%; SECO while the chloroform (>99.5%) extract was 85.5 ± 1% SECO. These experiments demonstrated the utility of liquid-liquid partition to recover SECO from aqueous solution. This method could be applied in industrial SECO production for non-food purpose as it requires simple equipment and operation and produces relatively high-quality SECO. However, in this study, since these organic solvents were not food-safe, other methods such as resin enrichment were considered.

4.4.2.2 Extraction and Enrichment of SECO by Resin Enrichment

Yield of SECO by resin enrichment was 66.6 ± 2%; SECO content was 86.4 ± 1%. Resin was recycled more than 10 times. When compared with liquid-liquid partition, resin enrichment gave acceptable yield and afforded comparatively high-quality products. Most importantly, resin enrichment could be used to produce SECO for food applications.

4.4.3 Yield and Content of SECO from Pilot-Scale Enzymatic Hydrolysis

Yield of SECO in pilot-scale experiments was 81.5% and its content was 84.3%. The higher yield in pilot-scale experiment might be due to recycled washing process. SECO absorbed on resin had less leakage compared to bench experiments.

Enriched SECO product was light yellowish power with molecular weight of 362.4 g/mole. Product was subjected to NMR and IR to determine its structure. Results are matched with literature reports (Li et al., 2015). SECO spectra are attached as appendix A.

4.4.4 Design of an Improved Process of SDG Enzymatic Hydrolysis

The improved process was designed based on achieved pilot-scale enzymatic hydrolysis of SDG, and it is rendered by Aspen Plus V10.0 (Aspen Technology, Inc., Bedford, MA, USA). Figure 4.15 shows ideal industrial process design of SDG enzymatic hydrolysis. SDG products from ammonium hydroxide hydrolysis are reacted with cellulase; hydrolyzed SDG is mixed with resin; anhydrous ethanol is used to elute SECO from the resin; resin is recycled for continuous SECO enrichment processing; ethanol containing SECO is evaporated under vacuum; recovered ethanol is recycled; SECO is dried and collected as the product.

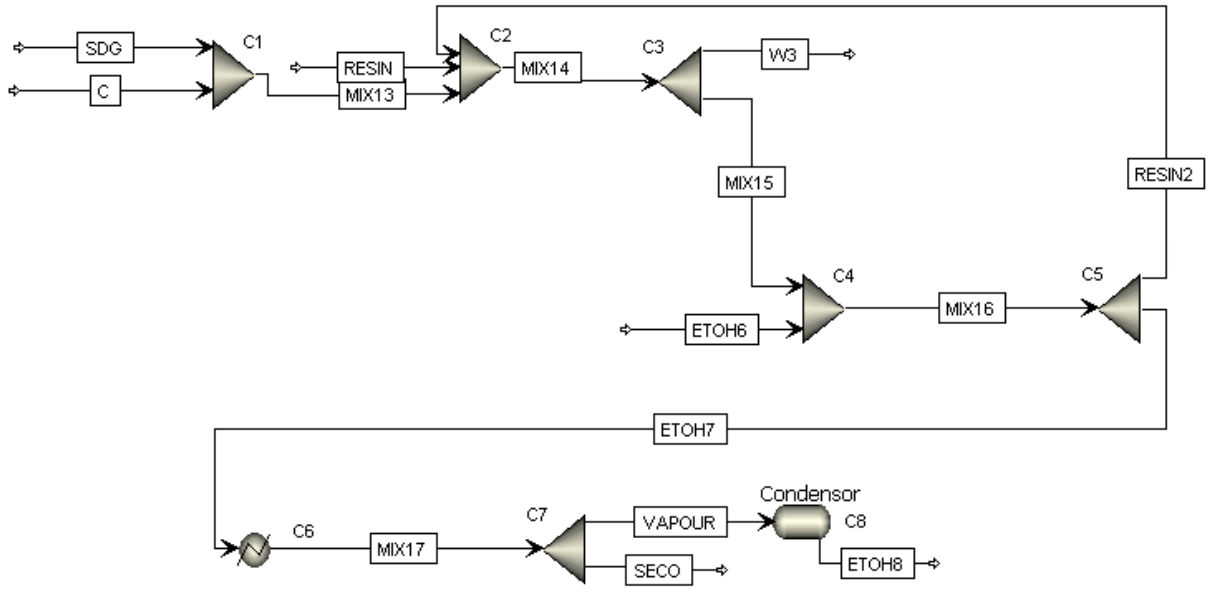


Figure 4.16. Conceptual design of industrial process of SDG enzymatic hydrolysis.

Where, SDG is SDG product from ammonium hydroxide hydrolysis, SECO is dried SECO product, C is cellulase, ETOH6 is anhydrous ethanol, ETOH7 is ethanol containing SECO, ETOH8

is recycled ethanol, RESIN is pretreated resin, RESIN2 is recycled resin, MIX13 is mixture of SDG, SECO and cellulase, MIX14 is mixture of cellulase, SDG, SECO and resin, MIX15 is mixture of resin, SDG and SECO, MIX16 is mixture of resin, SECO, SDG and ethanol, MIX17 is mixture of SECO and ethanol vapor, W3 is waste, C1 is reaction chamber, C2 is mixing chamber, C3 is separator, C4 is mixing chamber, C5 is separator, C6 is evaporator, C7 is separator, and C8 is condenser.

5. SUMMARY AND CONCLUSIONS

The central hypothesis of this research was that flaxseed lignan, which includes SECO, SDG and a polymer composed of SDG and 3-hydroxyl-3-methyl glutaric acid (HMGA), can be produced in one assembly line with acceptable yield and quality for food applications. To validate our hypothesis, SDG polymer was extracted from defatted flaxseed meal using aqueous ethanol; SDG was released from the polymer by ammonium hydroxide hydrolysis; in turn, SECO was released from SDG by cellulase hydrolysis.

The specific outcomes of this study are as follows:

1) A pilot scale method with few procedures to extract 1 kg SDG polymer per batch from defatted flaxseed meal has been established; preferred conditions was to use 70% ethanol (v/v) to extract defatted flaxseed meal twice at room temperature; yield of SDG polymer was 2.7% and content of SDG polymer was 65.8 ± 2%.
2) An environmentally friendly method to release SDG from SDG polymer by ammonium hydroxide has been established; preferred conditions was to use 29% (w/w) ammonium hydroxide solution to hydrolyze SDG polymer at ratio of 4:1 (w/w) at 80 °C for 2 h and followed by resin enrichment; yield of SDG was 51.9 ± 3% and content of SDG was 89.5 ± 1%.
3) An environmentally friendly method to hydrolyze SDG to SECO by cellulase has been established; preferred conditions was to use 30 U/mL cellulase from *Trichoderma viride* to hydrolyze 20 mg/mL SDG in sodium acetate buffer solution in ratio of 5:7 (v/v) at 60 °C for 48 h; yield of SECO was 66.6 ± 2% and content of SECO was 86.4 ± 1%.

As described in this research, a continuous pilot-scale process that produce SDG polymer, SDG and SECO was developed. Comparing to lab scale experiments, which is usually yield milligrams to grams, each pilot scale production can produce 1kg SDG polymer, 100 g SDG and 10 g SECO, respectively. With further improvement, target scale of extracting 1-2 kg SDG polymer, 500 g SDG and 100 g SECO per batch can be achieved. The SDG polymer, SDG and SECO had acceptable yield and quality. Further enrichment could be applied onto each component for specific purpose. The results demonstrated here would help to design industrial processing of flaxseed lignan production.

6. FURTHER DIRECTIONS

Results from this study demonstrate the possible path for industrial production of flaxseed lignan and its derived products. However, the current study does not specify details of flaxseed lignan industrial processing, which including the equipment, parameters, conditions, and efficiency. Further studies could focus on investigating the design of desirable equipment such as reaction chambers, processing parameters such as flow rate and resin column depth, processing conditions and production efficiency. In addition, economic evaluation including costs of raw materials, processing equipment, reagents, and energy input could be investigated.

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APPENDIX A

Picture and Spectrum of SDG and SECO

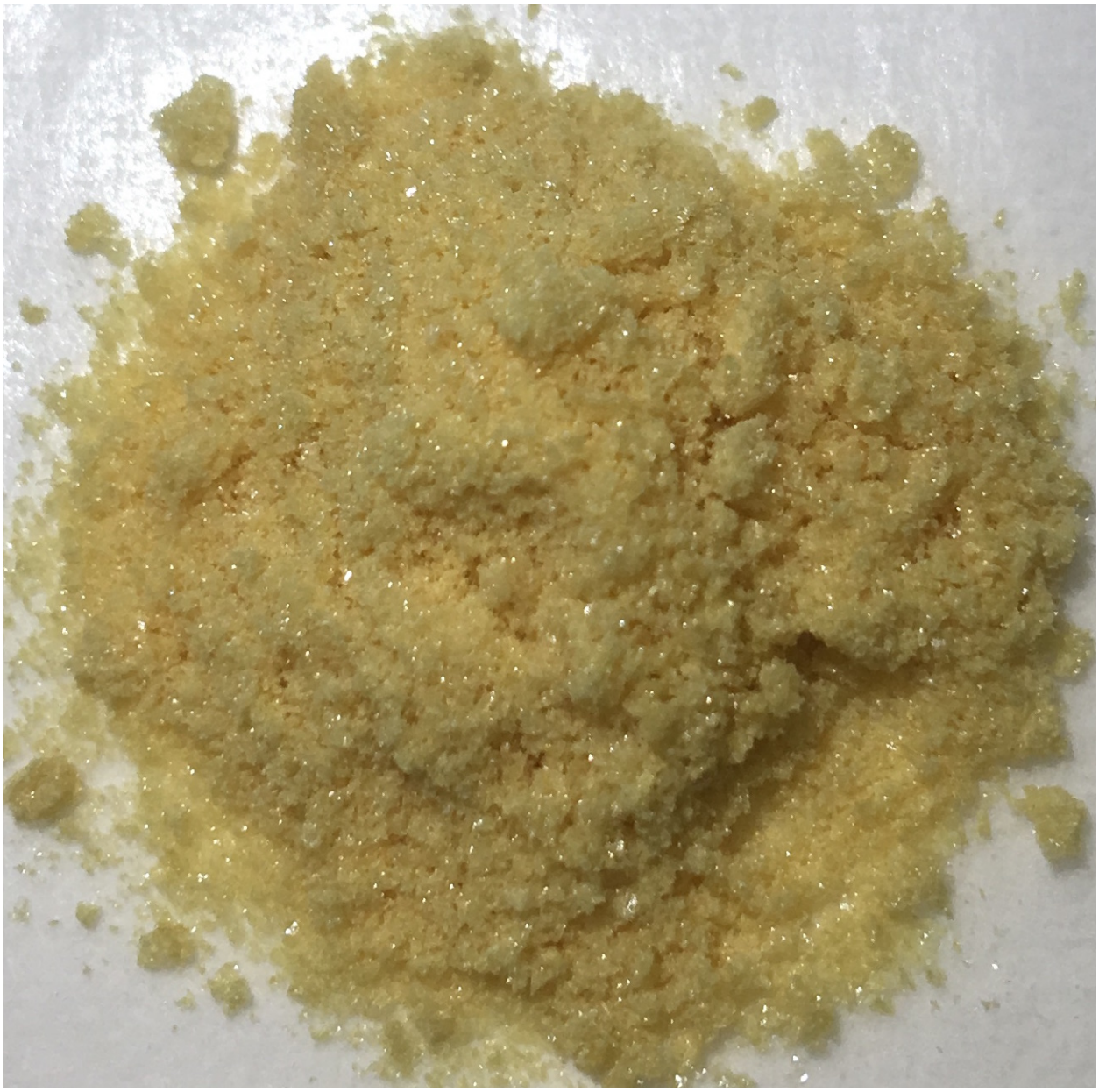

Figure A.1. Picture of enriched SDG powder.

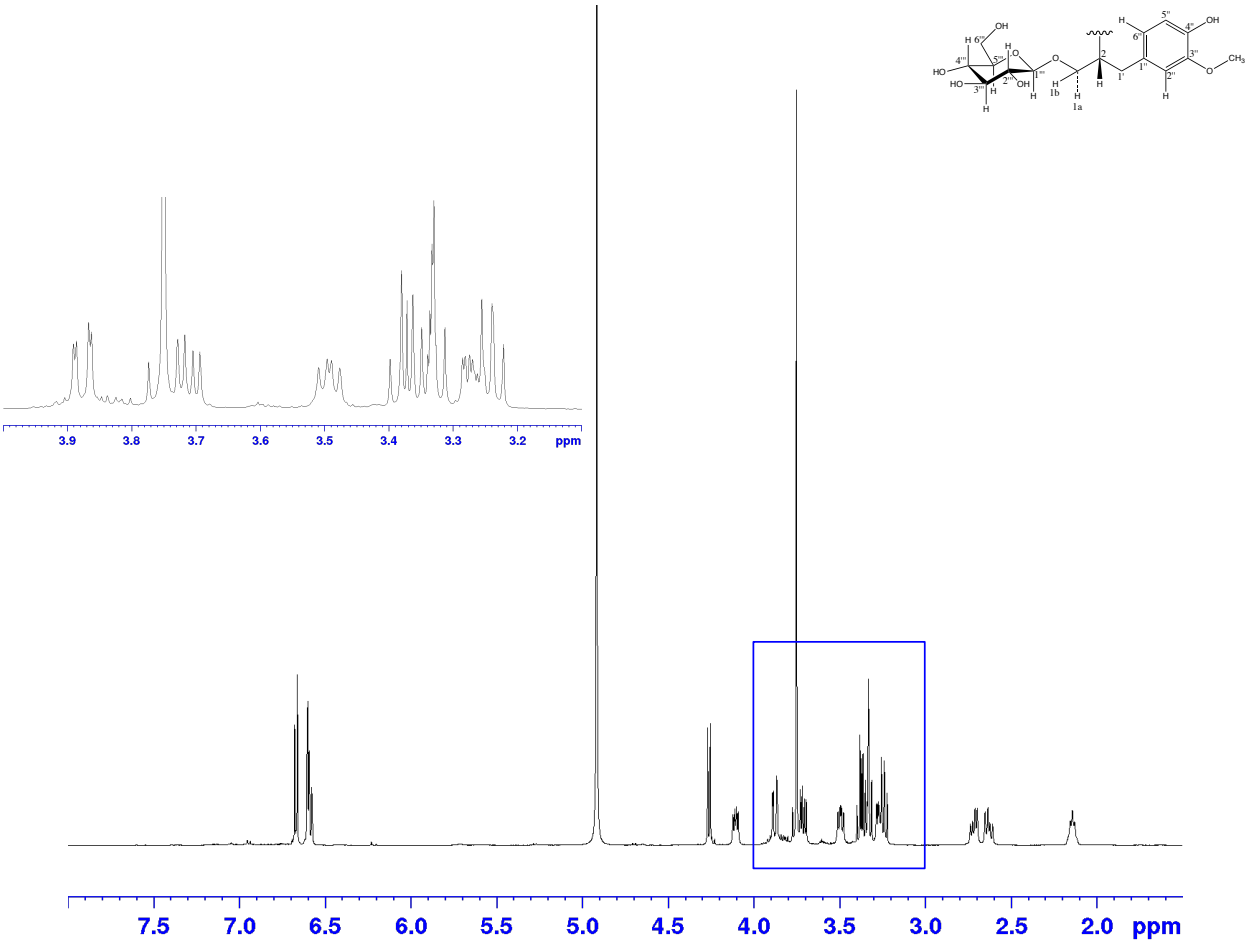

Figure A.2. ^{1}H-NMR spectra of enriched SDG (CD_3OD, 600 MHz).

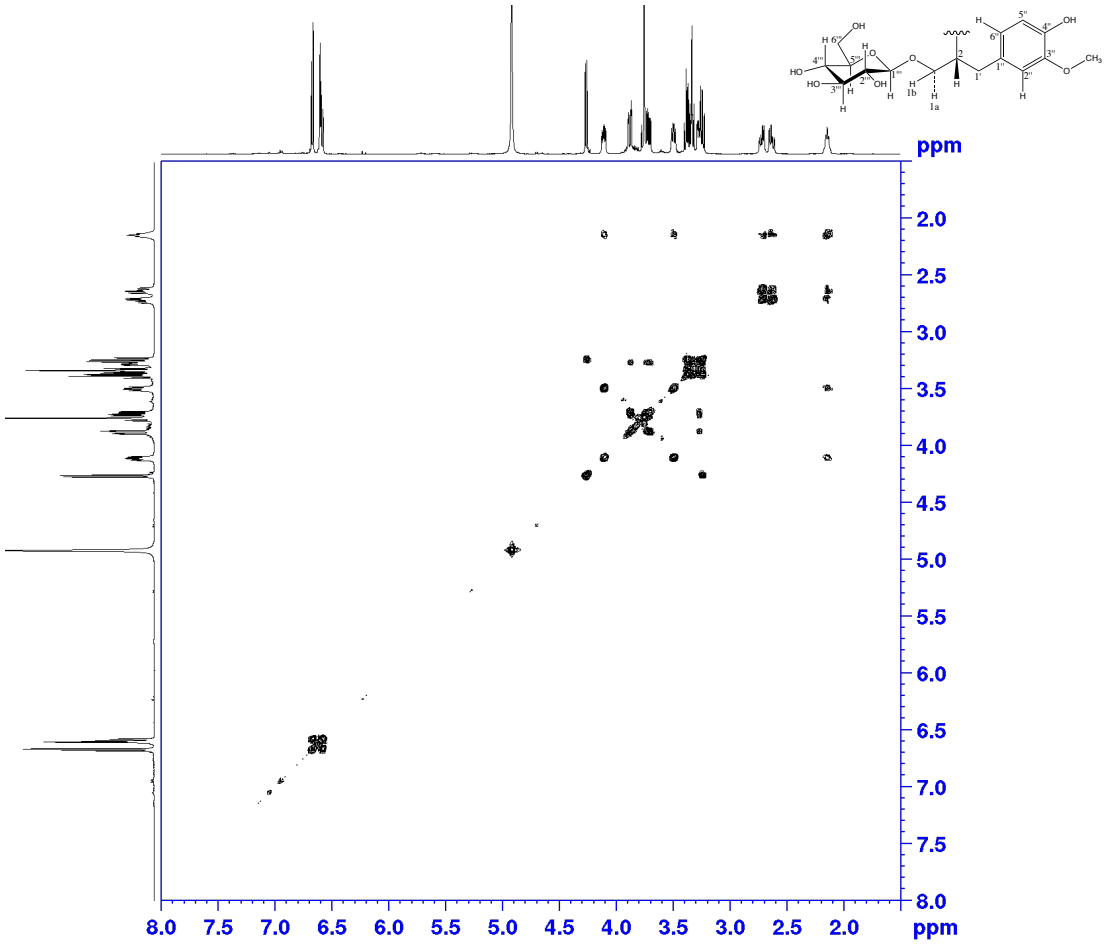

Figure A.3. ^{1}H-^{1}H Correlation Spectroscopy (COSY) NMR spectra of enriched SDG (CD_3OD, 600 MHz).

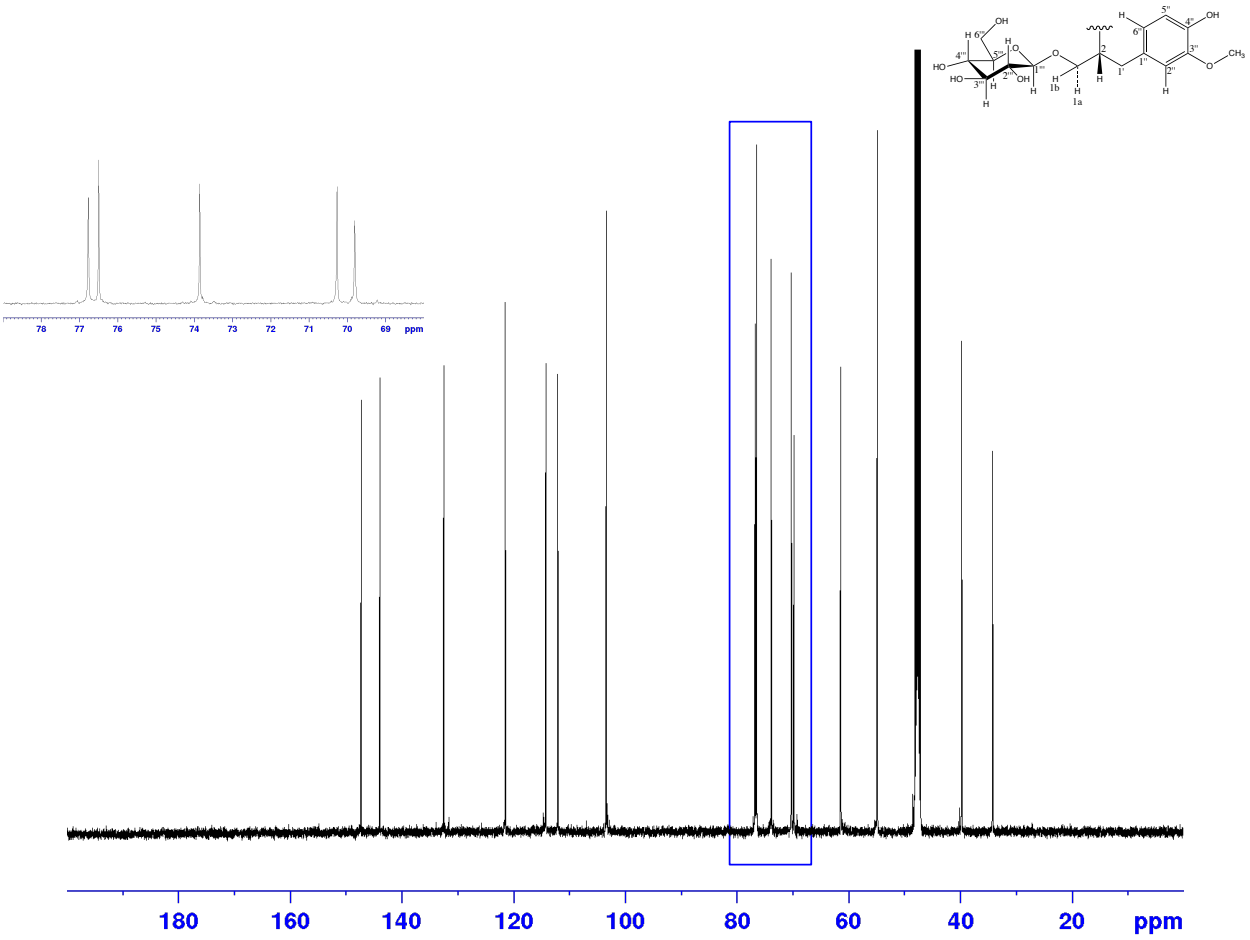

Figure A.4. ^{13}C-NMR spectra of enriched SDG (CD_3OD, 600 MHz).

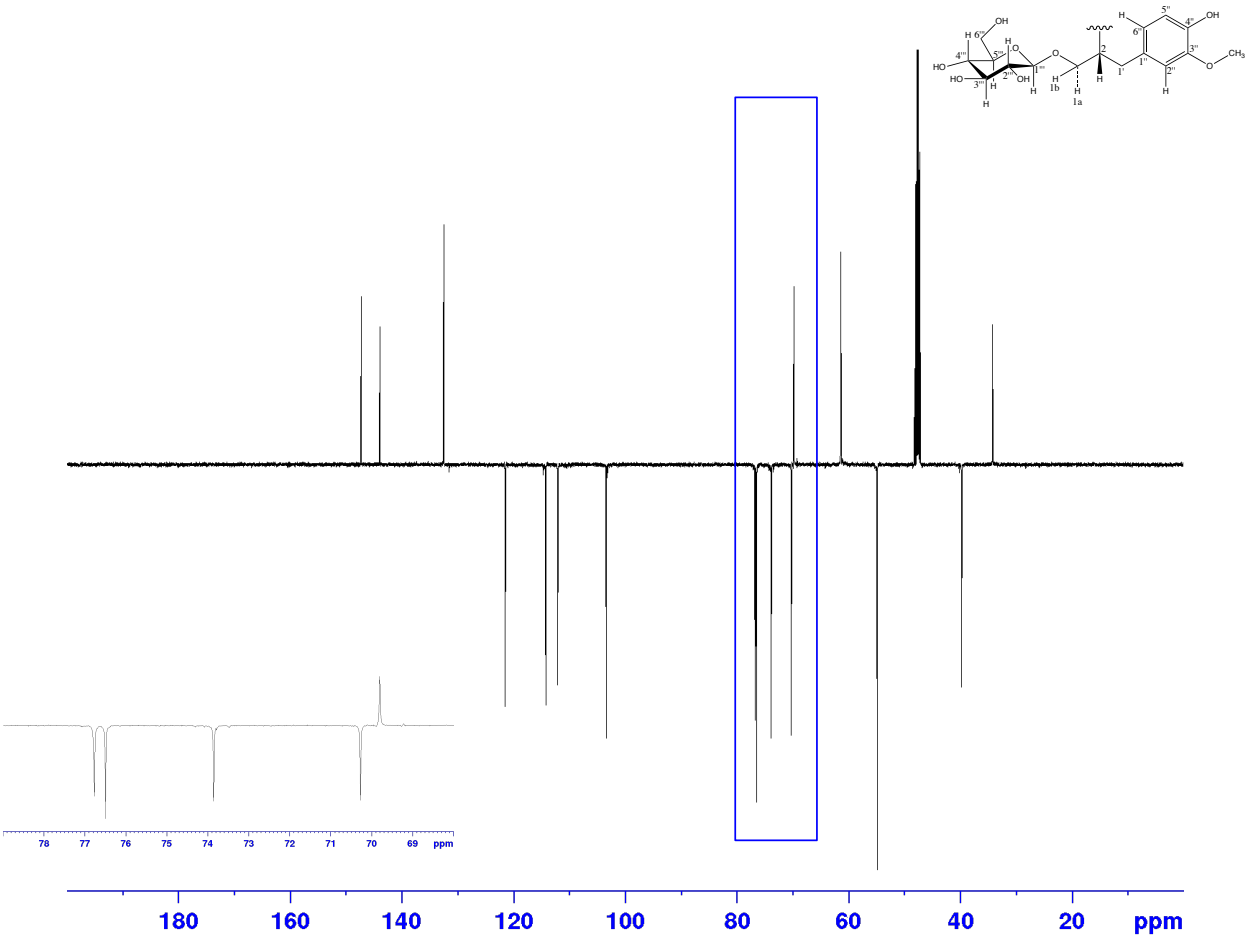

Figure A.5. ^{13}C Attached Proton Test (APT) NMR spectra of enriched SDG (CD_3OD, 600 MHz).

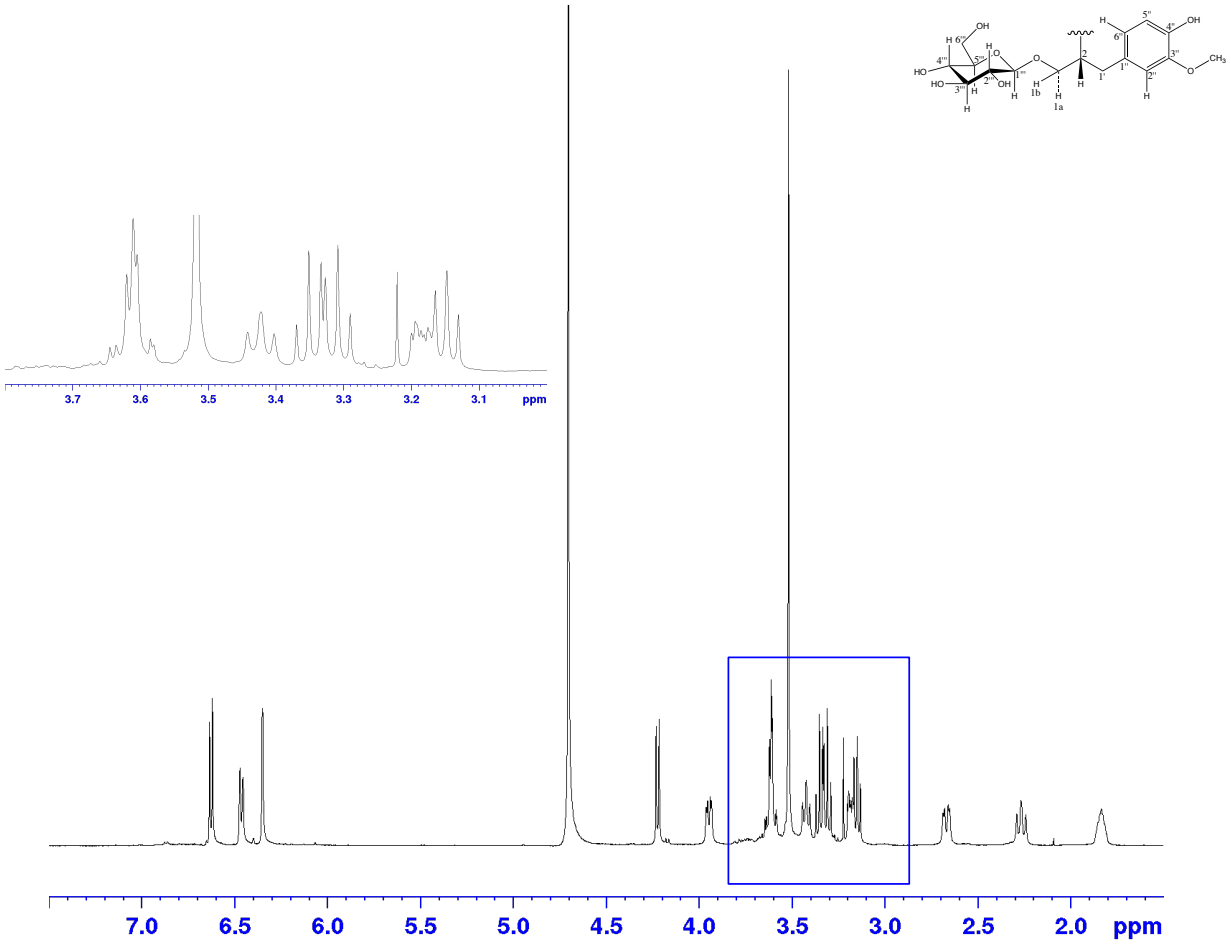

Figure A.6. ^{1}H-NMR spectra of enriched SDG (D_2O, 600 MHz).

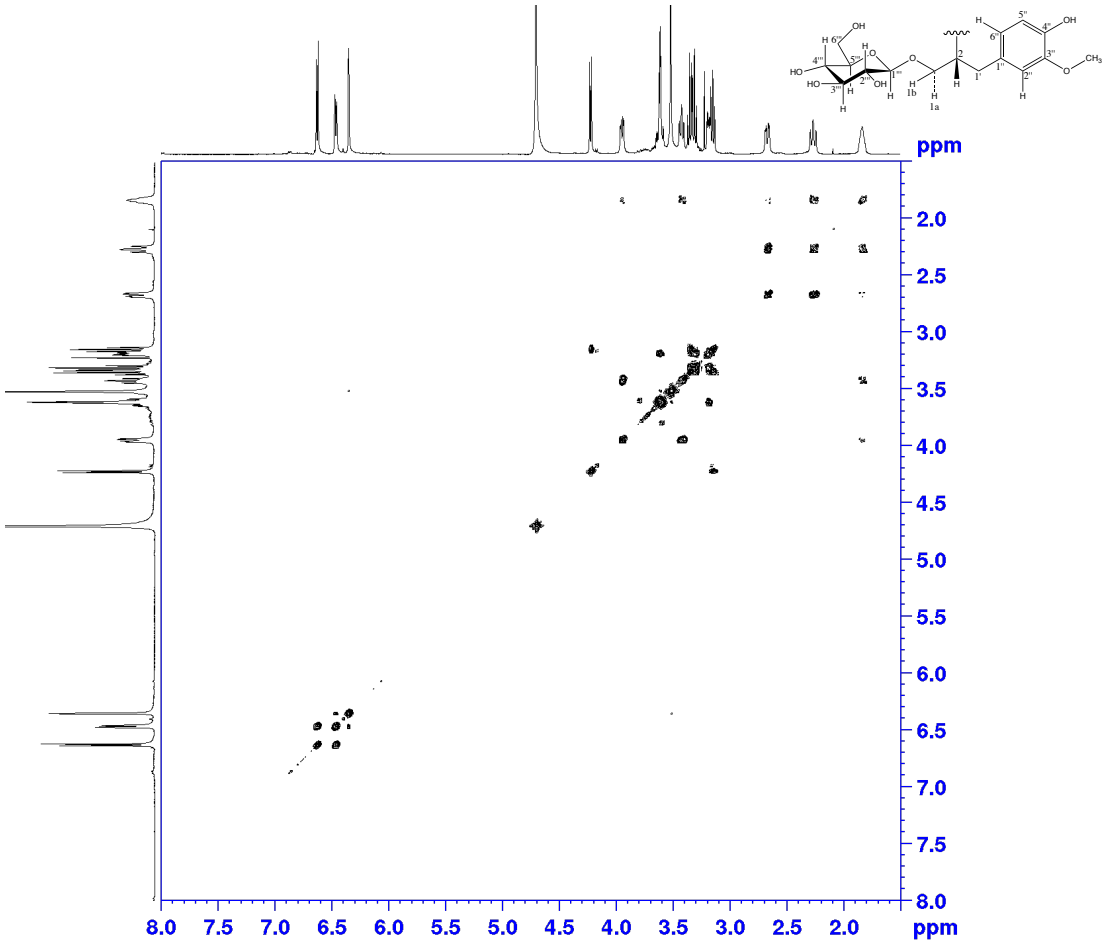

Figure A.7. ^{1}H-^{1}H Correlation Spectroscopy (COSY) NMR spectra of enriched SDG (D_2O, 600 MHz).

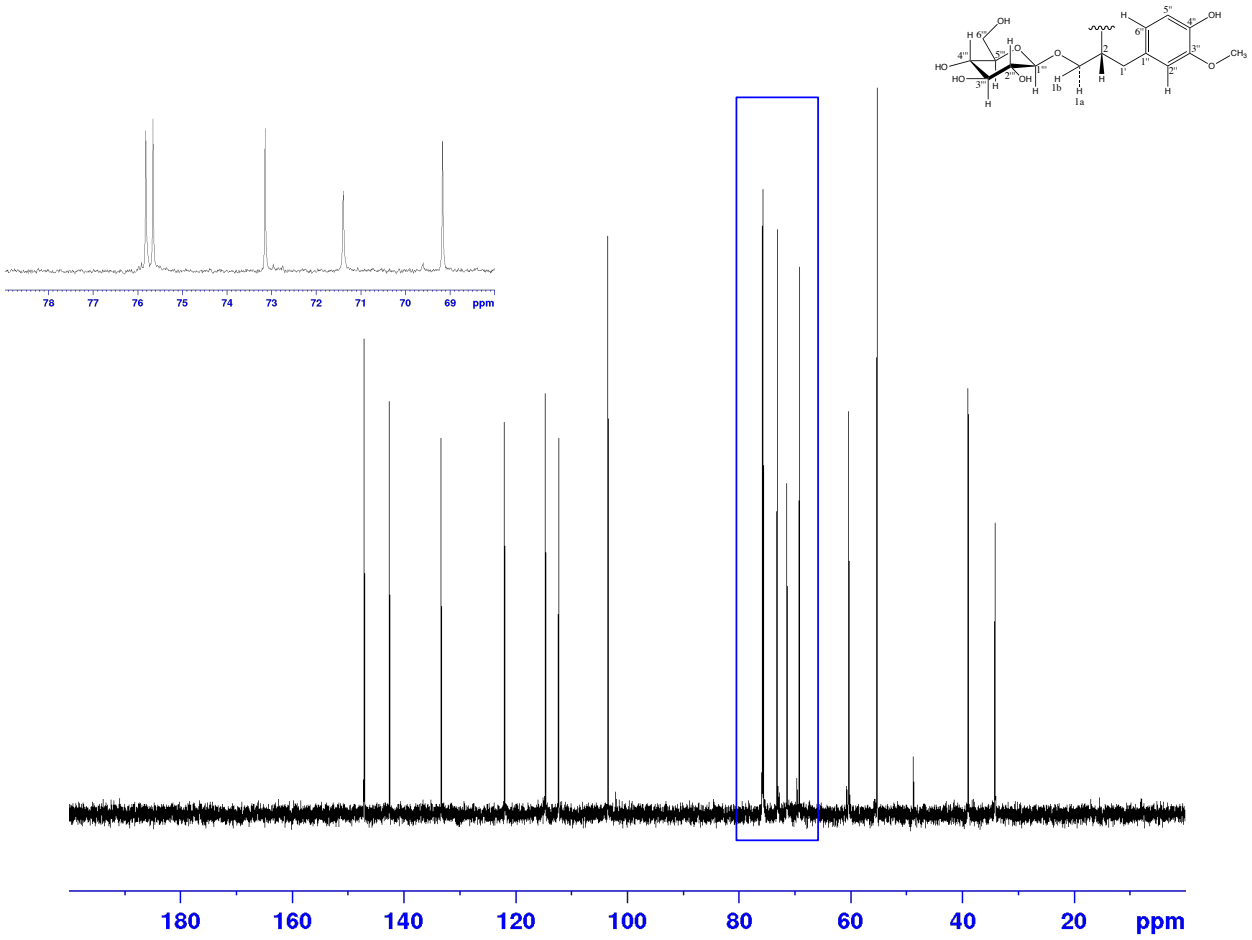

Figure A.8. ^{13}C-NMR spectra of enriched SDG (D_2O, 600 MHz).

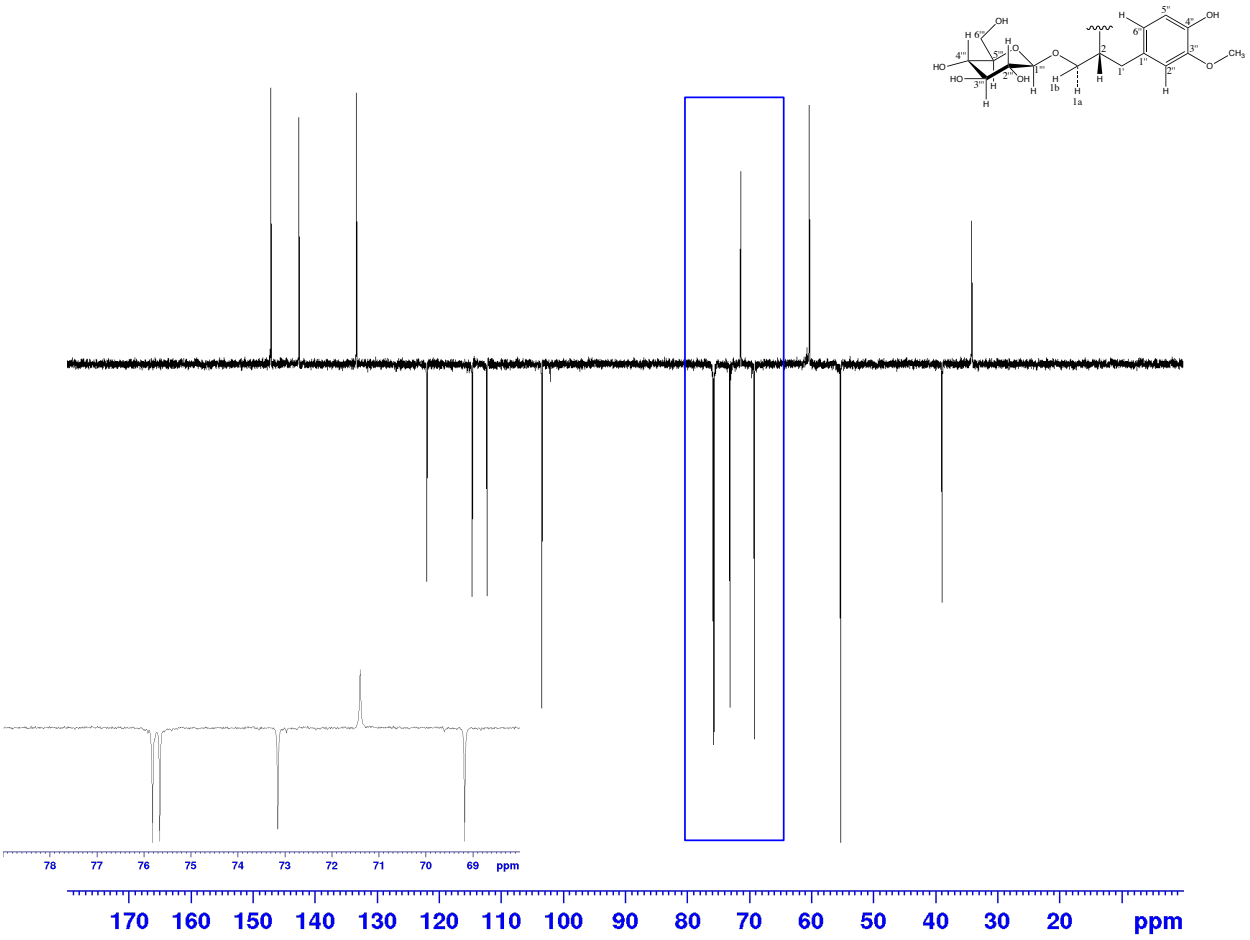

Figure A.9. ^{13}C Attached Proton Test (APT) NMR spectra of enriched SDG (D_2O, 600 MHz).

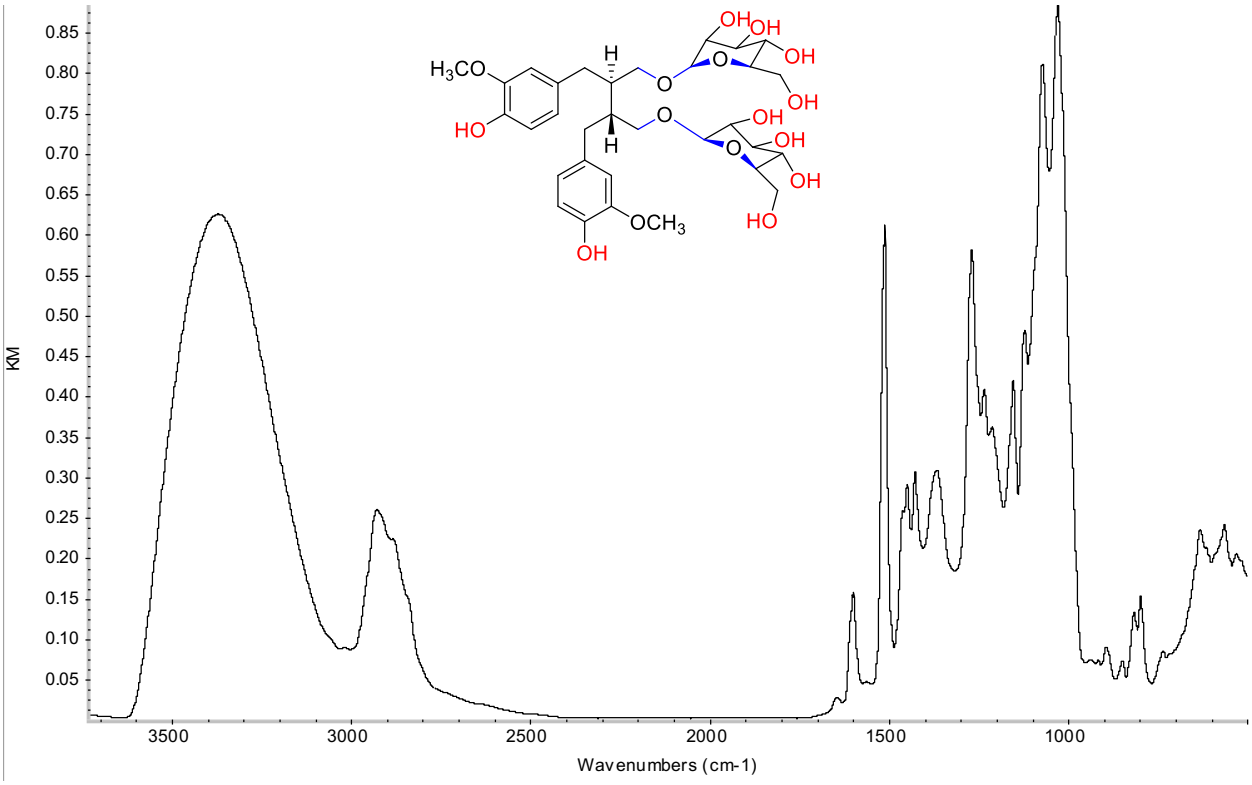

Figure A.10. Infrared spectroscopy (IR) spectra of enriched SDG.

Figure A.11. Picture of enriched SECO powder.

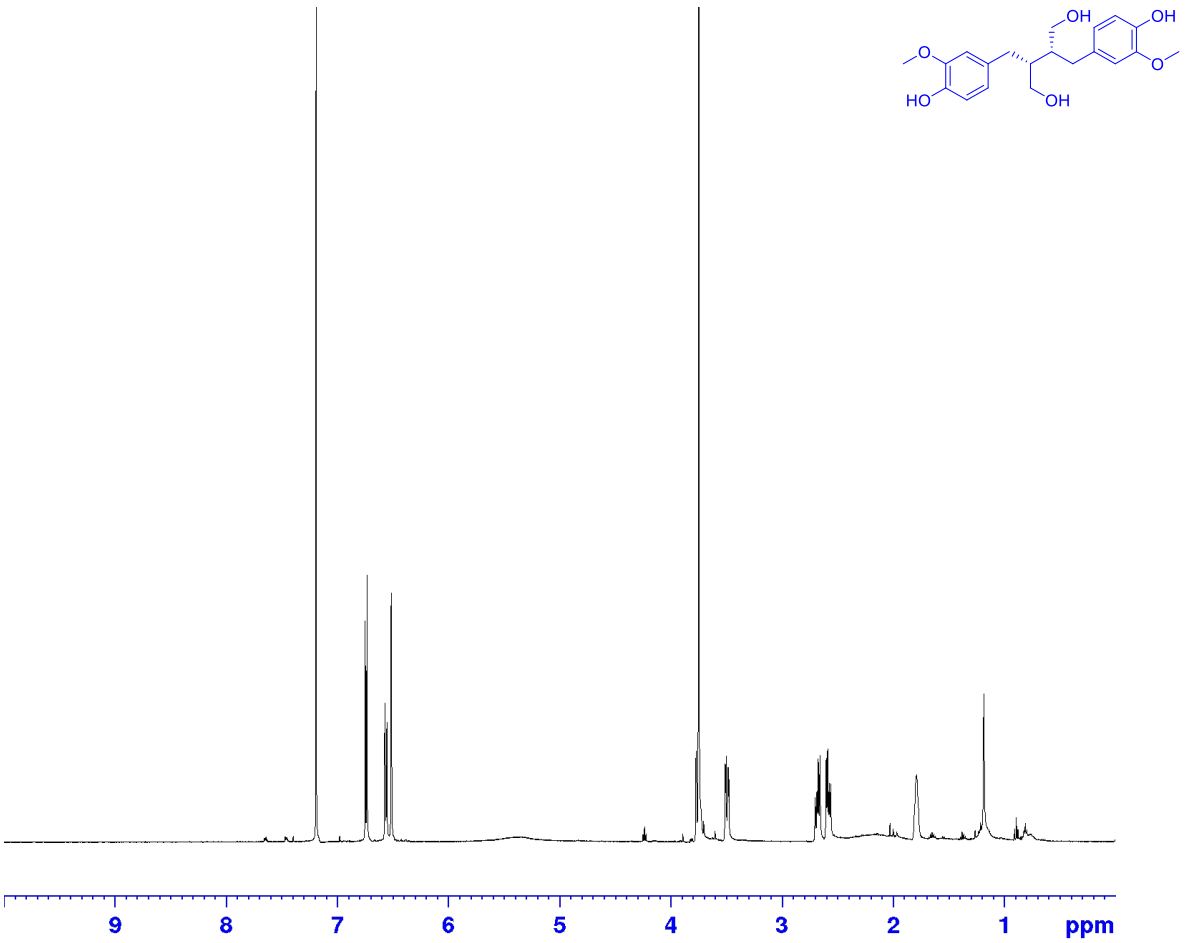

Figure A.12. ^{1}H-NMR spectra of enriched SECO (CD_3OD, 600 MHz).

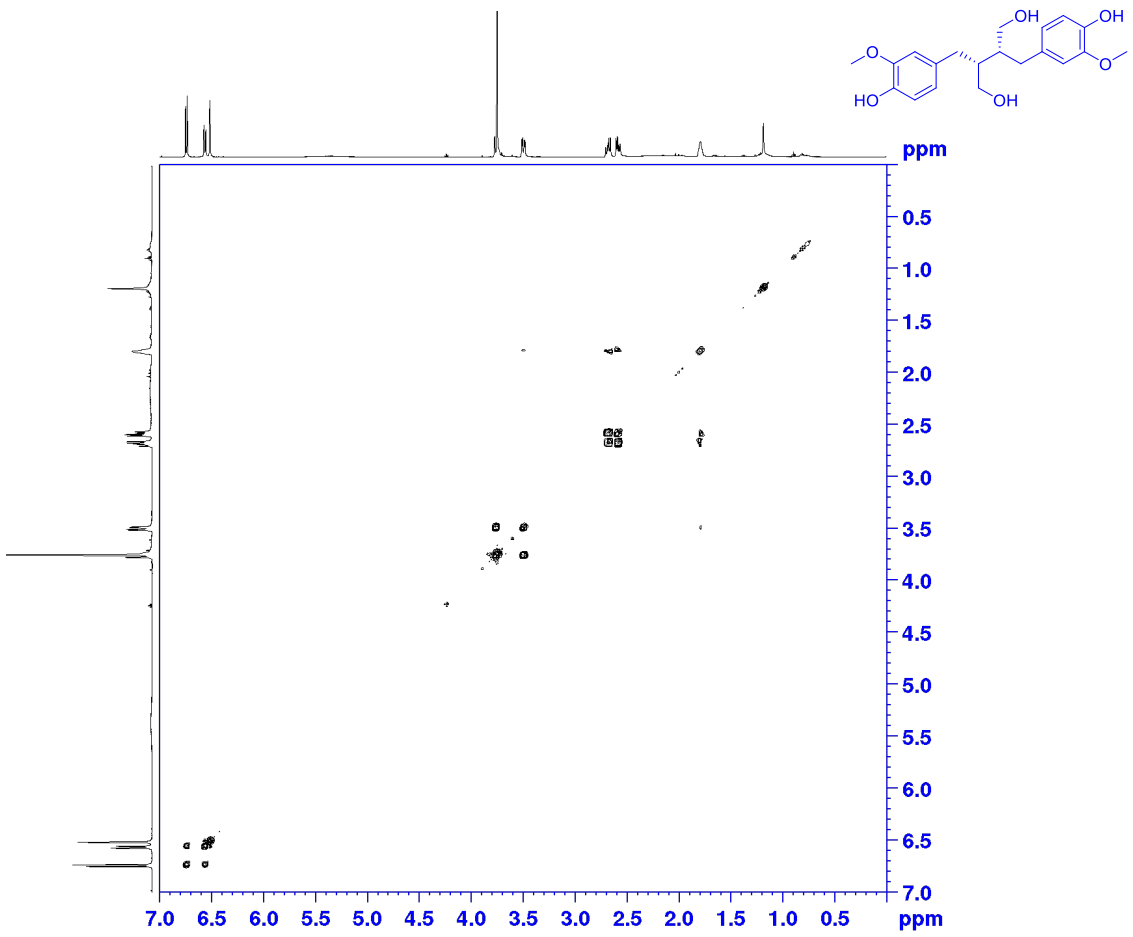

Figure A.13. ^{1}H-^{1}H Correlation Spectroscopy (COSY) NMR spectra of enriched SECO (CD_3OD, 600 MHz).

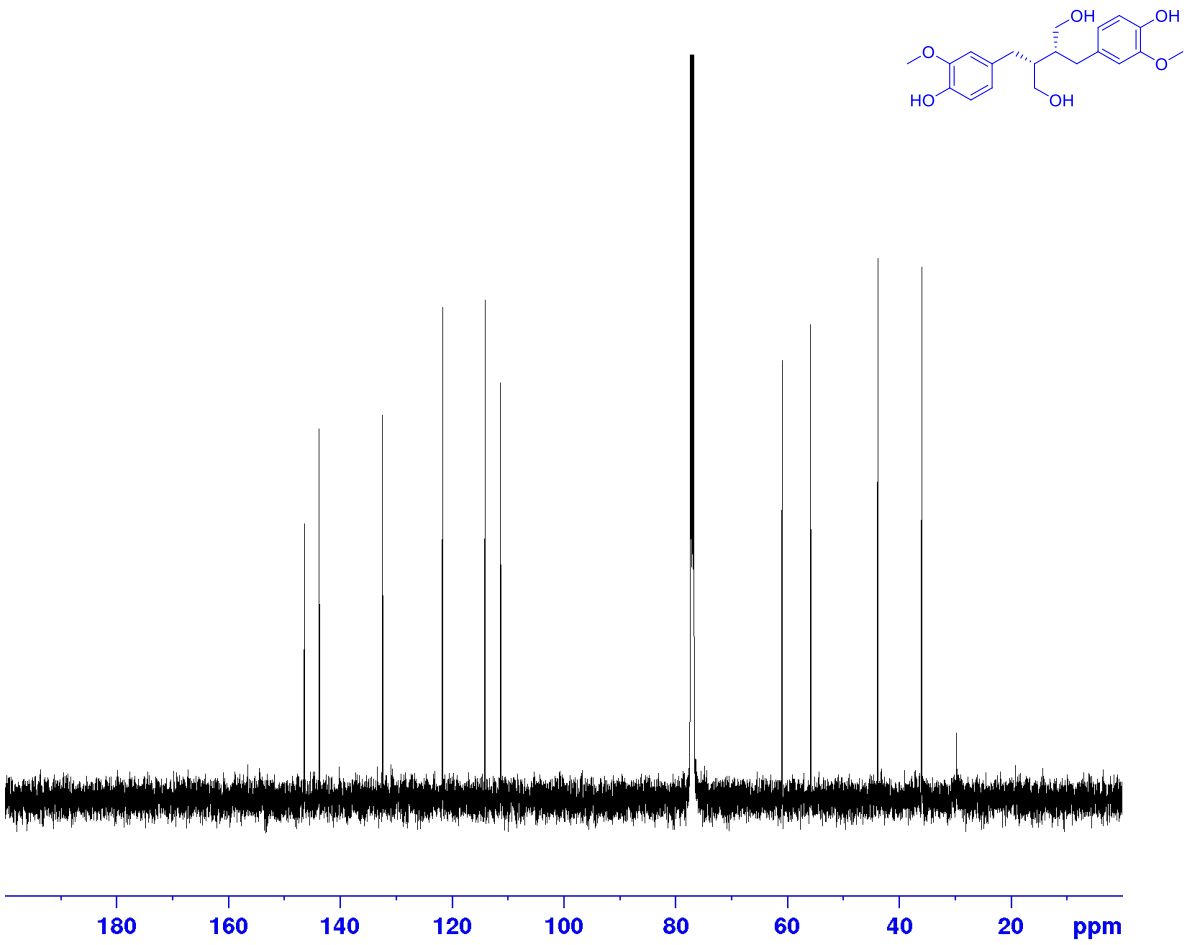

Figure A.14. ^{13}C-NMR spectra of enriched SECO (CD_3OD, 600 MHz).

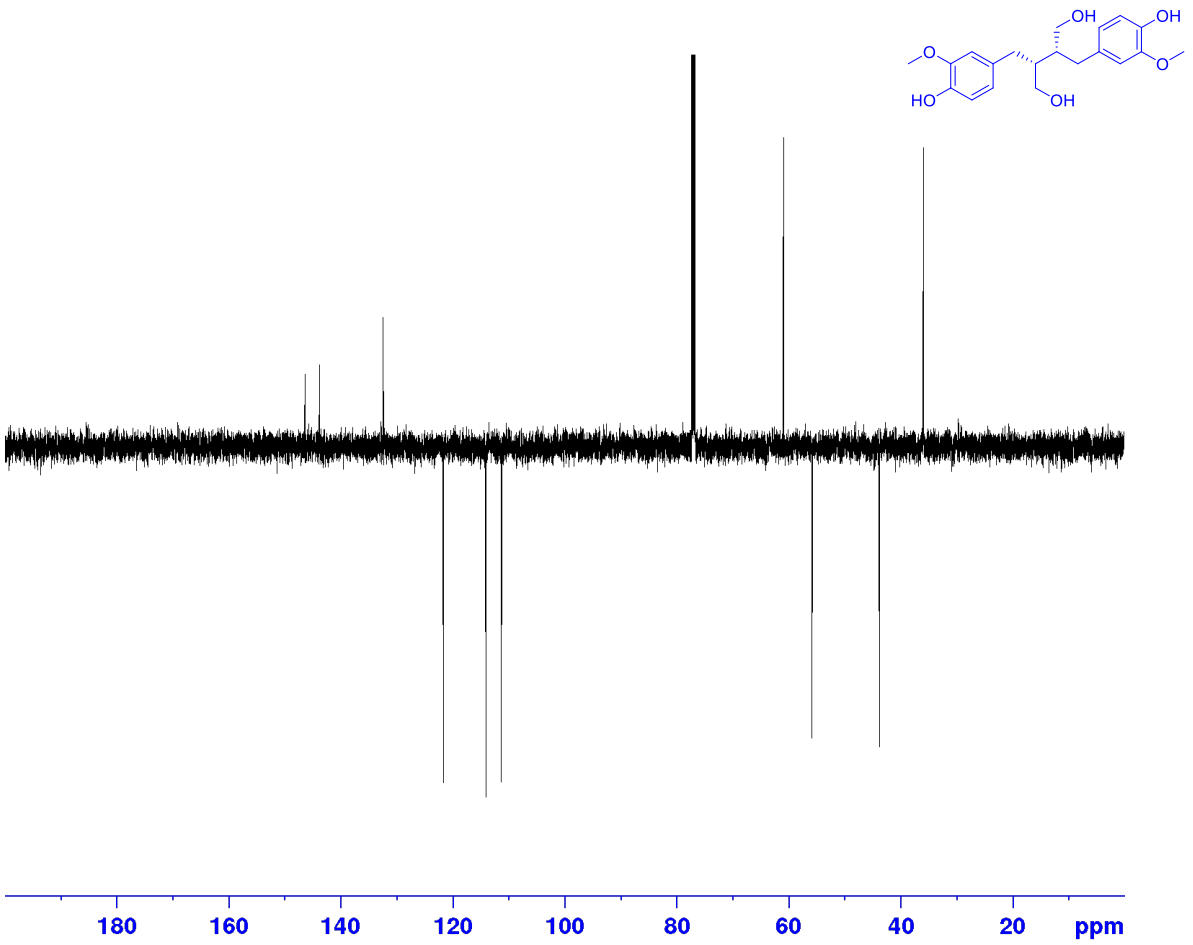

Figure A.15. ^{13}C Attached Proton Test (APT) NMR spectra of enriched SECO (CD_3OD, 600 MHz).