Pressurized Low Polarity Water Extraction of Lignans, Proteins and Carbohydrates from Flaxseed Meal

M. Sc. Thesis

Submitted to the Faculty of Graduate Studies

by

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In Partial Fulfillment of the Requirements

for the Degree of

Master of Science

Department of Food Science

Faculty of Graduate Studies

The University of Manitoba

August, 2006

ACKNOWLEDGEMENTS

I would like to express my sincerest thanks to my advisor, Dr. G. Mazza, for providing me the opportunity to pursue graduate studies and for his gracious support and continuous guidance. I would also like to extend my appreciation to Dr. R. Holley, Dr. M. Scanlon, and Dr. D. Jayas for their suggestions and participations on my advisory committee. I also like to give my deepest thanks to Dr. J. Han, Dr. H. Sapirstein, and Dr. G. Crow for their exceptional and dedicated teachings with a high calibre of professionalism which help transform into gratifying and rewarding learning experiences. Their dual role as distinguished professors and mentors helped me immensely, and allowed me to draw on their extensive experiences to shape my own identity. The generous financial assistance from the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

Special thanks are extended to all staff at the Department of Food Science for their kind assistance. To my lab mates at the Pacific Agri-Food Research Centre, Eduardo Cacace, Lana Fukumoto, Rod Hocking, Tony Cottrell, John Drover, Tom Kopp and David Godfrey, I am grateful for their valuable technical advice and friendship. I would also like to express my gratitude to Dr. B. Girard and Dr. K. Usher for introducing me to food science and teaching me how to improve and advance the quality of scientific research. I am thankful to Guangzhi Zhang, Jin Kim and Jun Kim for their intellectually stimulating conversations.

A heartfelt thank you to my talented mother for her endless trust, faith and encouragement. Her innate conventional wisdom and commitment to excellence are the

constant sources of enrichment and inspiration for my success. She also helps me reckon that the sky's the limit and lead me how to split the seemingly insurmountable goal into smaller reachable targets in an unprecedented manner. To my father, grandma and my sister for their love, patience and understandings. Without their tremendous love and unconditional contributions, my studies would not have been possible. I thank my other friends, Trisha Cho and Kevin Wong, for the fun and joy they gave to me that made graduate life enjoyable and memorable.

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C	solute concentration in the extractor at any time during the extraction process, (mg/mL)
C_i	initial solute concentration at the beginning of extraction (mg/mL)
C_{eq}	equilibrium solute concentration in the solution (mg/mL)
D_e	effective diffusion coefficient or diffusivity (m^2/s)
E_a	activation energy for diffusion (kJ/mol)
F	fraction of solute released quickly (dimensionless)
d_p	diameter of solid particle (m)
k_1	rate constant for fast extraction stage (min^{-1})
k_2	rate constant for slow extraction stage (min^{-1})
K_s	mass transfer coefficient (m/s)
L	bed depth (m)
M_t	total amount of diffusing substance extracted after time t (mg solute/g meal)
M_∞	equilibrium solute concentration in solution, maximum amount of solute that can migrate (extracted) after infinite time (mg solute/g meal)
R	universal gas constant, J mol^{-1} K^{-1} (1.987 cal/K mol)
r	radius of solid particle (m)
S/S	solvent to solid ratio (mL/g)
T	temperature (°C or K)
t	extraction time (min)
u	superficial velocity (m/s)
ρ	density of solution (kg m^{-3})
μ	viscosity of solution (kg $m^{-1}s^{-1}$)

Dimensionless numbers

Bi Biot number $Bi = \frac{K_s r}{D_e}$

Re Reynolds number $\mathrm{Re} = \frac{\rho u d_p}{\mu}$

Sc Schmidt number $Sc = \frac{\mu}{\rho D_e}$

Sh Sherwood number $Sh = \frac{K_s d_p}{D_e}$

ABSTRACT

The physiological benefits of flaxseed against pathological disturbances, such as cancers and heart diseases, are mainly attributed to its high lignan content. This study (Experiment 1) examined the application of pressurized low polarity water (PLPW) for extraction of lignans, proteins and carbohydrates from defatted flaxseed meal. Key processing conditions included temperature (130, 160, 190°C), solvent pH (4, 6.5 and 9), solvent to solid ratio (S/S) (90, 150 and 210 mL/g) and introduction of co-packing material (0 and 3 g glass beads). The addition of 3 g glass beads as co-packing material facilitated extraction by enhancing surface contact between the liquid and solid thus shortening extraction time. Elevated temperature accelerated the extraction rate by increasing the solid diffusion coefficient thereby reducing the extraction time. The maximum yield of lignans (99 %) was obtained at temperatures ranging from 160°C to 190°C, with solvent volume of 180 mL (90 mL/g meal) at pH 9. Optimal conditions for protein extraction (70 %) were pH 9, extraction volume of 420 mL (210 mL/g meal) and 160°C. Total carbohydrates yield was maximized at 50% recovery at pH 4 and 160°C with 420 mL solvent (210 mL/g meal). Increased temperature accelerated extraction, thus reducing solvent volume and time to reach equilibrium. For the extraction of proteins, however, a temperature of 130-160°C is recommended, as proteins are vulnerable to thermal degradation due to heat decomposition.

The effects of flow rate and geometric dimensions for extraction of lignans and other flaxseed meal bioactives were further investigated in Experiment 2, based on the variables optimized in the previous experiment. Defatted flaxseed meal was extracted

with pH 9 buffered water with meal to co-packing glass beads ratio of 1:1.5 at 5.2 MPa (750 psi) and 180°C. The aqueous extracts were analyzed for lignan, protein and carbohydrate using HPLC and colorimetric methods. The optimal extraction yields for lignan, protein and carbohydrate were found at flow rates of 1 to 2 mL/min with bed depth between 20 and 26 cm and a S/S ratio of 40 to 100 mL/g. The combination of low flow rate and high bed depth allowed the use of lower S/S ratio with reduced total solvent volume consumption.

This study also evaluated the mass transfer kinetics governing the process of lignan extraction from flaxseed meal in a fixed bed extraction cell. Diffusion of solute into the continuously flowing solvent was mainly responsible for the mass transfer mechanism as flow rate did not increase proportionally with the yield and rate of extraction. The extraction kinetics were studied on the basis of two approaches: Fick's diffusion equation and a two-site exponential kinetic model. The proposed two-site exponential kinetic model corresponding to the two-stage extraction (rapid and slow phases) successfully described the experimental data. Diffusivities attained from Fick's diffusion model ranged from 2×10^{-13} to 9×10^{-13} m^2s^{-1} while mass transfer coefficients were between 4.5×10^{-8} and 2.3×10^{-7} ms^{-1} for extraction of lignans at 180°C, pH 9 with 1:1.5 meal to co-packing material ratio.

CHAPTER 1

Introduction

In recent years, the agri-food sector and consumers have begun to look at food providing not only basic nutrition and enjoyment of eating, but also for health and medicinal benefits. Nutraceuticals and functional foods fit into this niche market as they are regarded as nutrients that provide unique beneficial effects through reducing the risk of chronic disease, above and beyond their basic nutritional functions. A primary force in the market for nutraceuticals and functional foods is a growing consumer belief in the link between diet and disease (Oomah and Mazza, 1999). Besides, aging populations and rising health care costs are the major reasons for governments to pay more attention to the development of the functional foods sector. Diseases, such as coronary heart disease, cancer, and diabetes are correlated to dietary habits and can be an economic strain on the government sponsored health care system. In the U.S, coronary heart disease alone contributes to a $259 billon economic loss, which along with other diseases could be reduced with dietary changes (Milner, 2000; Tucker and Miguel, 1996). In addition, elderly and middle-aged consumer groups specifically have increased their spending on functional foods (Roberts, 2002).

In order to meet this growing demand, government and industries are developing new methods for extracting natural plant components with potential disease prevention attributes. Oilseed crops grown in Canada offer considerable potential for value-added processing due to their content of nutritionally valuable constituents in them. One of the most promising crops is flaxseed which contains phytochemicals such as lignans, phenolic acids and proteins (Oomah, 2001). Therefore, flaxseed incorporation into the

diet is particularly attractive from the perspective of development of foods with specific health advantages.

In view of this growing popularity, functional food and nutraceutical development is increasingly focused on scientifically validated health claims and technology development. For example, Canadian companies and researchers specializing in the standardization of herb and plant extracts have developed extraction, isolation and purification expertise to manufacture herbal products to pharmaceutical standards. Companies have refined analytical methods to verify the potency and bio-activity of herbal extracts and other compounds (Agri-Food Trade Service, 2003). Undoubtedly, dietary improvement through functional foods and nutraceuticals are critical as it is directly relate to a healthy population. At the same time, however, consumers are more aware of food security, safety and quality, and are demanding more information about how their food is produced. More than ever, consumers want to know that their food is safe and that it has been produced in an environmentally responsible manner. Natural food components extracted by organic solvents are common industrial products developed due to their high recovery and relatively low cost of preparation (Frank et al., 1999). Organic solvents, however, have an added disposal cost burden because of tighter environmental compliance requirements (Barwick, 1997). Despite technological advances, little progress has been made toward the development of clean and economically viable extraction techniques. As a result, there is an urgent need and an emerging challenge for industries to comply with the tightened environmental regulations by finding alternatives to reduce organic solvent generation. Hence, intensive research

effort is needed to develop new extraction techniques that could produce high-value co-products from flaxseed with a net positive environmental impact.

New extraction methodology such as pressurized low polarity water (PLPW) extraction is considered to be a 'green' alternative to organic solvents. PLPW provides similar solvent strength and could even exceed extraction efficiency and product recovery compared to organic solvent under specific extraction conditions (Cacace and Mazza, 2005; Yang et al., 1998; Ong, 2005; Hawthorne et al., 1999). The present study examined a variety of processing parameters including temperature, pH, flow rate, solvent to solid ratio and co-packing materials for their ability to optimize extraction of bioactives from flaxseed meal using pressurized water as a solvent.

The objectives of this research were:

1. To optimize extraction of lignan, protein and carbohydrates from flaxseed meal in terms of yield using response surface methodology; and
2. To identify and determine mass transfer and extraction kinetics of lignan in a PLPW fixed bed extraction column.

CHAPTER 2

Literature review

2.1 Functional foods and nutraceuticals

The terms "nutraceutical" and "functional food" are used commonly around the world, but there is no consensus on their meaning. Consequently, the Bureau of Nutritional Sciences, of the Food Directorate of Health Canada, had proposed the following definitions. A functional food is similar in appearance to a conventional food, is consumed as part of a usual diet, and it is demonstrated to have physiological benefits or reduce the risk of chronic disease beyond basic nutritional functions. A nutraceutical is a product isolated or purified from foods that is generally sold in dosage or medicinal forms not usually associated with food (Health Canada, 1998). In both cases, the active components occur naturally in the food. In 2001, the value of the functional food and nutraceutical global market was $56.6 billion (Agri-Food Trade Service, 2003). The industry estimated that the global market for functional foods and nutraceuticals is growing faster than the processed food market as a whole, especially in the United States, Europe, Japan and Canada. Canada produces a wide variety of grains and oilseeds. Among the representative crops is flaxseed which serves as one of the rapidly growing top 10 supplements in terms of appreciable dollar sales (Marra, 2002). Canada plays a dominating role as the world's largest flaxseed producer, contributing about 40% of total world production and 75% of world export (Oomah and Mazza, 1998). Exports of oilseed products such as oil and meal total $667 million (Agriculture and Agri-Food Canada, 2003).

2.2 Flaxseed

2.2.1 Characteristics of flaxseed

Flaxseed has been used in the diets of humans for thousands of years. The Babylonians cultivated flaxseed as early as 3000 B.C. In 650 B.C., Hippocrates used flaxseed for the relief of intestinal discomfort (Flax Council of Canada, 1998a). The ancient Greeks and Romans valued flaxseed for its laxative effects and its ability to relieve gastric distress (Tolkachev et al., 2000).

Flaxseed is mainly grown in cool, northern climates in the midwestern region of United States and Canada. The major growing areas in Canada are in the prairie provinces Saskatchewan and Manitoba. The botanical name of flax is *Linum usitatissimum*. The term flaxseed and linseed are often used interchangably. Flaxseed is used to describe flax when it is eaten by humans. Linseed is to describe flax when it is used for industrial purposes, such as linoleum flooring, kitchen counters, cupboards, car door panels, brake linings or inks (Flax Council of Canada, 1998b).

Flax is grown in Canada essentially for industrial (linseed) oil used to manufacture industrial products, especially paints and plastics. Apart from that, flaxseed provides essential nutrients, including protein, essential fatty acids, vitamins and minerals. It also contains both soluble and insoluble dietary fibre as well as lignan, a type of phytoestrogen (Shahidi and Naczk, 2004). Flaxseed is comprised of 30-45% oil, including omega-3 fatty acids; 20-25% protein; 30-35% carbohydrates, 10% fiber; 4% ash and 6% moisture (Bhatty and Cherdkiatgumchai, 1990; Bhatty, 1995; Budavari, 1996; Daun et al., 2003). The content and composition of flaxseed is significantly

affected by the cultivar, year of harvest and growing location, the types of seed processing and analytical methods used (Westcott and Muir, 1996). Flaxseed also contains significant quantities of complex phenolics known as lignan. The lignan component in flaxseed of particular interest is secoisolariciresinol diglucoside (SDG) due to its abundance in flaxseed and its health benefits related to its estrogen-like actions in animals and humans (Mitchell, 2001). Flaxseed can be used long term as a bulk laxative and as a nutritional supplement. The demonstration of clinical activity associated with the consumption of flaxseed has led the U.S. National Cancer Institute to target flax as one of the six plant materials for study as cancer-preventive foods (Caragay, 1992). Flaxseed is one of the most concentrated sources of the lignan precursor SDG and contains 75-800 times the amount found in other foods as shown in Figure 2.1 (Mazur et al., 1998; Thompson et al., 1991). Flaxseed was found to be the champion plant species for lignan production when fed to rats (Figure 2.1). In spite of the health benfits from the major components, flaxseed contains two minor antinutrients cyanogenic glucoside and phytate. The amount of cyanogenic glycosides was found to be around 0.1% of dry weight of seed (Oomah and Mazza,1998). They have the ability to release hydrogen cyanide (HCN) upon acidic or enzymatic hydrolysis. An adult can detoxify 30-100 mg of cyanide per day. Studies have shown that when calculated in cyanide equivalent, the amount of cyanide may vary from 190-1000 mg HCN/kg of flaxseed. In other words, an adult can consume no more than 100 g of flaxseed per day before becoming susceptible to acute cyanide toxicity (Daun et al., 2003). It is believed that when flaxseed is used only as a minor ingredient in food products such as flax bread, muffins, or cereals, the cyanogenic glycosides are not really a problem for human consumptions.

Figure 2.1. Mammalian lignan production from various foods (Thompson et al., 1991)

2.2.2 Flaxseed meal

Flaxseed meal is a byproduct of flaxseed oil extraction. The defatted meal is normally underutilized as feed or discarded. Flaxseed meal is largely used in livestock feeds, particularly for ruminants. Although the defatted flaxseed meal is rich in lignan, very little flaxseed meal is used in human foods except for specialty foods. Flaxseed meal is generally obtained by cleaning, flaking, cooking and pressing of the seed followed by solvent extraction and solvent removal steps (Oomah and Mazza, 1997). Composition of the meal changes after various processing steps. For instance, the lignan and protein contents increase and the oil content decreases when flaxseed is processed

into oil and meal (Oomah and Mazza, 1995;1993a). Flaxseed meal has a protein content of up to 40% after oil extraction (Oomah and Mazza, 1993b). The lignan SDG content improves from about 10 mg/g in flaxseed to 20 mg/g in flaxseed meal (Johnsson et al., 2000; Eliasson et al., 2003). Carbohydrate is also present in the meal at a much higher concentration than in the seed (Mazza and Biliaderis, 1989; Mazza and Oomah, 1995). Flaxseed meal can be made from full-fat dehulled flaxseed. Full-fat flaxseed contains fat in excess of 30%, while the oil content of defatted flaxmeal is usually less than 10%. In addition to the nutritional characteristics, flaxseed protein provides prominent functional roles in foods. These functional characteristics include solubility, emulsifying, foaming and whipping ability (Oomah and Mazza, 1993a).

2.3 Bioactive compounds in flax

2.3.1 Lignan

Lignan is one of the widely distributed phenolics in the plant kingdom, being found in most unrefined grains such as barley, buckwheat, millet, oats and some vegetables such as broccoli, carrots, cauliflower and spinach (Thompson et al., 1991). In particular, the richest source of lignan is flaxseed. Secoisolariciresinol diglucoside (SDG, $C_{32}H_{46}O_{16}$) shown in Figure 2.2 has been identified as a major lignan of flaxseed (Bakke and Klosterman, 1956; Meagher et al., 1999). The minor lignan components are isolariciresinol, pinoresinol, and matairesinol (Meagher et al., 1999).

Figure 2.2. Structure of secoisolariciresinol diglucoside (SDG; 2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butane-diglucoside) (Cacace and Mazza, 2005)

Chemically, lignans are phenolic compounds formed by the union of monomeric units hydroxyl- and hydrox-methoxy derivatives of cinnamic and benzoic acids (Budavari, 1996). Cinnamic, caffeic, p-coumaric, ferulic and sinapic acids represent the cinnamic group. The benzoic, hydroxybenzoic, protocatechuic, vanillic and syringic acids belong to the benzoic group. By definition, lignans are dimers of phenylpropanoid (C6-C3) units linked by the central carbons of their side chains. Plant lignans possess multiple oxygenated substituents in the aromatic rings and notably in the para-position that make them different in structure from mammalian lignans (Oomah and Mazza, 1998). Lignans act as defensive substances in plants (Davin and Lewis, 1992). The lignan pinoresinol is formed when the plant is wounded and is toxic to microorganisms. Indeed, the pharmacological effects of lignans are related to their antiviral, antimitotic and antioxidant activity (Ayres and Loike, 1990; Setchell, 1995). Likewise, they may play a predominant role as anticancer agents in humans (Setchell et al., 1987). Dinkova-Kostova et al. (1996) and Ayres and Loike (1990) also reported that lignans play a

proactive role in plant growth and in defense against predators owing to their antifungal and insecticidal properties.

2.3.2 Protein

Flaxseed as a source of vegetable protein is commercially available in the form of seed, full-fat flour (i.e. milled flaxseed), and meal (Oomah and Mazza, 1998). Flaxseed meal usually contains between 30% to 32% protein (Oomah et al., 1994). Variability in the protein content of flaxseed has been attributed to genetic and environmental factors (Oomah and Mazza, 1995). Cool growing conditions usually result in lower protein but higher oil content (DeClercq et al., 1995). Nutritional studies have shown that flaxseed proteins have well-balanced amino acid composition (Oomah and Mazza, 1998). The protein fraction contains a favorable ratio of amino acids with lysine, threonine and tyrosine as the limiting amino acids as shown in Table 2.1. The table presents the amino acid profile of seed from a brown-seeded cultivar NorLin together with the amino acid composition of commercial meal as reported by Oomah and Mazza (1993a) and Bhatty and Cherdkiatgumchai (1990), respectively. Flaxseed is a good source of the sulfur amino acids methionine and cystine. It is particularly high in aspartic acid, glutamic acid, leucine and arginine. Arginine has been shown to provide cardioprotective effects as a precursor for the vasodilating substance nitric oxide and may retard atherogenesis (Nittynen et al., 1999).

Table 2.1. Amino acid composition of flaxseed and flaxseed meal (g/100g protein)

Amino acids	Flaxseed cv. NorLin[a]	Commercial Flaxseed Meal[b]
Alanine	4.4	5.5
Arginine	9.2	11.1
Aspartic acid	9.3	12.4
Cystine	1.1	4.3
Glutamic acid	19.6	26.4
Glycine	5.8	7.1
Histidine	2.2	3.1
Isoleucine[c]	4.0	5.0
Leucine[c]	4.0	7.1
Lysine[c]	4.0	4.3
Methioine	1.5	2.5
Phenylalanine[c]	4.6	5.3
Proline	3.5	5.5
Serine	4.5	5.9
Threonine	3.6	5.1
Tryptophan[c]	NR[d]	1.7
Tyrosine	2.3	3.1
Valine[c]	4.6	5.6

[a]Data from Oomah and Mazza, 1993a
[b]Data from Bhatty and Cherdkiatgumchai, 1990
[c]Essential amino acid
[d]NR = not reported

Proteins can be classified by their composition, structure, biological function, or solubility properties. Nitrogen is the most distinguishing element present in proteins. However, nitrogen content in various food proteins ranges from 13.4 to 19.1 percent due to variation in the specific amino acid composition of proteins (Sikorski, 2002). In general, proteins rich in basic amino acids contain more nitrogen. Proteins have unique conformations that can be altered by denaturants such as heat, acid, alkali, organic solvents and detergents (Nielsen, 1994). Thus, their solubility and functionality can be altered by denaturants. The poor water solubility of flaxseed proteins was confirmed in

experiments using a nitrogen extractability curve (Dev and Quensel, 1988; Dev and Quensel, 1986; Madhusudhan and Singh, 1985a). Flaxseed meal proteins were demonstrated to be only 20-24% solubility between pH 2 and 6. The buffer capacity of flaxseed protein is maximal at an acid pH below the isoelectric region (pH 4-6) and minimal in the alkaline region (Madhusudhan and Singh, 1985a). Therefore, alkaline pH favors extraction of protein. Flaxseed products generally exhibit favorable water absorption, oil absorption, emulsifying activity and emulsion stability compared with the corresponding soybean products (Dev and Quensel, 1986). Modification of flaxseed proteins by heat treatment effectively improves water absorption, but reduces fat absorption, nitrogen solubility, foaming and emulsion characteristics (Madhusudhan and Singh, 1985b).

2.3.3 Carbohydrate and dietary fibre

There are two groups of carbohydrates: those digestible by human enzymes (simple sugars and starch) and those resistant to human digestive enzymes (dietary fibre). Flaxseed contains only a small percentage (<2%) of soluble sugars but it is an excellent source of dietary fibre (Bhatty and Cherdkiatgumchai, 1990; Flax Council of Canada, 1998c). Flaxseed gum (fibre) or mucilage is a water soluble polysaccharide. Flaxseed gums can be extracted from whole flaxseed with hot water (85-90°C) followed by precipitation with 80% ethanol and then freeze-dried (Mazza and Biliaderis, 1989; Mazza and Oomah, 1995). Cui et al. (1994) reported the optimum conditions for the extraction of flaxseed gum are temperatures of 85-90°C; pH of 6.5-7.0 and a water/seed ratio of 13:1. The hypocholesterolemic effects of flaxseed gum may be partly attributed to the

high production of short-chain fatty acids from its fermentation in the colon (Berggren et al., 1993). Wolever and Jenkins (1993) concluded that the underlying mechanism for flaxseed function in reducing acute blood glucose response is by slowing carbohydrate digestion and absorption. Soluble fibers are believed responsible for causing a decline in glycemic response (blood sugar levels 3-4 h after eating) by increasing the viscosity of the small intestinal content and delaying the digestion and absorption of carbohydrates (Jenkins et al., 1978; Blackburn et al., 1984; Edwards et al., 1987). Flaxseed has the potential to increase laxation because it provides dietary fibre which stimulates bowel activity by binding with water. The mucilage then swells and increases stool volume thus facilitating passage of feces accordingly.

2.4 Health effects of flaxseed lignans

There are numerous reports showing beneficial health effects associated with flaxseed consumption. The most frequently investigated flaxseed functions are its protection against cardiovascular diseases and hormone sensitive cancers. Table 2.2 summarizes the various benefits of three flaxseed components: omega-3 fatty acids, lignans and soluble fibre. Roles of protein and carbohydrates are briefly discussed in previous sections. The increasing number of reports on the health benefits of lignans led us to focus this research on the extraction of lignans.

Table 2.2. Health benefits of flaxseed components

Thompson, 2003

Lignan functions as a phytoestrogen and an antioxidant. Phytoestrogens may protect both men and women against certain cancers, particularly hormone-sensitive cancers such as breast and prostate by interfering with sex hormone metabolism. Thompson et al. (2000) reported a significant reduction in tumor cell proliferation in postmenopausal women fed flaxseed. Additional work in Dr. Thompson's laboratory showed that mammary tumor size decreased by more than 50% and the number of tumors decreased by more than 37% (Thompson et al., 1996a; 1996b). Furthermore, flaxseed addition affected the initiation and promotional stages of mammary cancers (Serraino and Thompson, 1992). In men, 30 g per day flaxseed consumption in combination with a low fat diet were found to delay prostate cancer cell proliferation rates and promoted apoptosis (tumor cell death) (Denmark-Wahnefried, 2001). Kitts et al. (1999) postulated that the total anti-carcinogenic activity of flaxseed could be due to the *in vivo* antioxidant

properties of the lignans. Haggans et al. (1999) followed the consumption of 10 g of ground flaxseed daily for seven weeks in 28 postmenopausal women. The urinary excretion of 2-hydroxyestrogen increased significantly (2-hydroxyestrogen is a metabolite of estrone that may protect against breast cancer). In another case-controlled study, women with a high dietary intake of lignans tended to have a lower breast cancer risk (Thompson et al., 2000).

Phytoestrogens may help prevent osteoporosis (Kuzer and Xu, 1997). Osteoporosis is a disease caused by low bone mass leading to increased risk of bone fracture (Brown and Josse, 2002). Following research conducted by Arjmandi (2001), it was reported that flax may have positive effect on bone in postmenopsusal women by enhancing antioxidant activity. They noted that free radicals generated in bone tend to cause bone resorption which increases bone loss. Flax lignans may help prevent bone loss and osteoporosis by blocking the production of prostaglandins and decreasing the rate of bone resorption by its antioxidant activities (Arjmandi et al., 1998).

2.5 Flaxseed potential as a functional food source

Flaxseed has been proposed as a functional food because (i) it is a conventional food, (ii) it is eaten as part of a usual diet, and (iii) it offers physiological benefit or protection against disease (Clifford, 2002; Cui and Han, 2003). The incorporation of flaxseed in functional foods is expanding its market share. For instance, nutritionally enhanced eggs from hens fed flax are available in supermarkets in North and South America, Europe and Asia (Clifford, 2002). Flax has gained popularity in the

mainstream market in many forms including raw seeds and expressed oils, and as an ingredient in breads, bagels, muffins, cereals, and breakfast bars (Blumenthal et al., 2000). The addition of flaxseed to cereal-based foods has been widely accepted (Hyvarinen et al., 2006; Muir and Westcott, 2000). Flax was added to pancakes, waffles, energy bars, granola and other snack foods. Maris Foods (Boxboro, MA) combines milled flaxseed with soy and a low glycemic sweetener into a health food snack to provide possible hormone replacement therapy (HRT) benefits (Anonymous, 2002). In addition, flax is added to the diets of livestock and pets to maintain or improve animal health (Clifford, 2002).

2.6 Concentrating bioactive compounds

The first step in the analysis of phytochemicals in a raw or processed plant matrix usually begins with extraction. The extraction procedure depends on the type of food to be analyzed, compounds to be measured, and the chemical properties of the compounds such as polarity, acidity, and hydrogen-bonding capacity. Extraction starts with increasing sample surface area by crushing, milling or grinding the food thus allowing better contact of the extracting solvent with the sample (Lee, 2004). Then the ground biomass is ready for extraction.

2.6.1 Extraction and quantification of lignan

The most common method of extracting lignans from flaxseed is solvent extraction due to its ease of operation and high recovery of target compounds. An alternative method is supercritical carbon dioxide extraction modified with ethanol (Pihlava et al., 2004). In the case of lignans, the step followed by extraction is

hydrolysis. Acid or alkaline hydrolyses are the main techniques used to prepare samples for chromatographic analysis of lignans (Lee, 2004).

2.6.1.1 Solvent extraction

Lignans and most other phenolic compounds are normally extracted with alcohol (methanol or ethanol) or alcohol-water mixtures such as aqueous ethanol, but it is necessary to extract the sample several times to obtain satisfactory results.

Extraction of lignans from defatted flaxseed powder was performed by Harris et al. (1994) with various solvents or blends of solvents. Solvent mixtures included ratios of methanol and chlorinated solvents which produced similar chromatographic profiles. The solvents which give the best results were high purity methanol or 95% ethanol:1,4 dioxane (1:1, v:v). After the samples were refluxed for 8 h, the cooled mixtures were filtered, collected and transferred to 5 mL volumetric flasks using small amounts of methanol, and then brought to volume with methanol (Harris et al., 1994). Westcott and Muir (1998) patented an alcohol-based method for isolating flaxseed lignans in greater than 90% purity. Extraction described in their patent can be performed at 65-75% ethanol at 4-30:1 alcohol to meal ratio for 1-4 h at room temperature. The lignan can then be liberated from the liquid fraction by base-catalyzed hydrolysis (0.06-2.5% wt/vol sodium methoxide or 1.25% wt/vol triethylamine or 1N potassium hydroxide 3-7% wt/vol for 4-24 h). The hydrolyzed extract can be further purified to above 95% by anion exchange chromatography. The yield of SDG from this process is about 20 mg/g of defatted flaxseed meal (Westcott and Muir, 1998). Jerkins (1995) described an extraction method for obtaining an SDG-rich extract from defatted flaxseed meal. Flaxseed meal

initially defatted by cold pressing was extraced by supercritical carbon dioxide to further remove the oil; ethanol (80%) extraction was performed subsequently on the CO_2 extracted meal (Jerkins, 1995). Lignan can also be readily obtained from an aqueous ethanol (85%) extract of flaxseed meal (Empie and Gugger, 2002). The main difference between their method and the previously mentioned method is the concentration and purification step after the extraction. The aqueous fraction was ultrafiltered (5000 Mwt, cut-off), and the permeate was fractionated by absorbent resins (XAD-4) to enrich the SDG concentration from 1.9 to 13.4 mg/g meal.

2.6.1.2 Hydrolysis

Acids and bases can alter the lignan complex and release SDG through hydrolysis. In most of the literature on lignans, the reported systems for the separation of lignans and their glycosides in foods are carried out by reversed phase high performance liquid chromatography (RP-HPLC) on silica-based C-18 bonded-phase columns. Muir et al. (2002) compared the lignan levels of a commercial flax meal sample by HPLC analysis of extracts between the base hydrolysis method published by Liggins et al. (2000) and the acid hydrolysis method of Meagher et al. (1999). Both methods required 3 h of extraction prior to 3 h of hydrolysis. Muir et al. (2002) concluded that both acid and base hydrolysis methods generate samples which can be easily analyzed by RP-HPLC and yield similar lignan concentrations. Johnsson et al. (2000) also developed a method for analyzing SDG in flaxseeds. Their analytical method involved extraction of defatted flaxseed flour with dioxane/ethanol followed by aqueous base hydrolysis and then by solid-phase purification of the SDG-containing fraction, and finally quantitative

analysis is undertaken by HPLC. Although the above method (Johnsson et al., 2000) gave reliable results, it involved a very lengthy procedure (16 h solvent extraction plus 2 days of alkaline hydrolysis) and it was highly dependent on the use of large amount of solvents. Muir and Westcott (2000) extracted SDG from flaxseed meal using a methanol/water mixture prior to base hydrolysis, but the whole process took 9 h to complete.

A simple, fast and reliable method was invented by Eliasson et al. (2003) for the quantitative preparation of SDG in flaxseed meal. This method eliminated the alcoholic extraction step by using direct alkaline hydrolysis. They found that direct extraction by 1M NaOH for 1 h at 20°C resulted in a higher yield than that obtained by hydrolysis of alcoholic extracts due to inefficient extraction of oligomers from flaxseed matrix with alcohol. Thus, direct alkaline hydrolysis is the method employed in the present experiments and will be described in detail in Chapter 3.

2.6.2 Extraction and quantification of protein

Plant-based proteins found in oilseeds have been the focus of process and product development for food and industrial protein products as it is believed that the supply of vegetable proteins must be increased to sustain the ever growing population (Marcone, 1999). Solubility has traditionally been used as the principal parameter for classifying proteins (Osborne, 1924). Albumins are typically soluble in water at pH 6.6, globulins in dilute salt solutions at pH 7.0, prolamines in 70% ethanol, and glutelins in acid at pH 2 or alkali at pH 12 (Marcone, 1999; Portyanko et al., 1997; Waggle et al., 1989). Heat

decreases the viscosity of water but can increase viscosity of protein as well as their gelation functionality (Cybulska and Doe, 2002).

Available commercial grade flaxseed meals contain 30-40% protein (Bhatty and Cherdkiatgumchai, 1990). Several attempts have been made to extract flaxseed protein with buffered salt solutions. Vassel and Nesbitt (1945) described the isolation of protein from fat-free flaxseed meal with a 0.2 M phosphate buffer (pH 7.2) - ethylene glycol mixture. Sosulski and Bakal (1969) isolated protein from hexane-defatted ground flaxseed by extraction with 0.2% sodium hydroxide (1:20 meal to solvent ratio) followed by precipitation by adjusting the pH to 4.5. Dev and Sienkiewicz (1987) extracted total proteins from oil-free flaxseed meal using 0.66M sodium phosphate buffer (pH 7.6) containing 1.0M NaCl with a meal to solvent ratio of 1: 20. Madhusudhan and Singh (1985b) extracted protein from degummed, defatted and dehulled flaxseed meal with 1 M NaCl at pH 7.0 and obtained approximately 85% total protein. In their experiments, five different factors including pH, solvent to meal ratio, solvent composition, salt concentration and heat treatment were tested separately on protein solubility of defatted flaxseed meal (Madhusudhan and Singh, 1985b). The possible interactions among the five parameters were not evaluated in their experiments. Later, Oomah et al. (1994) maximized the solubility of protein in flaxseed meal by optimizing three operating conditions (pH, solvent to solid ratio and salt concentration). The optimum yield was 97% of flaxseed meal proteins using sodium phosphate buffer with 0.8M NaCl at pH 8.0. Also, Wanasundara and Shahidi (1994a) studied extraction of protein using various solvent systems, including hexane or 95% (v/v) alkanol (methanol, ethanol, isopropanol)

with or without 10% (w/w) ammonia. Most of the methods mentioned above are time-consuming procedures; thus, an objective of this work was to explore the possibility of co-extracting protein when extracting lignan using PLPW.

The quantification of total protein in food can be achieved by a colorimetric dye-binding protein assay or by a combustion method determining total organic nitrogen followed by conversion of total nitrogen into crude protein content using a suitable conversion factor (Camp and Dierckx, 2004).

The working principle of the combustion method involves converting all forms of nitrogen into gaseous nitrogen oxides by combustion in an oxygen-rich atmosphere at about 1000°C. The final step is the quantification of nitrogen gas by thermal conductivity (Buckee, 1994; Wiles et al., 1998). The total protein present can be calculated from the nitrogen content by multiplying percent nitrogen by a factor of 6.25, a factor corresponding to the average protein nitrogen content of 16% (Heidelbaugh et al., 1975; Hyvarinen et al., 2006; Oomah et al., 2006). This combustion technique has received official approval for cereal grains, barley and oilseeds, meat and dairy products (Bicsak, 1993; King-Brink and Sebranek, 1993; Buckee, 1994). Conversion of nitrogen into protein can only occur accurately if the nitrogen content of the protein fraction is known and if the food product includes no other nitrogen-containing matter besides protein. The presence of nonprotein nitrogen (NPN) compounds in foods, like ammonia, urea, and trimethylamine oxide, might cause overestimate of true protein nitrogen content

as derived from the current nitrogen determination methods. Hence, we also used a dye-binding method for comparing the results.

Dye binding-based procedures are official methods for dairy products, cereal grains, oil seeds, legumes and forage analyses (Osborne, 1986; Lakin, 1978). Bradford (1976) introduced a direct type of dye-binding assay. The working principle is the binding of Coomassie Brilliant Blue (CBB) G250 to protein which results in a change in colour of the dye from a reddish to a bluish colour. Since the colour reagent is unstable, controlling the precise incubation time for reagent addition is important if reproducible results are desired. The change in absorbance at 595 nm is proportional to the protein concentration of the sample (Lewis et al., 1980). With the selection of an appropriate standard (bovine serum albumin, BSA, 2 mg/mL), the assay can be both accurate and sensitive with a usable working range of 0.2-1.4 mg protein /mL for the standard assay and 5-100 µg protein/mL for the microassay (Sapan et al., 1999).

2.6.3 Extraction and quantification of carbohydrate

Flaxseed contains high levels of dietary fiber consisting of soluble and insoluble types (9 and 20%, respectively) (Daun et al., 2003). The soluble fiber, also referred to as flaxseed gum or mucilage, has been extracted with water at 85-90°C, a pH 6.5-7.0 and a water to seed ratio of 13:1 (Cui et al., 1994). This process yields a polysaccharide fraction of about 8% by weight of flaxseed. Kankaanpaa-Anttila and Anttila (1996) described a process for producing water-soluble carbohydrates from flaxseed meal. In

this process, the cold-pressed flaxseed meal was alkali extracted, the insoluble fiber was then separated and the mucilage was precipitated by ethanol.

The phenol-sulfuric acid method is a simple, rapid and reliable colorimetric technique to quantify the total soluble carbohydrates (Dubois et al., 1956). In this sugar determination method, oligosaccharides are hydrolyzed by concentrated sulfuric acid. Monomers such as glucose, fructose and galactose are formed and become the principal compounds measured in the sugar assay. The test is based on the reaction of carbohydrate with phenol in the presence of a strong acid (Nielsen, 1994). The phenol-sulfuric method is a colorimetric assay which involves two stages. The first step is the dehydration of carbohydrates to form furfural and hydroxyl methyl furfural with concentrated sulphuric acid. The second step is the condensation of these compounds with phenol to produce a yellowish orange colour. The colour intensity is proportional to the carbohydrate concentration. The absorbance is read at 490 nm. Standard curves were prepared and the result calibrated against a 1g/mL glucose standard solution equivalent. Different sugars give rise to different intensities of color, depending on the way in which they are dehydrated by the acid (Peris-Tortajada et al., 1992; Peris-Tortajada, 2004). The results obtained are then expressed as total carbohydrates.

2.7 Pressurized low polarity water extraction technique

Phytochemicals typically are not soluble in water under ambient conditions due to their organic nature and the preponderance of non-ionic/covalent bonds in their architectures (Gertenbach, 2002). However, they are readily soluble in various organic

solvents such as aliphatic alcohols, hexanes, dioxanes, acids, ethers, methyl chloride, trichloroethylene and acetonitrile (Wakelyn and Wan, 2003). Some methods use alkali or alkaline solvents in combination with organic solvents for extraction efficiency but extracts must be further processed to remove all trace of organic solvents. As mentioned earlier, use of organic solvents represents an environmental challenge and is not popular with government or environmental lobbying groups. On the other hand, water is a non-hazardous, non-toxic, chemically and thermally stable, non-flammable, consumer acceptable and inexpensive solvent. Also, in contrast to other solvents, water has special properties such as, ionic, hydrogen bonding, dipole-dipole forces that are strongly sensitive to temperature and pressure (Lewicki, 2004). Temperature can disrupt the strong solute-matrix interaction caused by hydrogen bonding and dipole attractions of the solute molecules. First, at elevated temperature, hydrogen bonding of water is weak. Second, dipole-dipole forces are attractive forces between the dipoles but can also be weakened dramatically with an increase of temperature (Cybulska and Doe, 2002).

At room temperature, water is a good solvent for charged or polar compounds but a relatively poor solvent to extract non-polar or hydrophobic compounds (eg. hydrocarbons) because water is a polar solvent and its dielectric constant is quite high (Mackay and Mackay, 1981; Clifford and Vandenburg, 2004). Hence, water favours the solubility of ionic and very polar compounds near room temperature. Dielectric constant (ε_r) can be harnessed as a measurement of polarity. The dielectric constant is a macroscopic property of the solvent that indicates the ability to reduce the interaction of particles with opposite charges and also determines the solvation characteristics of a

solvent (Carey and Sundberg, 1984; Carey, 1987). The dielectric constants of some common solvents (at 25°C) range from 2 for hexane to 21 for acetone, 24 for ethanol and 33 for methanol (Skoog and West, 1982; Lide, 1992). However, the polarity of pressurized water can be manipulated by temperature. The dielectric constant of water decreases significantly at elevated temperature and pressure (Basile et al., 1998: Clifford et al., 1999; Kubatova et al., 2001b). For instance, pure water at ambient temperature and pressure has an $\varepsilon_r = 79$ at 25°C while increasing the temperature to 250°C at a pressure of 5 MPa reduces ε_r to 27 as shown in Figure 2.3. This value is similar to that of ethanol at 25°C and 0.1 MPa (Vematsu and Franck, 1980). The ε_r value at 200°C is similar to that of methanol or acetonitrile (Richter et al., 1996; Yang et al., 1998). Thus, between 100 and 200°C, pressurized water (at 5 MPa/ 50 bar) behaves like a water-methanol mixture. As a result, the wide range of dielectric constants that can be generated using pressurized water makes it more attractive than non-polar supercritical CO_2 (ε_r= ~1 to 2) (Hawthorne et al., 2000a). The change in the ε_r value can be explained by the decrease in hydrogen bonding and the weaker intermolecular forces between water molecules at elevated temperature. The weakened hydrogen bond can affect the electric permittivity in water and hence affect the dielectric constant. Owing to this special property of water at temperatures above 100°C, selectivity for different analyte compound classes can be achieved by temperature selection (Kubatova et al, 2001a). On the contrary, organic solvent extractions generally show little or no compound class selectivity and fractionation is usually required after the extraction is complete (Hawthorne et al., 2000b). Figure 2.4 shows the effect of temperature on solvent surface tension for pure water at 5 MPa (50 bar) and for water mixed with methanol or acetonitrile at 25°C and

ambient pressure. Heating water to 250°C reduces its surface tension to about the same as 100% methanol or acetonitrile. Similarly, increasing temperature lowers viscosity of water as shown in Figure 2.5 (Yang et al., 1998). Therefore, change in temperature under pressure can affect the dielectric constant, viscosity and surface tension of water which modify its extraction capacity and renders it similar to that of methanol/water or acetonitrile/water mixtures.

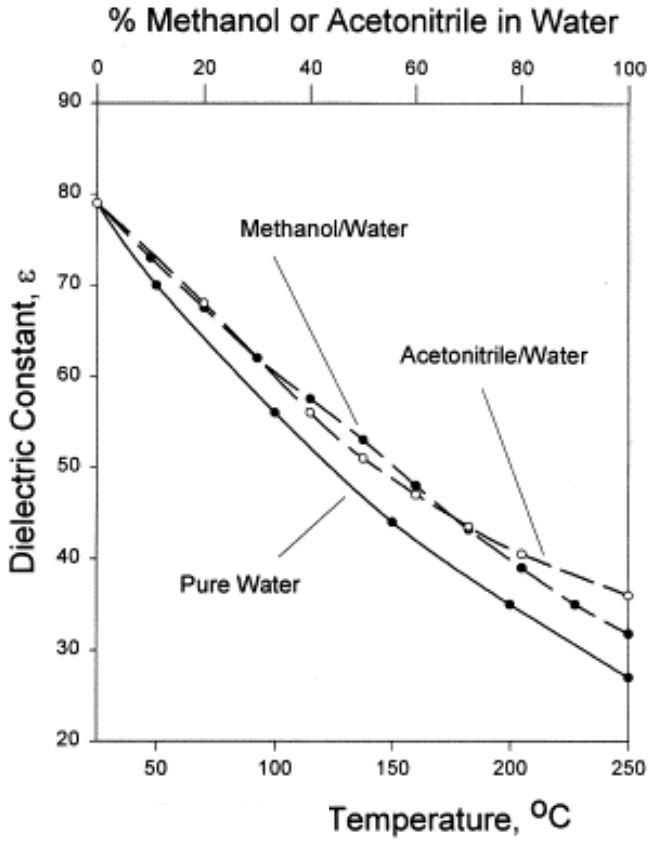


Figure 2.3. Dielectric constant of water, acetonitrile/water or methanol/water mixture as a function of temperature (adapted from Yang et al., 1998). Wata data from Haar et al. (1984), and the mixed solvent data from Melander and Horvath (1980).

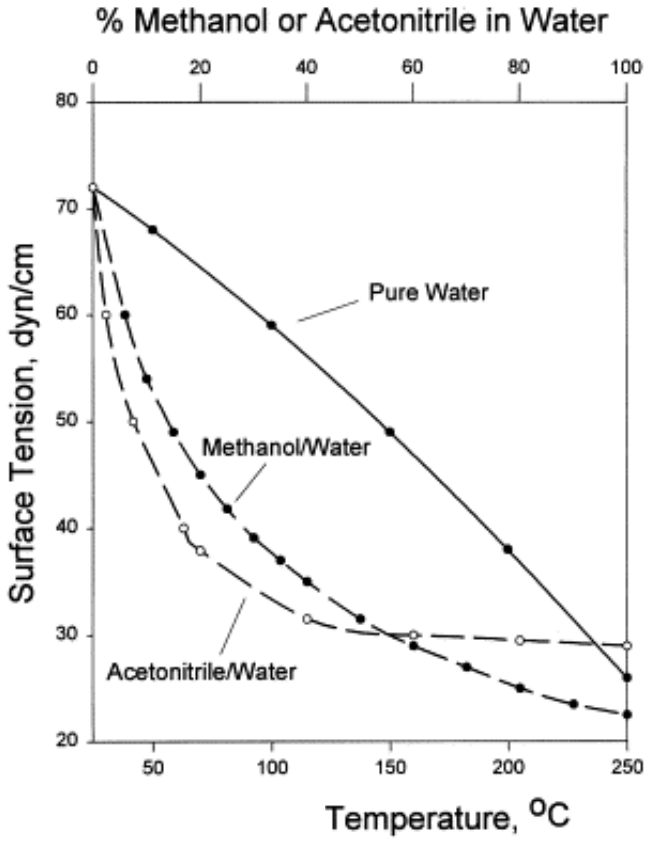


Figure 2.4. Surface tension of water, acetonitrile/water or methanol/water mixture as a function of temperature (adapted from Yang et al., 1998). Water data from Haar et al. (1984), and the mixed solvent data from Melander and Horvath (1980).

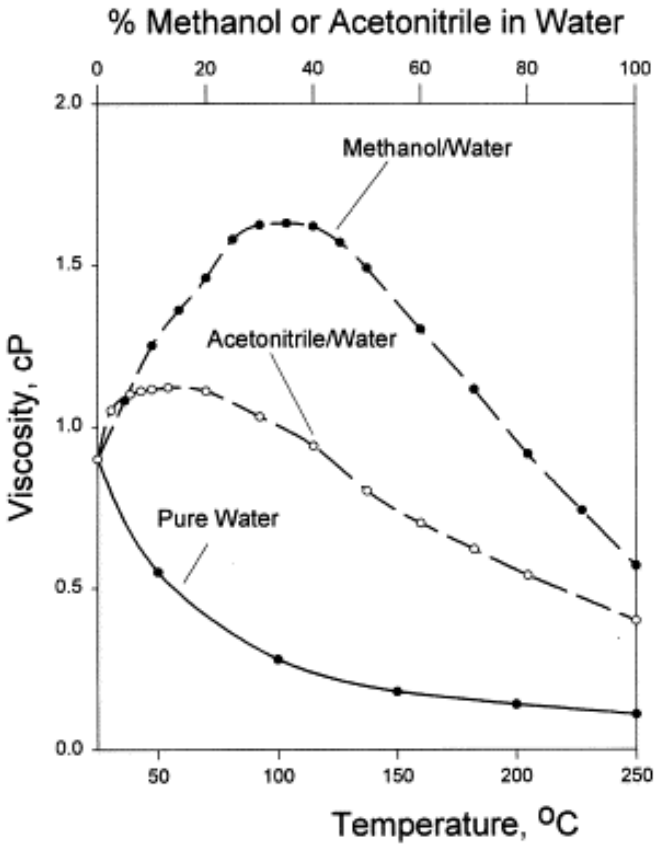


Figure 2.5. Comparison of viscosity of water, acetonitrile/water or methanol/water mixture by changing temperature (adapted from Yang et al., 1998). Water data from Haar et al. (1984), and the mixed solvent data from Melander and Horvath (1980).

Pressurized low polarity water (PLPW) extraction, also known as subcritical water extraction (or hot water extraction, pressurized hot water extraction, superheated water extraction or high-temperature water extraction) is a promising extraction and fractionation technique that uses liquid water under pressure. The critical temperature and pressure of water are shown as a phase diagram in Figure 2.6 (Tc=374°C, Pc=221 bar or 22 MPa). PLPW can be maintained in the liquid form up to a temperature of 374°C and a pressure of 22.1 MPa (221 bars) (as shown in the highlighted region in Figure 2.7) after which it becomes supercritical water (Haar et al., 1984; Hawthorne et al., 2002). A pressure of 5MPa (50 bars) would be high enough to prevent the water from vaporizing at temperatures from 100 to 250°C. A higher extraction pressure may be detrimental for the process because of a slight increase in the dielectric constant and a considerable increase in the cost of the equipment. For example, changing the pressure from 0.1 to 10 MPa gives an increase of ε of only 0.37 (Vematsu and Franck, 1980). Despite this, elevated pressure facilitates extractions by forcing the fluid into areas of the matrices (solubilization of air bubbles or pores) that would not normally be contacted by solvents under atmospheric conditions (Richter et al., 1996). Once pressure is high enough to keep water in liquid state, additional pressure is not necessary as it has limited influence on solvent characteristics of water as long as water remains in liquid state (Ayala and Castro, 2001). This phenomenon helps to simplify the optimization procedures since pressure can be skipped as a potential variable. Increasing the water temperature from 25°C to 250°C causes similar changes in dielectric constant, surface tension, and viscosity as those achieved by conventional mixing of methanol or acetonitrile with ambient water as illustrated in Figures 2.3, 2.4 and 2.5. Consequently, PLPW can easily

solubilize organic compounds such as phytochemcials, which are normally insoluble in ambient water.

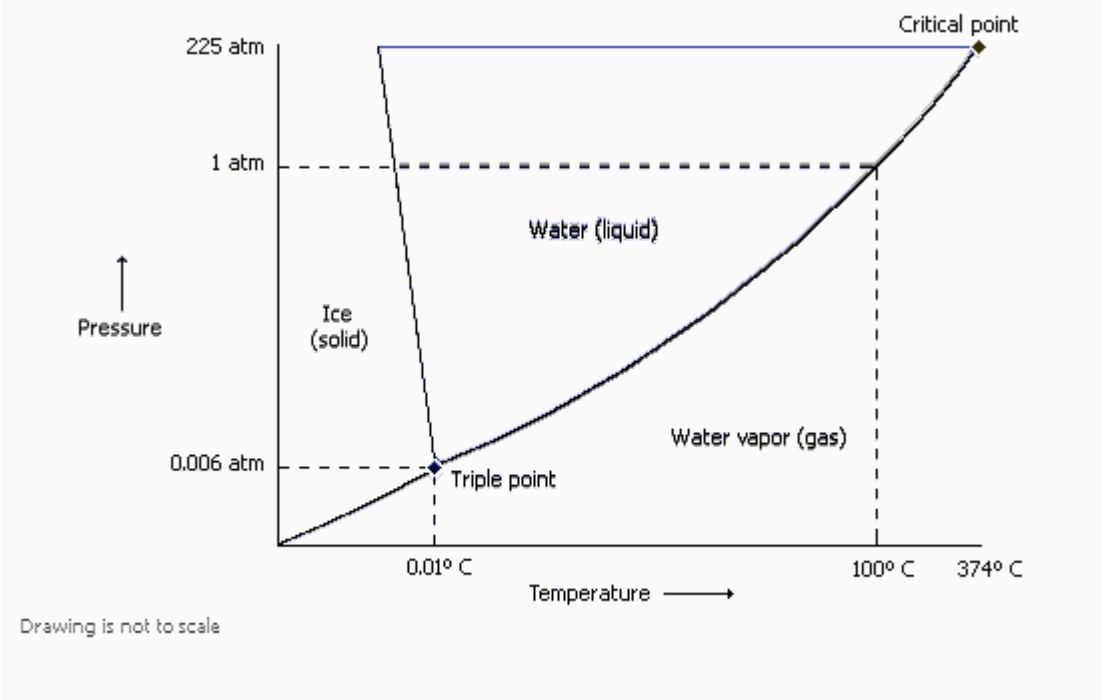


Figure 2.6. Phase diagram of water

Adapted from Haar et al., 1984

Figure 2.7. Pressure-enthalpy chart of water.

In functional foods and nutraceutical applications, pressurized low polarity water is an environmentally friendly solvent, in contrast with other organic solvents often used in extraction techniques. At present, more attention is being paid to functional foods development. Therefore, the demand for efficient methods to extract naturally occurring substances from food has arisen. This includes the extraction of biologically active components from plants and one of the recently studied materials is rosemary. Ibañez et al. (2003) described the use of subcritical water to extract antioxidant compounds from rosemary over a temperature range from 25 to 200°C. They found that the polar compound, rosmanol, was the main compound extracted at low temperatures while the capability of water to dissolve the relatively non-polar compounds increased at 200°C. Chen et al. (2004) also reported PLPW extraction of non-polar antioxidants from Taiwan Yams.

Ozel et al (2003) studied the extraction of essential oil from *Thymbra spicata*. The influence of several factors, such as temperature (100, 125, 150 and 175°C), pressure (2, 6 and 9 MPa) and flow rate (1, 2 and 3 mL/min) were investigated. It was shown that the best extraction yields (3.7%) were obtained at 150°C and 6 MPa, using a flow rate of 2 mL/min for 30 min. The essential oils of *Thymbra spicata* were found to inhibit mycelial growth of several fungi species (Ozel et al., 2003).

Ju and Howard (2003, 2005) studied the effect of temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin. Results showed that pressurized liquid extraction with water yielded recoveries of total phenolics and total anthocyanins at > 80°C comparable to those extracted by methanol at 60 and

128°C. Another study by Ong and Len (2004) showed that thermally liable components tanshinone I and IIA from *Salvia miltiorrhiza* used in Chinese medicine could be extracted using pressurized hot water.

The use of PLPW provides a number of advantages over traditional extraction techniques (i.e. hydrodistillation, organic solvent extraction). Some of the benefits include higher selectivity, cleanness, speed, low extraction times, higher quality of extracts (mostly essential oils) and lower costs of the extracting agent. PLPW demonstrated an advantage over traditional organic solvents in both extraction rate and energy cost in separating kava lactones (Kubatova et al., 2001a). Kubatova et al. (2001a) revealed that subcritical water yielded quantitative recovery of oxygenated flavour and fragrance compounds in less than 12 min compared to 4 h of hydrodistillation. Kubatova et al. (2001b) also compared the extraction of kava lactone efficiencies using organic solvents and subcritical water. Forty min of subcritical water extraction yielded essentially the same recoveries of kava lactones as 18 h of extraction with acetone, methylene chloride or methanol. Jimenez-Carmona et al. (1999) performed a comparison of continuous subcritical water extraction and hydrodistillation of marjoram leaf essential oil. They concluded that the subcritical water method was quicker and allowed substantial savings in terms of energy and plant material costs. Furthermore, a continuous subcritical water method was proposed by Ayala and Castro (2001) to isolate oregano essential oil. The yields from the extracts were higher after 15 min of subcritical water extraction than after hydrodistillation for 3 h. In addition, Ayala and Castro (2001)

mentioned that with subcritical water extraction, the composition of the oil extract may be manipulated by altering parameters such as temperature and flow rate.

Extraction using PLPW has also been compared with and was reported to be superior than supercritical carbon dioxide extraction. In this regard, Clifford et al. (1999) compared the extraction of clove bud oil by supercritical carbon dioxide and subcritical water. They found that total yields from both processes were approximately the same. Based on their results, supercritical carbon dioxide extraction consumed less solvent but the rate of extraction was higher using subcritical water. In the same study, Clifford et al. (1999) showed that the amount of eugenol and eugenyl acetate recovered using subcritical water at 150°C was similar to that achieved using Soxhlet extraction and hydrodistillation.

2.8 Modeling of PLPW extraction of bioactives from plant materials

Two models have been applied to describe the extraction mechanisms obtained with PLPW extraction of essential oil and polycyclic aromatic hydrocarbons (Kubatova et al., 2002). The first of these methods is based on the thermodynamic distribution coefficients (K_D), which assumes extraction is mainly governed by partitioning of solutes from matrix to water rather than by diffusion in the solid matrix. The second model is a two site kinetic model which emphasizes diffusion in the particle and assumes that extraction is limited by the diffusion of solutes from the matrix, and is not limited by the thermodynamic (K_D) partition that occurs during elution. The two models are defined by the following equations:

Thermodynamic model:

$$\frac{S_b}{S_0} = \frac{(1-\frac{S_a}{S_0})}{\left[\frac{K_D m}{(V_b - V_a)d}+1\right]} + \frac{S_a}{S_0} \qquad (2.1)$$

Kinetic model:

$$\frac{S_t}{S_0} = 1 - \left[Fe^{-k_1 t}\right] - \left[(1-F)e^{-k_2 t}\right] \qquad (2.2)$$

S_a: cumulative mass of the analyte extracted after certain amount of volume V_a (mg)

S_b: cumulative mass of the analyte extracted after certain amount of volume V_b (mg)

S_0: total initial mass of analyte in the matrix (mg)

S_t: mass of the analyte removed by the extraction fluid after time t (mg)

S_t/S_0: cumulative fraction of the analyte extracted after the time t

S_b/S_0 and S_a/S_0: cumulative fraction of the analyte extracted by the fluid of the volume V_b and V_a

t: extraction time (min)

F: fraction of the analyte released quickly

(1-F): fraction of the analyte released slowly

K_D: distribution coefficient; concentration in matrix/concentration in fluid

k_1: first-order rate constant describing the quickly released fraction (min^{-1})

k_2: first order rate constant describing the slowly released fraction (min^{-1})

d: density of extraction fluid at given condition (mg/mL)

e: exponential function

m: mass of the extracted sample (mg)

The two site kinetic model does not include solvent volume, but relies solely on extraction time. Therefore, doubling the extractant flow rate should have little effect on the extraction efficiency when plotted as a function of time. On the contrary, the thermodynamic model is only dependent on the volume of extractant used. Therefore, the extraction rate can be varied by changing the flow rate. Hence, the mechanism of thermodynamic elution and diffusion kinetics can be compared simply by changing the flow rate in PLPW extraction. If the concentration of bioactive compounds in the extract increases proportionally with an increase in flow rate at given extraction time when the solute concentration is plotted versus extraction time, the extraction mechanism can be explained by the thermodynamic model. However, if an increase in flow rate has no

significant effect on the extraction of the bioactive compounds, with the other extraction parameters being kept constant, the extraction mechanism can be modeled by the two site kinetic model (Cacace and Mazza, 2005; Kubatova et al., 2002). The mechanism of control and hence the model valid for PLPW extraction may be different depending on the raw material, the target analyte and extraction conditions.

Apart from kinetic modeling, the solute migration model also obeys Fick's law as a governing equation when internal diffusion is the rate limiting step. The diffusion of solute into the solvent can be modelled with Fick's second law for spherical particles (Hulbert et al., 1998):

$$\frac{\partial C}{\partial t} = D_e\left(\frac{\partial^2 C}{\partial r^2}\right) \qquad (2.3)$$

where C is concentration of solute in the flaxseed meal (mg/mL), D_e is effective diffusion coefficient (m^2/s) and r is radial distance from the centre of the particle (m).

The model is based on the hypothesis that there is no axial dispersion in the bed and therefore, a linear gradient of concentration exists along the extraction vessel. This model supposes the bed is formed by spherical particles of uniform size. Other assumptions include: the particle diameter corresponds to the average diameter of sample particles; the diffusion of individual solute and other compounds are carried out in parallel and there are no interactions between them; the diffusivity of the extracted compound is independent of particle radius; the controlling stage is internal diffusion; D_e is constant with the concentration.

Crank (1975) has given the solution to Fick's second law for spherical particle:

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=1}^{\infty} \frac{6}{\pi^2 n^2} \exp(-\frac{\pi^2 n^2 D_e t}{r^2}) \qquad (2.4)$$

where: M_t = total amount of solute (mg solute/g meal) removed from flaxseed meal after time t, M_∞ = maximum amount (mg solute/g meal) of solute extracted after infinite time. M_t/M_∞ = ratio of total migration to the maximum migration concentration, r = average radius of a flaxseed meal particle.

Two site kinetic model and Fick's second law were used to fit experimental data obtained from experiments 1 and 2. Results from diffusion and two site kinetic models will be presented in Chapter 5.

CHAPTER 3

Pressurized low polarity water extraction of lignans, proteins and carbohydrates from flaxseed meal: optimization of temperature, pH and solvent to solid ratio and amount of co-packing materials[1]

3.1 Introduction

Phenolic compounds are reducing agents that protect the body's tissues against diseases associated with oxidative stress, such as cancers and coronary heart diseases. Dietary sources of phenolics are beneficial for disease prevention. Flaxseed is one dietary source which contains a considerable amount of phenolics, namely lignans, that have beneficial health effects (Thompson, 2003). Lignans are important plant chemicals that have been shown to reduce the risk of certain cancers (Thompson et al., 1996a). Therefore, incorporation of flaxseed lignans into foods is particularly attractive from the perspective of development of functional foods with specific health advantages.

Organic solvents are commonly used industrially to extract natural food components. In many food processing operations, organic solvents are employed based on the polarity, solubility and mass transfer characteristics of the compounds to be extracted. Flaxseed lignans have traditionally been extracted using organic solvents, particularly ethanol and acetone (Westcott and Muir, 1998), and proteins and carbohydrates have been extracted with NaCl solutions and water, respectively (Mazza and Biliaderis, 1989; Oomah et al., 1994).

[1]Part of this chapter has been submitted for publication: Ho, C. H. L., Cacace, J. E., and Mazza, G. (2006). Extraction of lignans from flaxseed meal with pressurized low polarity water. *Lebensmittel Wissenschaft und Technologie.* (submitted).

The use of solvent extraction has the disadvantages of long extraction time, labour intensive procedures and toxic waste generation (Choi et al., 2003). Industry is being challenged to comply with tightened environmental regulations and is responding by finding alternatives to reduce organic solvent consumption. In addition, the public is more sensitive to food security, safety and quality, and is demanding more information about how food is produced and processed.

Water can be used to replace organic solvents. It can be treated as a multi-polarity solvent by manipulating its temperature and pressure. Pressurized low polarity water (PLPW) is a promising extraction and fractionation technique that uses hot liquid water under pressure. Working temperatures below the critical value of water (374°C) but above 100°C are usually employed. At this temperature range, water is not highly compressible and the pressure does not have much effect as long as it is high enough to prevent water from vaporizing (Hawthorne et al., 2002). Increasing the water temperature from 25°C to 250°C causes similar changes in solvent polarity, surface tension, and viscosity as those achieved by conventional mixing of methanol or acetonitrile with water (Yang et al., 1998). Water dielectric constant is also reduced with increasing temperature resulting in reduction of polarity (Hawthorne et al., 1999). Hence, PLPW can improve the extraction of both polar and relatively non-polar compounds from plant matrices by altering temperatures of water.

PLPW has shown potential for the extraction of selected plant components; and it has been used as a HPLC analytical solvent to extract and quantify caffeine, chlorophenols and anilines (Li et al., 2000). PLPW has also been applied as an analytical technique to extract flavour and fragrance compounds from rosemary, clove (Basile et al.,

1998; Rovio et al., 1999); isoflavones from defatted soybean flakes (Li-Hsun et al., 2004); ginsenosides from American ginseng (Choi et al., 2003), catechins, epicatechin and proanthocyanidins from tea leaves and grape seeds (Piñeiro et al., 2004; Garcia-Marino et al., 2006); cedar oil from eastern red cedar (Eller and Taylor, 2004); essential oil from oregano (Ayala and Castro, 2001); floral oil from *Rosa canina* (Ozel and Clifford, 2004); anthraquinones (antibacterial, antiviral and anticancer compounds) from roots of *Morinda citrifolia* (Shotipruk et al., 2004); and the low polarity pharmaceutical compound, nifedipine, for use in coronary artery-relaxing tablets (Richter et al., 2006).

Recently, Cacace and Mazza (2005) successfully extracted lignans and other phenolics from whole flaxseed with PLPW at temperatures ranging from 100 to 160°C. However, there are no published studies that have utilized PLPW for the extraction of lignans from flaxseed meal (which has been shown to contain about twice as much lignans as that in whole flaxseed) (Eliasson et al., 2003; Johnsson et al., 2000).

Flax is a major Canadian crop grown essentially for industrial (linseed) oil. The material remaining after the extraction of oil from flaxseed is flaxseed meal which is largely used as livestock feed. Flaxseed is one of the most concentrated sources of the plant lignan precursor secoisolariciresinol diglucoside (SDG), which is present at 75-800 times the amount found in other foods (Mazur, 1998).

Variables such as temperature, pH, flow rate, sample quantity, solvent to solid ratio were reported to affect the efficiency of the extraction process (Pinelo et al., 2005; Turker and Erdogdu, 2006). The objective of the present study (Exp 1 Chapter 3) was to optimize the extraction of lignan SDG from flaxseed meal using PLPW. The selectivity and recovery of PLPW were tested at several different temperatures, pHs, amount of co-

extraction packing materials and solvent to solid ratio. The effects of extraction conditions on other co-extracted components such as proteins and carbohydrates were also investigated by measuring their concentrations in the extracts. Since optimum process values for extractions of SDG, protein and carbohydrate were different, only optimal process values obtained from Exp 1 (Chapter 3) for lignan SDG will be applied to the next optimization experiment (Exp 2; Chapter 4).

3.2 Materials and methods

Flaxseed meal (cultivar NorMan) was obtained from Flora Manufacturing and Distribution Ltd., Burnaby, B.C. The meal was produced by cold pressing flaxseed and was milled to pass through a 1.65 mm screen. The meal was sealed and stored in a freezer at -25°C before use. The proximate composition of defatted meal was determined. Residual oil in the defatted meal was determined by Goldfisch extraction with petroleum ether for 6 h. Moisture and protein (N*6.25) contents were determined by standard methods (Horwitz, 2000, 2003). Ash was determined based on the gravimetric loss by heating the meal at 525°C for 8 h (St John, 1943).

3.2.1 Reagents and standards

The chemicals and reagents used were: citric acid monohydrate, glucose standard solution 1mg/mL, Bradford reagent as a protein dye, sodium carbonate (Sigma, St. Louis, MO); sodium hydrogen phosphate, sodium hydroxide (Fisher Scientific, New Jersey); sodium hydrogen carbonate (The British Drug Houses Ltd, Poole, England); sulphuric acid, phenol (BDH Inc., Toronto, Ontario); albumin standard, BSA, 2mg/mL (Pierce, Rockford, IL); and SDG standard (Chromadex, Santa Ana, CA).

3.2.2 Pressurized low polarity water extraction

A schematic diagram of the pressurized low polarity or subcritical water extraction apparatus used for this study is presented in Figure 3.1. The system consisted of a HPLC pump (Model 510, Waters, Milford, MA), preheating coil, extraction cell, temperature-controlled oven (5700A Series, Hewlett-Packard, Palo Alto, CA), cooling coil, back pressure regulator with a cartridge of 5.17 MPa (750 psi or 52 bar) (Upchurch Scientific, Oak Harbor, WA), and a collection vessel. All connection tubing was made from stainless steel (1.59 mm o.d.x 0.762 mm i.d.). A 3 m preheating coil was put inside the oven leading to the extraction cell. A relief valve and a thermocouple were connected to avoid excess liquid pressure buildup and to monitor the actual solvent temperature, respectively. A 1 m length of tubing outside the GC oven that ran inside a beaker with room temperature water served as a cooling coil to reduce extract temperature. A shut-off valve (HIP 15-11AF1, High pressure Equipment Co., Erie, PA, USA) was placed outside the oven between the back pressure regulator and the tube outlet to maintain high pressure and avoid losses of extract during the heat-up period of the oven. All system components were rated for at least 34 MPa (4900 psi).

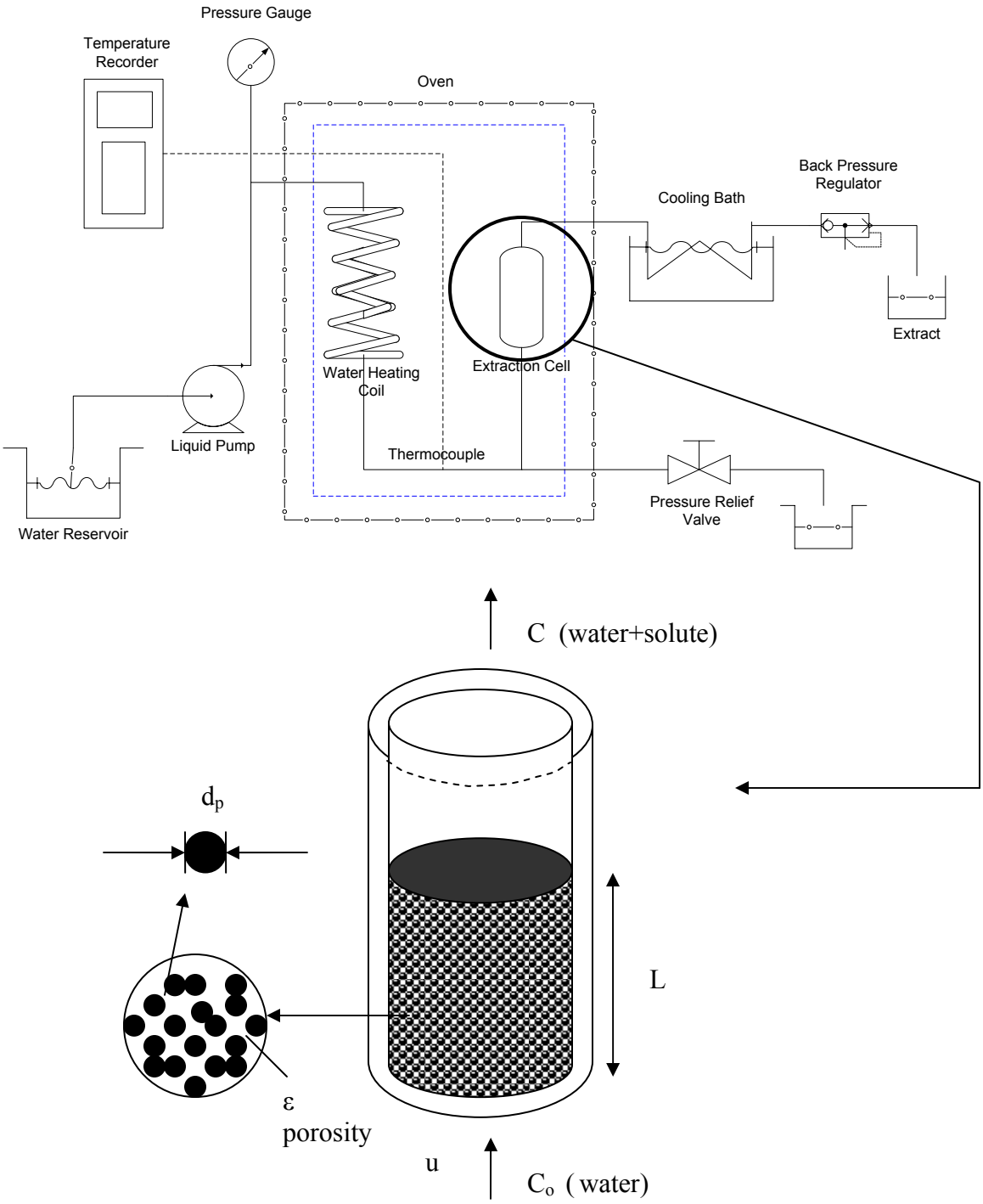


Figure 3.1. Pressurized low polarity water extraction diagram with characteristic dimensions and geometry of the packed bed extraction vessel

All extractions were carried out in a stainless steel cylindrical extraction cell of 10.6 mm i.d. (1/2 inch o.d.) and 10 cm length with chromatography-column end fittings (Chromatographic Specialties Inc, ON, Canada). The cell was equipped with a 10 μm stainless steel frit at the inlet, and a 50 μm frit at the outlet. The larger pore size frit was installed at the outlet to prevent flaxseed meal particle from clogging the frit. Layers of glass wool were placed inside the cell to keep the sample at a fixed bed depth and to avoid blockage of solvent flow under pressure. The extraction cell was loaded with 2 g flaxseed meal and mounted vertically in the oven with solvent flowing from bottom to top. A total of 3 g glass beads (3 mm in diameter) were mixed with the samples to incorporate 1:1.5 ratio of meal to glass beads in selected runs according to the experimental design (Table 3.1). In this experiment, buffered water was required to ensure pH stability. Citric acid and disodium hydrogen phosphate made up both pH 4 (0.1 M) and pH 6.5 (0.1 M) buffers. Sodium carbonate and sodium bicarbonate constituted the pH 9 buffer (0.25 M). The buffer concentration calculations are shown in Appendix 1.

Table 3.1. Optimization of four variables using a mixed level fractional factorial design

Run	Temp (°C)	Solvent to solid ratio (mL/g)	pH	Packing (g)
1	130 (-1) [a]	90 (-1)	4 (-1)	0 (-1)
2	130 (-1)	90 (-1)	4 (-1)	3.0 (+1)
3	130 (-1)	90 (-1)	9 (+1)	0 (-1)
4	130 (-1)	90 (-1)	9 (+1)	3.0 (+1)
5	130 (-1)	210 (+1)	4 (-1)	0 (-1)
6	130 (-1)	210 (+1)	4 (-1)	3.0 (+1)
7	130 (-1)	210 (+1)	9 (+1)	0 (-1)
8	130 (-1)	210 (+1)	9 (+1)	3.0 (+1)
9	160 (0)	90 (-1)	4 (-1)	0 (-1)
10	160 (0)	90 (-1)	4 (-1)	3.0 (+1)
11	160 (0)	90 (-1)	9 (+1)	0 (-1)
12	160 (0)	90 (-1)	9 (+1)	3.0 (+1)
13	160 (0)	210 (+1)	4 (-1)	0 (-1)
14	160 (0)	210 (+1)	4 (-1)	3.0 (+1)
15	160 (0)	210 (+1)	9 (+1)	0 (-1)
16	160 (0)	210 (+1)	9 (+1)	3.0 (+1)
17	190 (+1)	90 (-1)	4 (-1)	0 (-1)
18	190 (+1)	90 (-1)	4 (-1)	3.0 (+1)
19	190 (+1)	90 (-1)	9 (+1)	0 (-1)
20	190 (+1)	90 (-1)	9 (+1)	3.0 (+1)
21	190 (+1)	210 (+1)	4 (-1)	0 (-1)
22	190 (+1)	210 (+1)	4 (-1)	3.0 (+1)
23	190 (+1)	210 (+1)	9 (+1)	0 (-1)
24	190 (+1)	210 (+1)	9 (+1)	3.0 (+1)
25	160 (0)	150 (0)	6.5 (0)	0 (-1)
26	160 (0)	150 (0)	6.5 (0)	0 (-1)
27	160 (0)	150 (0)	6.5 (0)	0 (-1)
28	160 (0)	150 (0)	6.5 (0)	3.0 (+1)
29	160 (0)	150 (0)	6.5 (0)	3.0 (+1)
30	160 (0)	150 (0)	6.5 (0)	3.0 (+1)

[a] Numbers in parentheses are coded values of variables in the experimental design.

Extraction procedures began by filling the cell with water, pressurizing and then heating. Deionized ultrapure buffered water (Millipore Corporation, Milli-Q Plus, Bedford, MA) was degassed with nitrogen for 30 min prior to extraction to remove dissolved oxygen from the water. With the outlet valve closed, water was pumped through the preheating coil at a constant flow of 1mL/min to fill and build the pressure inside the system to around 10.3 MPa (1500 psi). After completing a leak check, the outlet was opened and the oven was heated to the desired temperature. The start of the extraction (time = 0) was set at the moment the oven reached the desired temperature and then the pump was started to deliver the solvent at the required flow rate. The system pressure at 5.2 MPa (750 psi) was controlled by the back pressure regulator throughout the extraction. After the extract started to come out of the oven, the lignan-rich extract was cooled and transferred to a collection vial. The extractions were dynamic and were performed to collect sequential extract volumes based on cumulative extraction time intervals. The total extraction time ranged from 180 to 420 min. A clear advantage of dynamic extraction is that the water is cooled outside of the extraction cell, avoiding the possibility of re-adsorption of the analytes on the solid matrix.

When the extraction was completed, outlet valves were closed to keep the pressure around 2.1 MPa (300 psi) while cooling the oven to prevent water from vaporizing when temperature decreased (while water was still above its boiling point of 100°C). The residual water trapped in the cell was then purged with nitrogen gas. At the end of the extraction, the cell was removed and the inside of the tubing was washed with about 50 mL of 50:50 (v/v) ethanol/tetrahydrofuran (EtOH/THF) solvent mixture, and then rinsed with 100 mL of Milli-Q water. The wash solvent collected was concentrated

by evaporation under nitrogen flow or by vacuum rotary evaporator. The wash extracts were kept separate from the main extracts. Extracts collected from each experiment were stored at -25°C. The solid residues (extracted flaxseed meal) were removed from the cell, weighed, dried in a vacuum oven at 60°C for 24 h, reweighed and ground before analysis. The average particle size of flaxseed meal was determined using a sonic sifter separator equipped with 6 sieves ranging from 38 to 850 μm in size.

3.2.3 Analysis of lignans

Lignan contents in extracts and residues were measured by the direct hydrolysis method of Eliasson et al. (2003) as modified and described by Cacace and Mazza (2005) (Fig 3.2). For the analysis of solid samples, 0.5 g of flaxseed meal or extracted residue was weighed out into 25 mL flasks and then mixed with 1mL of methanol, 4 mL of distilled water, and 5 mL of 2N NaOH. For the analysis of liquid samples, 4 mL of aqueous extract was used, and no distilled water was added to the methanol + 2N NaOH mixture. The flasks or test tubes were sealed and shaken at room temperature on an orbital shaker for 1 h. Each hydrolysate was subsequently acidified with the addition of 5mL of 2N H_2SO_4. Calculations used for preparation of standard NaOH and H_2SO_4 solutions are shown in Appendix 2. The neutralized mixtures were then centrifuged at 11000x g for 10 min and the supernatants were collected. To each of two microcentrifuge tubes, 0.6 mL liquid phase from the supernatant was added and then mixed with 0.9 mL of 100% methanol. The solution was allowed to sit for 30 min at room temperature before re-centrifuging for 5 min at 11000x g in order to precipitate and remove water-soluble polysaccharides and proteins. The supernatant was then filtered through a 0.45 μm filter and analyzed by HPLC.

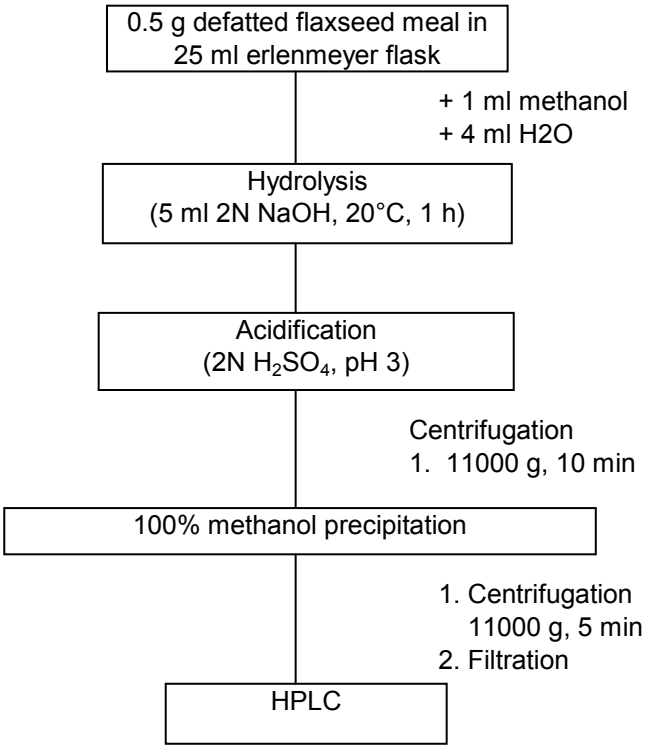


Figure 3.2. Direct alkaline hydrolysis procedure for flaxseed lignans

3.2.4 High performance liquid chromatography analysis of lignans

Analysis of lignan SDG was performed using a Waters HPLC system (Waters Corp., Milford, MA) equipped with a Waters Model 600 pump, a 717 plus autosampler, an Agilent 1100 degasser, and a 996 photodiode array detector. The system was run using Empower software. The procedure of Muir and Westcott (2000) was followed with minor modifications as described by Cacace and Mazza (2005). An injection volume of 20 µL of the hydrolyzed extracts was loaded and separated using a Luna C18, 5 µm, 100 Å, 250x3.00 mm column with a C18 Security Guard cartridge (Phenomenex, Torrance, CA, USA). The separation was done at 30°C. The mobile phases consisted of 0.025% TFA (trifluoroacetic acid) in water (Solvent A) and methanol (Solvent B). The solvent flow was 0.8mL/min, and a linear gradient elution was followed with 80% A and 20% B at t=0 min, 30% A and 70% B at t=44 min, 30% A and 70% B at t=46 min, 80% A and 20% B at t=52 min, and 80% A and 20% B at t=70 min. Peaks were detected at 280 nm. SDG was identified and quantified by comparison to an SDG standard obtained from ChromaDex (Santa Ana, CA).

3.2.5 Protein and total carbohydrate determinations

Protein analyses were performed by the Bradford method for liquid extracts (Bradford 1976), and by the total nitrogen method for solid residues and flaxseed meal samples (Sweeney and Rexroad, 1987). Percentages of total nitrogen were converted to protein values (%w/v) by multiplying measured percentages by a factor of 6.25 (Dev and Quensel, 1988; Hyvarinen et al., 2006). Total carbohydrates (in aqueous extracts) were determined by the phenol-sulphuric acid colorimetric assay method (Dubois et al., 1956).

The absorbance was read at 490 nm. Standard curves were prepared and the results were calibrated against a glucose standard solution up to 1 g/mL.

3.2.6 Experimental design

The selected experimental design was a mixed-level fractional factorial design for four factors (three factors at two levels and one factor at three levels) (Haaland, 1989) (Table 3.1). The four factors (independent variables) were temperature, pH, solvent to solid ratio, and amount of co-packing material. A uniform bed depth, constant velocity (flow rate/area) and identical weight of glass beads-to-flaxseed meal ratio were used in the extractions. The actual and coded (inside bracket) values of the factors used in the experimental design are given in Table 3.1. The experimental design consisted of 30 runs including three replications at the centre points for each packing and non-packing level. One or two runs were performed each day.

3.2.7 Statistical analysis

Data were analyzed using the (General Linear Model) GLM procedure of SAS (SAS Institute Inc. 1990) to fit the following second order polynomial equation.

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{1} \beta_{ii} X_i^{\,2} + \sum_{i=1}^{4} \beta_{ij} X_i X_j$$

where $\beta_0, \beta_i, \beta_{ii}, \beta_{ij}, \beta_{ijk}$ are constant and regression coefficients of the model. X_i and X_j are the independent variables in coded values. The model developed by a four factor factorial design was used to analyze the main effects, two factor interactions and one quadratic effect. PROC GLM was adopted as it included a CLASS statement that allows

differentiation in the order of explanatory variables analysis (0 and 3 g packing). PROC GLM produced the parameter estimates, their standard errors, and statistics to access the model fit. It also provided the coefficient estimates for the regression model. A goodness of fit test of the model was performed with a regression (REG) procedure by backward elimination to keep variables significant at the 0.1% level. Response surface plots were drawn using the predicted value from the fitted model by imposing a constant value on one of the least significant independent variables.

3.3 Results and discussion

3.3.1 Particle size distribution and composition

The average size corresponding to a passing weight probability of 50% in the plot of particle size distribution was found to be 223.5 μm (0.22 mm). The calculations for particle size determination are shown in Appendix 3.

Proximate composition of flaxseed meal is shown in Table 3.2. Protein, lipid, ash and moisture were 33.3, 12.4, 5.5, and 5.8% respectively. Carbohydrate content (43%) was calculated by difference, as the remaining proportion in flaxseed meal. Results are comparable to values reported by Bhatty and Cherdkiagtumachai (1990).

Table 3.2. Proximate composition of defatted flaxseed meal[a]

Sample	Protein [b] %	Lipid [c] %	Carbohydrates %	Ash [d] %	Moisture [e] %
1	33.34	12.30	43.07	5.47	5.82
2	33.25	12.38	43.27	5.53	5.58
3	33.34	12.38	42.84	5.49	5.95
4	33.30	12.40	42.97	5.50	5.84
5	33.32	12.52	42.90	5.50	5.76
Avg ± SD	33.31 ± 0.05	12.39 ± 0.08	43.01± 0.17	5.50 ± 0.02	5.79 ± 0.18

[a] Amounts are expressed in percentage of weights

[b] measured as total nitrogen percentage by combustion with a thermal conductivity detector multiplied by 6.25

[c] measured by Goldfisch lipid extraction

[d] measured by burning at 525°C for 8h

[e] measured by AOAC Official Method (925.09), moisture determination (Horwitz, 2000)

3.3.2 Effect of co-packing material

The use of co-packing glass beads had a significant effect on all dependent variables as determined by ANOVA ($p<0.1$) (Table 3.3). Extraction of lignans, proteins and carbohydrates with PLPW was positively affected by the addition of 3 g glass beads to 2 g flaxseed meal as packing in the cell compared to flaxseed meal without glass beads (Figures 3.3, 3.4 and 3.5A,B). During leaching of compounds from plant material in solid-liquid extraction, the mass transfer between two phases was found proportional to their interfacial area (Kirwan, 1987). The degree of increase in solute yield, however, differed depending on time and temperature of the extraction. The overall lignan yield increased from 10% (47 mg/g meal) to 50% (11 mg/g meal) at all temperatures tested with the addition of 3 g glass beads (Figure 3.3). These results are consistent with those of Bjorklund et al. (1998) who investigated the effect of different co-packing materials on mass transfer, and found that glass or stainless steel beads gave 20% better recovery than other packing materials such as sea sand. The inserted glass beads in this experiment maintained spacing and dispersal of the flaxseed meal throughout the vessel thereby preventing water channeling and migration of plant material against the outlet frits. Glass beads had a greater promoting effect at lower temperatures (130°C) as shown in Figures 3.3 and 3.4. This phenomenon could be explained by the fact that lower temperature reduced the mass transfer process and that co-packing glass beads compensated for the reduction by providing more contact surface area for solvent penetration and diffusion. Meanwhile, PLPW at higher temperatures (160-190°C) successfully extracted most of the analytes from the sample matrices, and under these conditions the effect of co-packing became minor. Given that co-packing material was treated as a categorical variable in

this experiment, and its effect on the yield of lignans, proteins and carbohydrates was significant (Table 3.3), the regression analysis was subdivided exclusively for runs with co-packing beads using a CLASS sorting statement within PROC GLM in SAS (SAS Institute Inc. 1990). The corresponding regression coefficients fitting the polynomial second-order model for yields of lignans, proteins and total carbohydrates obtained from extractions with 3 g co-packing materials are presented in Table 3.4. The coefficients shown in Table 3.4 were calculated by goodness of fit to remove the least significant parameters in the model; and these coefficients were then used to predict the outcome yields between the experimental range of the process variables (130-190°C, pH 4-9, solvent to solid ratio 90-210 mL/g). A response surface diagram at constant 3g packing was obtained using predicted values (estimable regression coefficients) from the fitted polynomial model (Table 3.4) to illustrate the effect of temperature, solvent to solid ratio and pH on quantitative recovery of the target compounds (lignans, proteins and carbohydrates). The HPLC chromatogram (Figure 3.6A) shows the base-hydrolyzed extract from raw flaxseed meal. The peak eluting at 30 min retention time was identified as SDG by comparison with the authentic standard. The peak showed the corresponding absorption maxima at 280 nm indicative of SDG (Figure 3.6B). Other compounds reported to be present in flaxseed include p-coumaric acid glucoside, ferulic acid glucoside, pinoresinol glucoside, isolariciresinol, matairesinol, and derivatives of other phenolic acids, eg., p-hydroxybenzoic, gentisic, vanillic, and sinapic acids in free and/or bound forms (Johnsson et al., 2000). Identifications of those compounds were not pursued in this study. The result of the HPLC analysis showed that samples extracted with 3 g co-packing glass beads provided a higher SDG peak area (Figure 3.7A) than the

glass beads-free flaxseed meal sample (Figure 3.7B). This indicates that higher amounts of SDG were extracted in the presence of glass beads.

Table 3.3. Extraction yields and analysis of variance for lignans, proteins and carbohydrates

Run	SDG		Proteins				Total carbohydrates	
	Amount [a]	Yield [b]	Amount [c]	Yield [b]	Amount [d]	Yield [b]	Amount [e]	Yield [b]
	mg/g	%	mg/g	%	mg/g	%	mg/g	%
1	7.5	35.7	104.9	31.5	95.6	28.7	112.5	26.2
2	10.7	51.0	120.1	36.1	132.4	39.8	148.7	34.6
3	6.4	30.4	113.0	33.9	80.5	24.2	54.3	12.6
4	13.0	61.9	167.3	50.2	151.8	45.6	125.0	29.1
5	13.3	63.3	48.4	14.5	185.8	55.8	171.4	39.9
6	16.5	78.6	59.4	17.8	210.4	63.2	205.0	47.7
7	11.3	54.0	123.8	37.2	190.0	57.1	120.2	28.0
8	18.5	88.4	196.9	59.1	259.8	78.0	190.3	44.3
9	16.5	78.7	98.9	29.7	227.3	68.2	191.1	44.4
10	18.4	88.0	101.5	30.5	259.2	77.8	201.9	47.0
11	12.9	61.8	135.8	40.8	181.0	54.4	112.5	26.2
12	15.7	74.9	189.0	56.8	227.5	68.3	135.2	31.5
13	14.7	70.0	19.6	5.9	223.5	67.1	114.0	26.5
14	17.6	84.3	30.9	9.3	199.7	60.0	208.7	48.5
15	19.2	91.9	225.9	67.9	283.1	85.0	143.9	33.5
16	20.8	99.1	222.6	66.8	294.0	88.3	164.2	38.2
17	16.8	80.2	15.9	4.8	276.7	83.1	135.5	31.5
18	17.3	82.4	97.2	29.2	258.0	77.5	161.5	37.5
19	18.7	89.5	288.4	86.6	290.2	87.2	125.0	29.1
20	20.3	96.9	261.3	78.5	300.1	90.1	154.2	35.9
21	17.8	85.1	17.4	5.2	204.1	61.3	123.4	28.7
22	18.0	86.0	20.6	6.2	119.1	35.8	138.1	32.1
23	20.1	96.0	191.1	57.4	235.7	70.8	145.4	33.8
24	20.5	97.9	208.7	62.7	220.9	66.3	153.5	35.7
25	16.9	80.7	116.3	34.9	264.6	79.5	139.8	32.5
26	17.8	84.8	167.4	50.3	285.8	85.8	148.6	34.6
27	18.3	87.4	143.5	43.1	291.9	87.7	162.7	37.8
28	19.8	94.6	203.2	61.0	318.2	95.6	176.0	40.9
29	19.5	93.3	203.7	61.2	313.4	94.1	181.6	42.2
30	19.5	93.1	190.3	57.2	314.5	94.4	183.3	42.6
Model	*** [f]		***		***		***	
R^2	0.9076		0.8613		0.8581		0.8080	
Effects								
X_1 [g]	***		NS		***		***	
X_2	***		NS		***		**	
X_3	*		**		NS		***	
X_4	**		*		**		*	
X_1^2	***		NS		***		***	
X_1*X_2	***		NS		***		***	
X_1*X_3	NS		***		NS		**	
X_1*X_4	***		NS		**		*	
X_2*X_3	*		*		**		**	
X_2*X_4	NS		NS		NS		NS	
X_3*X_4	NS		NS		NS		NS	

[a] Lignan amount in mg per g of flaxmeal expressed as SDG equivalents

[b] Compound yields in weight percentage of total content in flaxseed meal

[c] Protein amount in mg/g of flaxmeal measured by Bradford BSA dye-binding assay

[d] Protein amount in total nitrogen percentage by combustion (%N x 6.25)

[e] Total carbohydrate amount in mg/g flaxmeal expressed as glucose equivalents

[f] ***Significant at 0.01 level, **significant at 0.05 level, *significant at 0.1 level, NS non significant (p>0.1)

[g] X_1=Temp, X_2=Solvent to solid ratio, X_3=pH, X_4=Packing

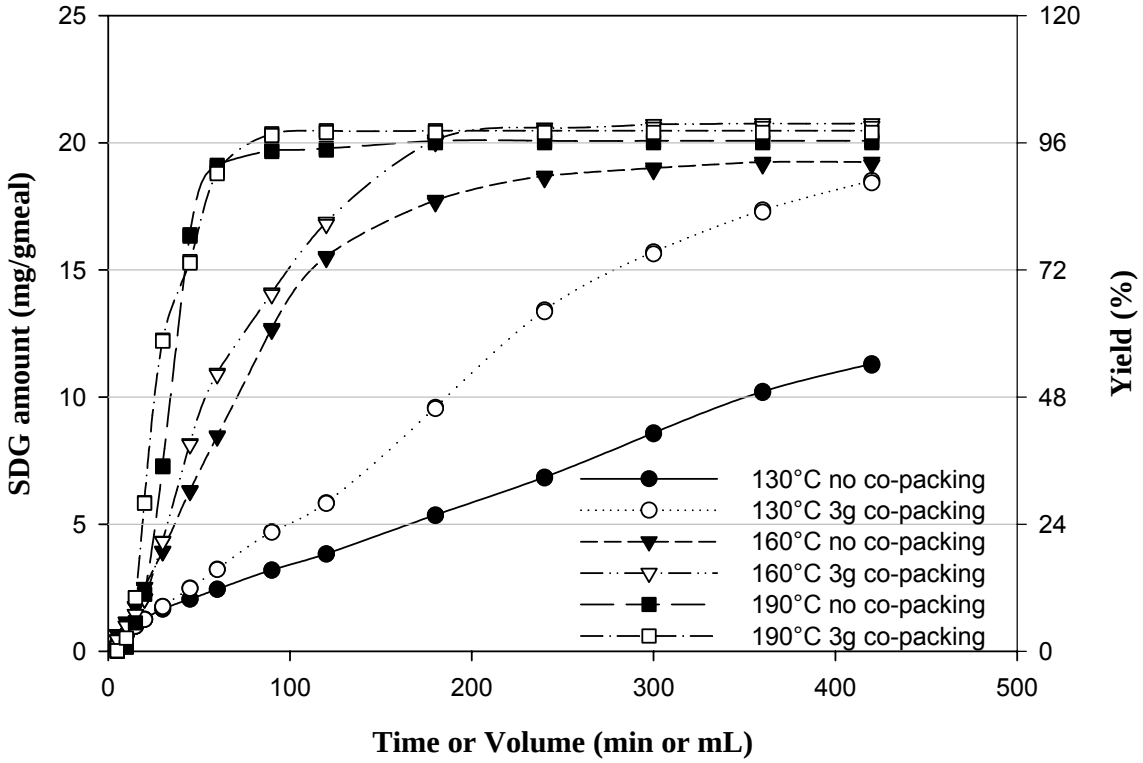


Figure 3.3. Effect of temperature and co-packing material on PLPW extraction of SDG from 2g of flaxseed meal with pH 9 buffered water at 1mL/min

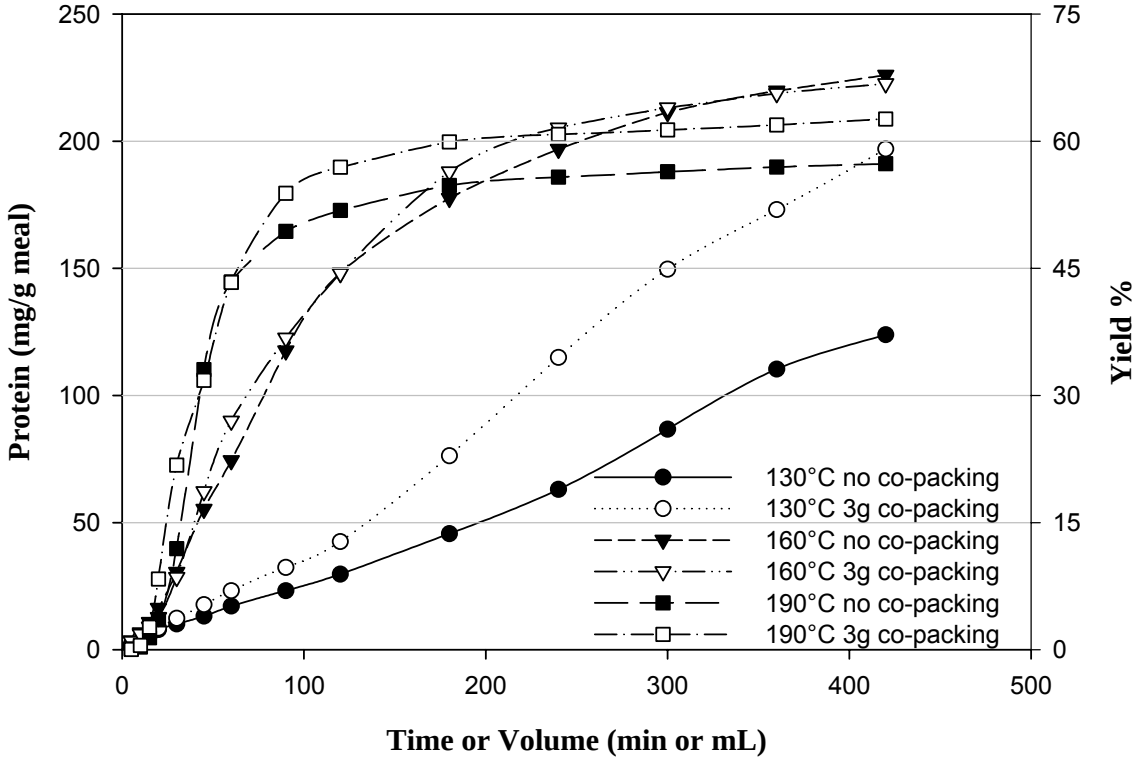


Figure 3.4. Effect of temperature and co-packing material on PLPW extraction of protein with pH 9 buffered water at 1mL/min from 2g of flaxseed meal. Protein was determined by Bradford BSA assay.

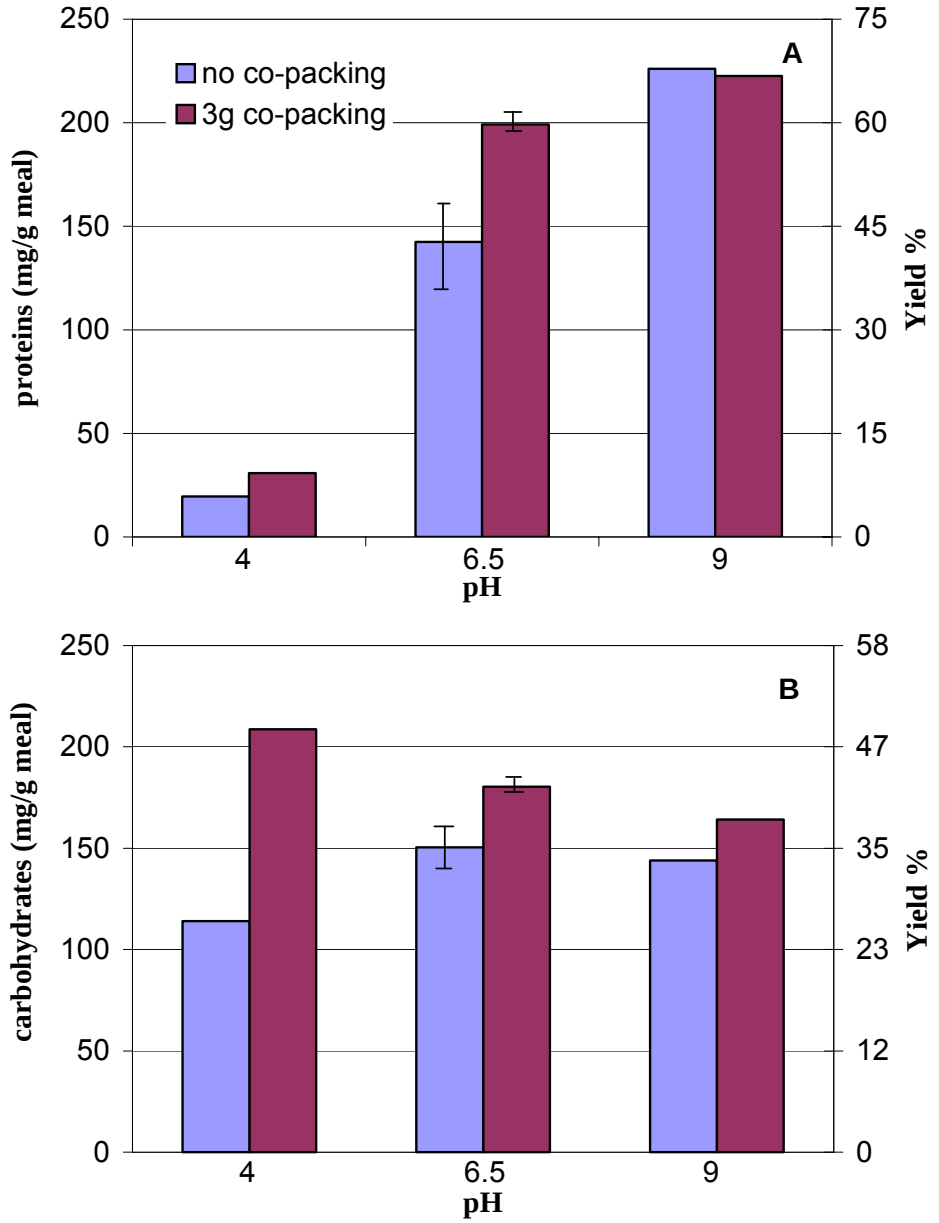


Figure 3.5. Effect of pH on extractions of proteins (A) and carbohydrates (B) from 2g of flaxseed meal at 160°C

Table 3.4. Regression coefficients and analysis of variance of the second order polynomial model for lignans, proteins and total carbohydrates of flaxseed meal extracts with 3g co-packing materials

	Lignan Coefficients	Protein Coefficients		Total Carbohydrate Coefficients
Variables[a]	**Yield**	**Yield [d]**	**Yield [e]**	**Yield**
Intercept	-68.14 ** [c]	201.68 *	-2384.32 ***	-485.0 *
X_1 [b]	0.87 ***		29.22 ***	7.57 **
X_2	0.060 **		1.84 **	0.90 ***
X_3	-0.084 [NS]	-39.81 [NS]	-8.79 [NS]	-17.11 [NS]
X_1^2	-0.0022 **	-0.0027 [NS]	-0.076 ***	-0.021 **
X_1*X_2	-0.00036 **	-0.0033 **	-0.014 ***	-0.0051 ***
X_1*X_3		0.31 *		0.077 [NS]
X_2*X_3	0.0014 [NS]	0.059*	0.060 [NS]	
Model	***	***	**	**
R^2	0.8718	0.8764	0.8248	0.8080

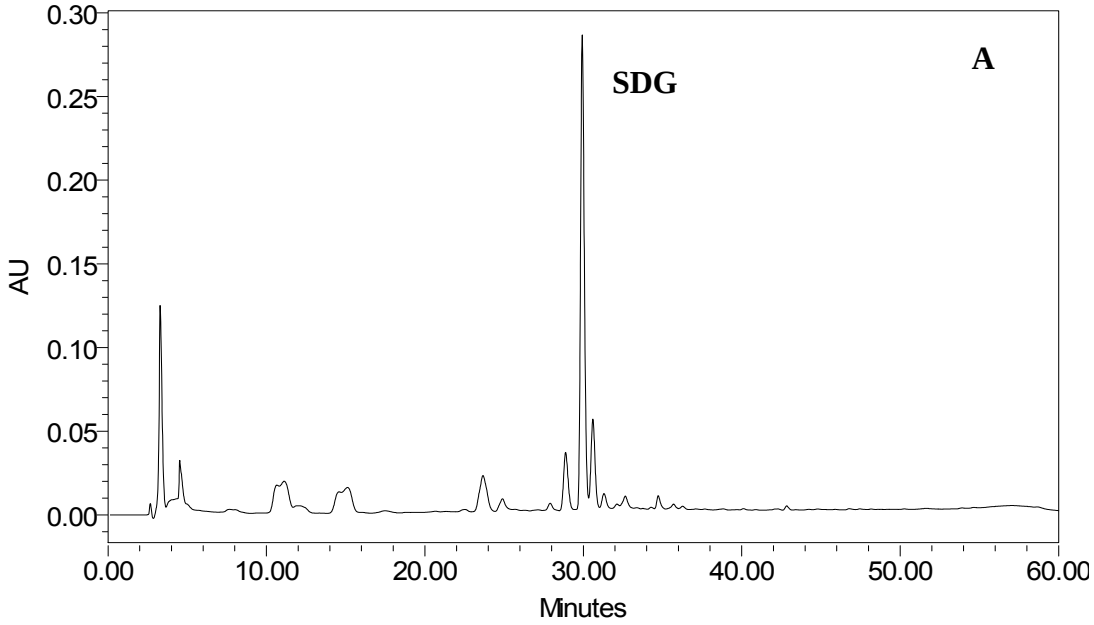
[a] Polynomial model $$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{1} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \beta_{ij} X_i X_j$$ adjusted by backward elimination with the goodness-of-fit test at the level of 0.1%

[b] X_1=Temp, X_2=Solvent to solid ratio, X_3=pH

[c] ***Significant at 0.01 level, **significant at 0.05 level, *significant at 0.1 level, NS non significant (p>0.1)

[d] Protein yields in mg/g of flaxmeal measured by Bradford BSA dye-binding assay

[e] Protein yields in total nitrogen percentage by combustion (%N x 6.25)



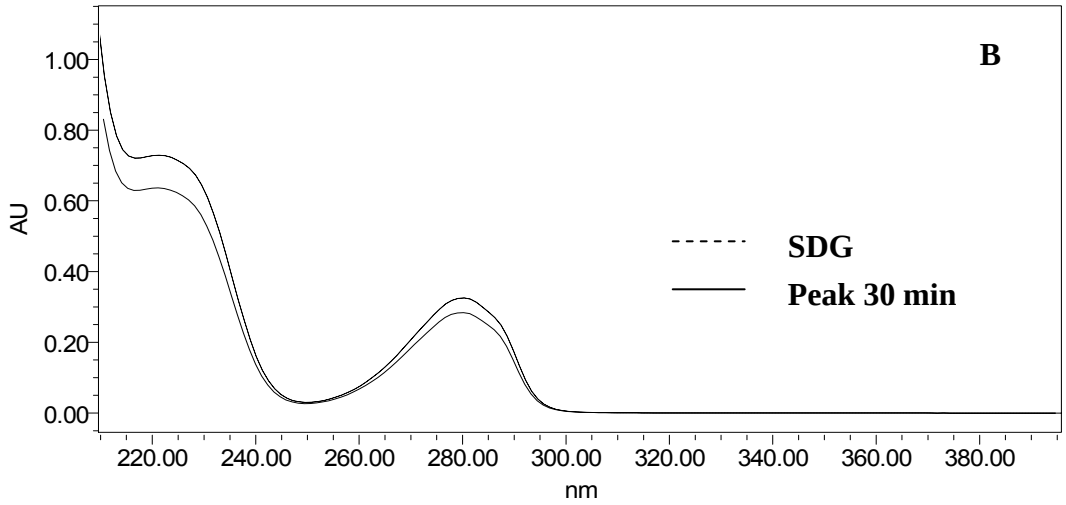


Figure 3.6. HPLC chromatogram of raw flaxseed meal (A); UV spectra of free SDG standard and SDG from raw flaxseed meal (B).

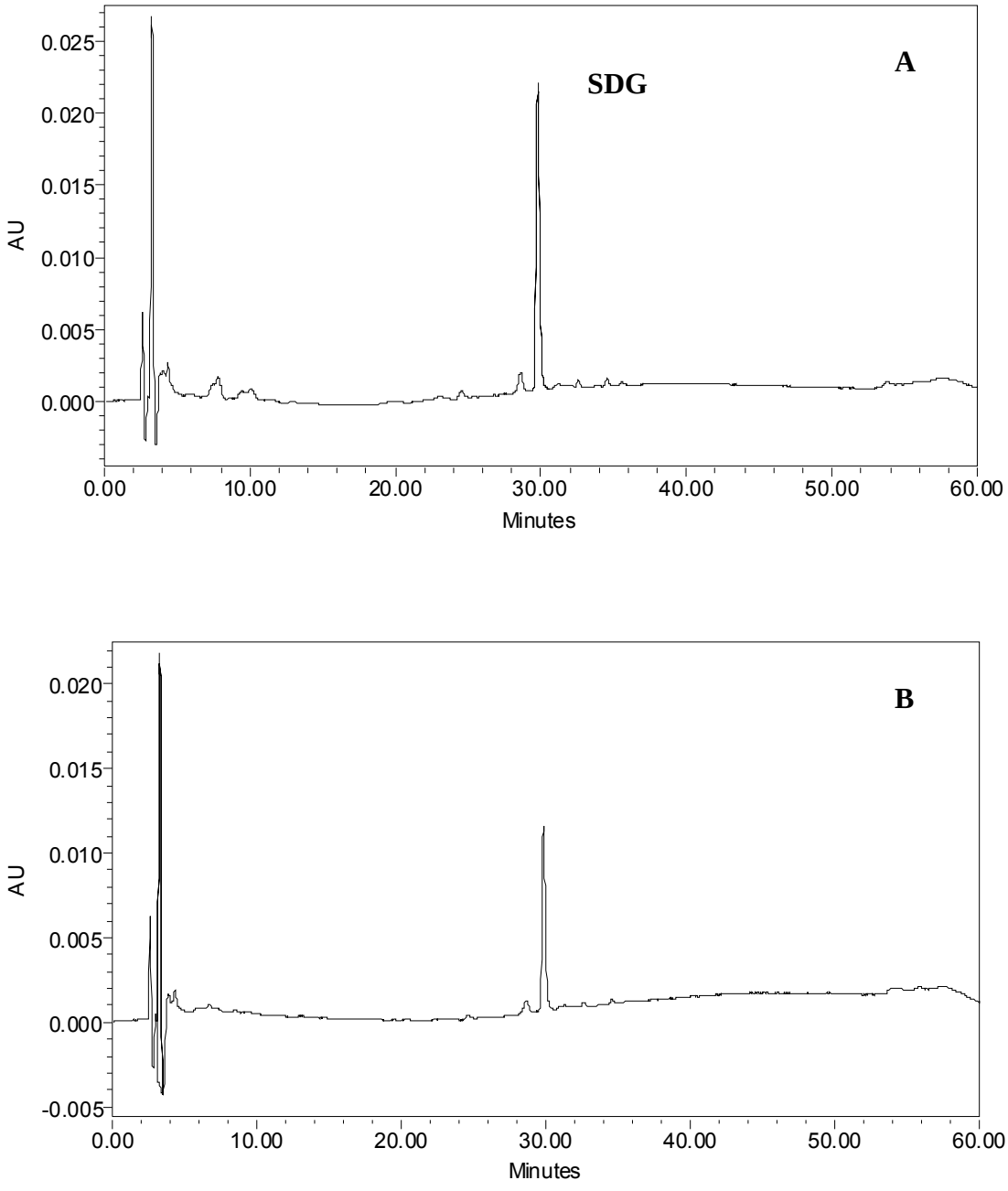


Figure 3.7. HPLC chromatograms of the PLPW extracts from 2g flaxseed meal at 130°C using 1 mL/min pH 9 buffered water collected at 180min 3g glass beads (A); no glass beads (B).

3.3.3 Effect of extraction temperature

The effect of temperature was statistically significant ($p<0.01$) for extraction yields of lignan SDG, proteins (nitrogen) and carbohydrates (Table 3.3). The amount of lignan SDG extracted increased with temperature (Figure 3.3). The highest yield (ratio of mg SDG/g meal in extract to mg SDG/g meal in raw meal) (99%) of SDG was obtained at 160-190°C giving a total SDG amount of 21 mg per g of flaxseed meal.

Extraction yield of SDG increased from 30% at 130°C to approximately 97% at 190°C and 120 min (Figure 3.3). In general, the extraction of SDG was more efficient at temperatures in range of 160 to 190°C. This shows that PLPW extraction efficiency is greatly affected by extraction temperature. High temperature decreases surface tension and viscosity of water which allow solvent cavities to form easily thus permitting analytes to be more rapidly dissolved in the water (Ramos et al., 2002). Elevated temperature also increased the extraction rate which in turn reduced the time to reach equilibrium and maximum recovery. These findings are in full agreement with the results of Cacace and Mazza (2005) who also obtained high yields of SDG from whole flaxseed extracted with PLPW at high temperatures; and with the results of Kubatova et al. (2001b) for extracting flavor and fragrance compounds. In addition, Yang et al. (1998) showed that increasing temperature favored PLPW extraction by increasing diffusion coefficient of organic solutes, which accelerated the rate of mass transfer and reduced extraction time. Thus, for SDG, times required to reach equilibrium and maximum yield were 120 min and 200 min at 190°C and 160°C, respectively. For extraction performed at 130°C, equilibrium was not reached even after 420 min extraction.

The use of high temperature may create the possibility of decomposition of thermally sensitive compounds. The slightly higher yields of lignans obtained at 160°C than at 190°C for S/S above 180 mL/g (Figure 3.3; Figure 3.8) indicate that there may have been thermal degradation of lignans at the higher temperature. This was confirmed from a visual examination of the extracts which revealed that extracts obtained between 130 and 160°C were light yellowish-orange in colour, but they turned to brown above 160°C. This is in agreement with Ju and Howard (2005) who also observed brown pigment formation when Maillard reactions took place at elevated temperature when extracting phenolics with PLPW.

The results of the HPLC analyses showed that the increase of SDG concentration at high temperature was due to the increase of the SDG peak. Chromatograms at 280nm of samples extracted at 190 and 160°C showed increased peak area in comparison with chromatograms of samples extracted at 130°C (Figure 3.9). These further show the effectiveness of elevated temperature in separating SDG from flaxseed meal. It is worthy to note that the three chromatograms in Figure 3.9 are referring to cumulative extracts collected at 180 min of extraction (S/S 90). Therefore, extracts at 190°C contain higher amount of SDG than those at160°C and no degradation was observed due to shorter extraction time.

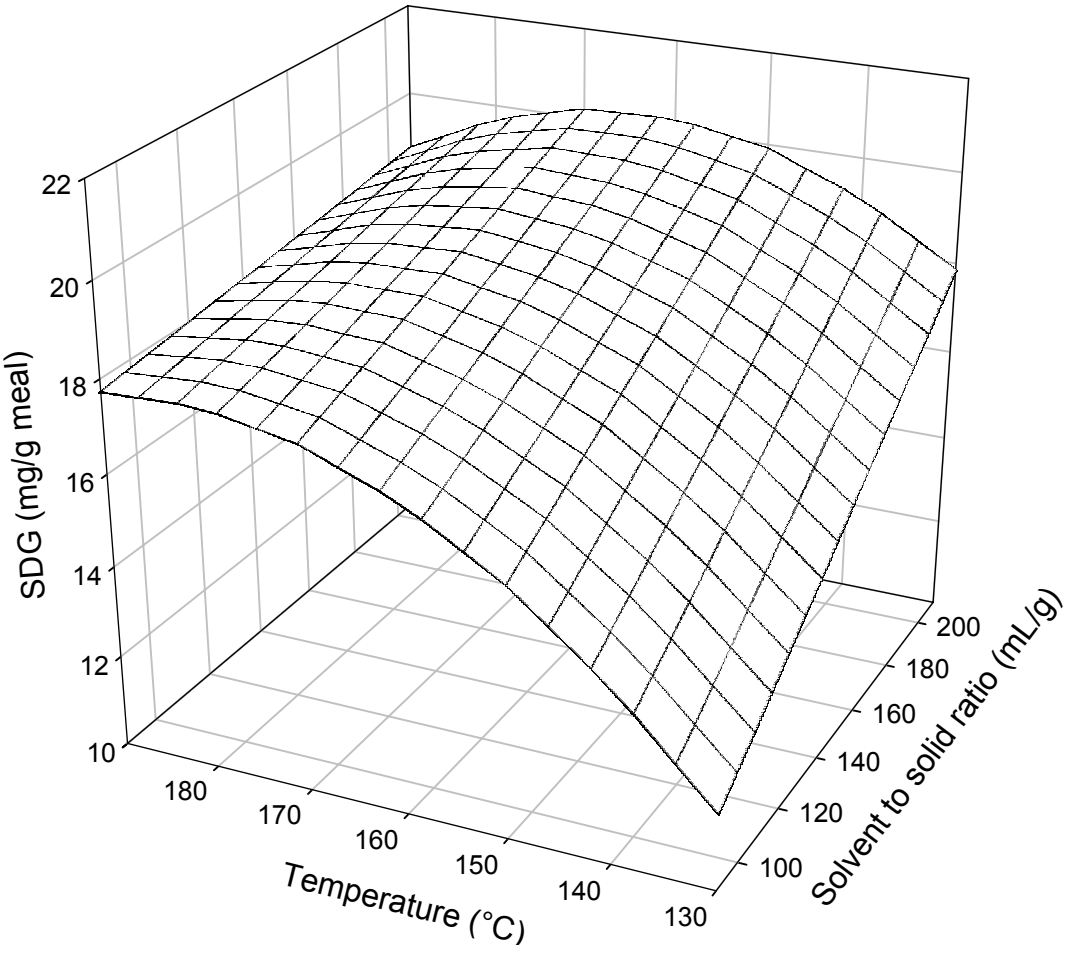


Figure 3.8. Response surface for the effects of temperature and solvent to solid ratio on SDG removed from flaxseed meal with 3 g co-packing material at a constant pH 9

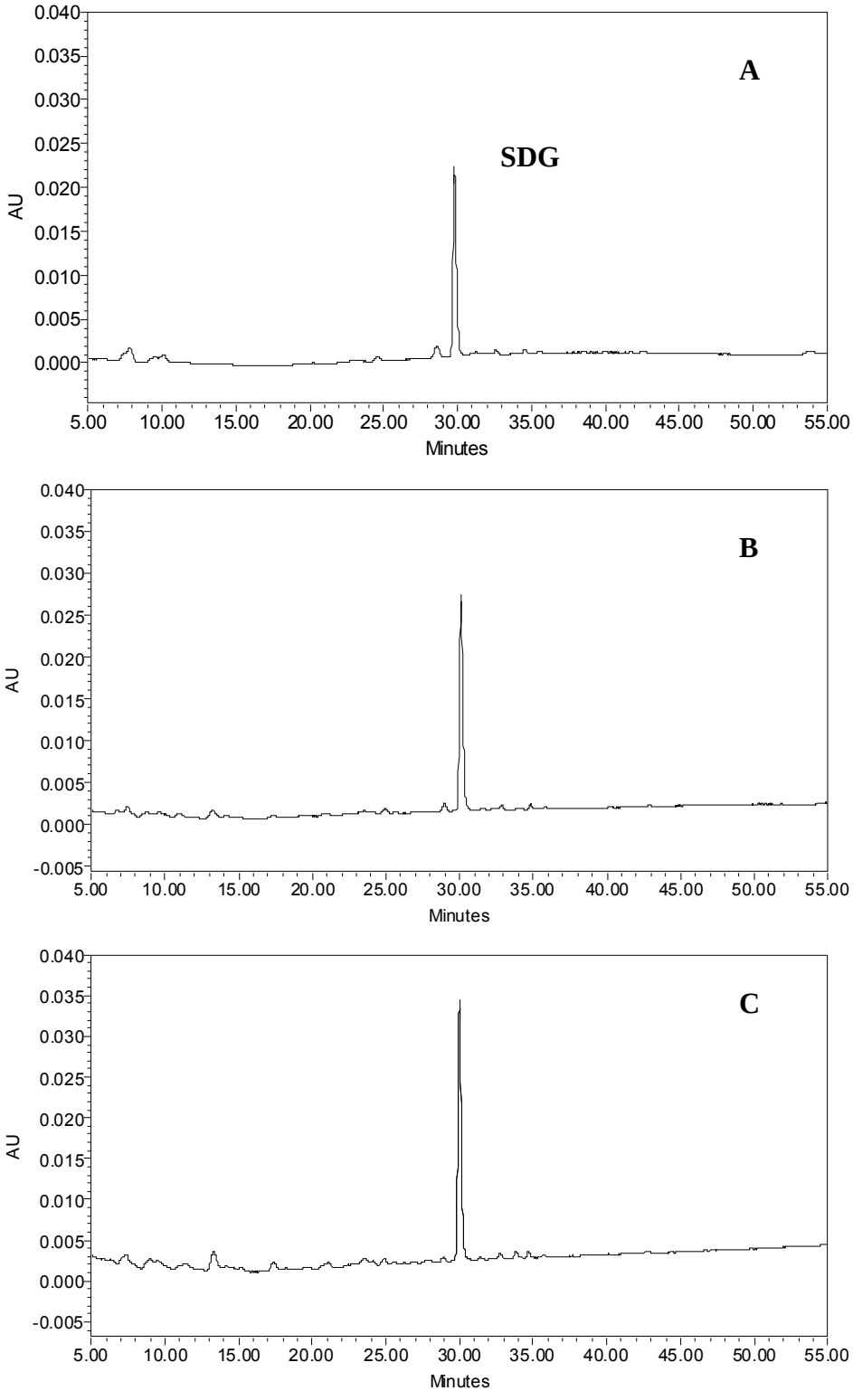


Figure 3.9. HPLC chromatograms of the PLPW extracts from 2 g flaxseed meal with 3 g glass beads using 1 mL/min pH 9 buffered water collected at 180 min at 130°C (A); at 160°C (B); at 190°C (C).

Protein yields reached a maximum value (68%) at 160°C, pH 9 and 400 min extraction (Figure 3.4). The increase in extraction temperature from 130°C to 160°C resulted in higher protein yield but the overall gain in percentage recovery was less than 20%. The positive effect of temperature on protein yield was more pronounced during the first 100 to 300 min since the equilibrium times needed were 200 min and 420 min at 190°C and 160°C, respectively. High temperature can reduce the polarity of water by weakening hydrogen bonds (Ramos et al., 2002). Generally, proteins which are rich in ionizable residues, and have low surface hydrophobicity, are soluble in water or dilute salt solutions (Sikorski, 2002). Subsequently, proteins with low surface hydrophobicity (more polar) would be more soluble at lower temperature (130°C) while less polar proteins (with high hydrophobicity) would be more soluble in pressurized water at high temperature of 190°C. Thus, the lower overall recovery at 190°C (62%) than at 160°C (67%) in Figure 3.4 was likely caused by protein denaturation. A cross-over effect was observed at around 200 mL between temperatures of 160 and 190°C. The unfolding of the molecules and exposure of the hydrophobic amino acids leading to denaturation may result at high temperatures. Therefore, the conformation, peptide bonds and cross-linking of proteins may be ruptured. The dark brown coloured extracts observed at temperature 190°C also had a burnt smell, suggesting that elevated temperatures may cause partial degradation of proteins and perhaps other compounds. Gogus et al. (2006) have reportedly produced flavor compounds associated with the presence of browning reaction products such as furfural, acetylfuran and 5-methylfurfural in superheated water extracts of flowers of *Achillea monocephala* at 175°C. Extractions performed at 130°C (with co-packing material) for over 420 min gave 60% (200 mg/g meal) protein yields,

comparable with runs carried out at 160°C (65%) and nearly double the yields obtained at 130°C with no co-packing material (Figure 3.4).

The recovery of carbohydrates was also affected by temperature at constant pH 4 (Figure 3.10). The optimal temperature was about 160°C which gave a yield of 210 mg per g of flaxseed meal. A rise in extraction temperature from 130°C to 160°C at S/S 100 mL/g enhanced the total carbohydrate yield by about 40%. However, there was a decline in carbohydrate content when the temperature was further increased from 160°C to 190°C at low S/S. At 180-200 S/S, extraction performed at low temperature (130°C) obtained the highest carbohydrate recovery. These effects may be explained if there were simultaneous superpositions of different effects of temperatures. Pineiro et al. (2004) explained the superposition of two different effects of a temperature increase. The first effect results from an increase in solubility which increases the rate of extraction at higher temperature due to weakening of the bonds between the carbohydrate and the matrix; the second effect is the degradation of thermally labile compounds at high temperatures. The degradation kinetics of monosaccharides (glucose, fructose) have been documented by Kabyemela et al. (1999, 1997) and Khajavi et al. (2005). Although elevated temperatures favored carbohydrates extraction by reducing extraction time, thermal degradation over-rode this benefit at temperatures above 160°C. Thus, high temperature can be detrimental in some cases due to thermal decomposition, and consequently care must be taken to select the temperature as high as possible to provide a fast extraction rate without destroying the thermally unstable components.

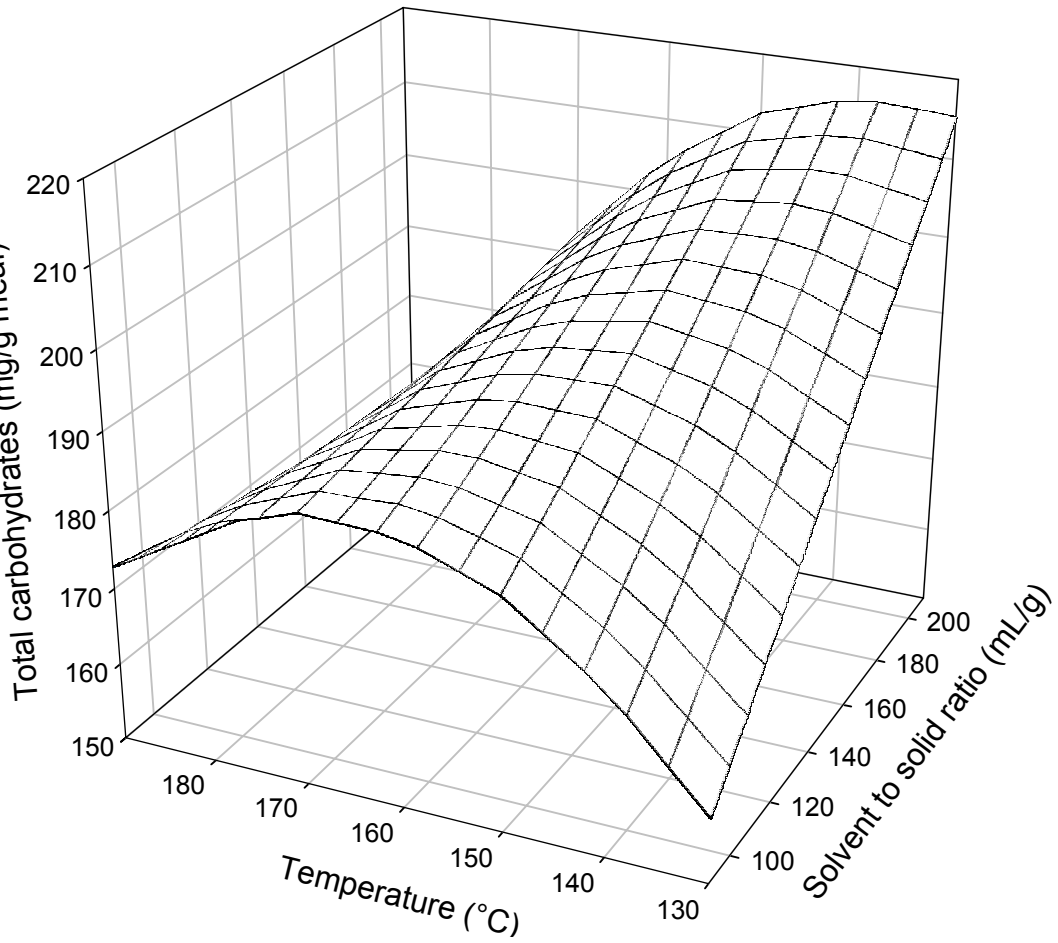


Figure 3.10. Response surface for the effects of temperature and solvent to solid ratio on carbohydrate recovery from flaxseed meal with 3 g co-packing material at pH 4

3.3.4 Effect of pH

In addition to temperature, a pH effect was also evaluated for the PLPW extraction of lignans, proteins and carbohydrates from flaxseed meal. Although pH was the least significant ($p<0.1$) among other independent variables for SDG (Table 3.3), it had a promoting effect on SDG extraction. Alkaline pH 9 showed an advantage in overall lignan recovery (Figure 3.11). The yield of lignans at 160 and 190°C increased by 20-25% with an increase of pH from 4 to 9, and pH was a controlling factor in determining the extraction yield of SDG. Thus, extractions carried out at 160 and 190°C resulted in a distinct increase in SDG recovery at a particular pH, with the equilibrium SDG concentration and recovery being higher at pH 9, irrespective of temperature (Figure 3.11). Metivier et al. (1980) and Crippa (1978) also used pH to maintain the stability of the active compounds when extracting phenolics such as anthocyanins and alkaloids, respectively.

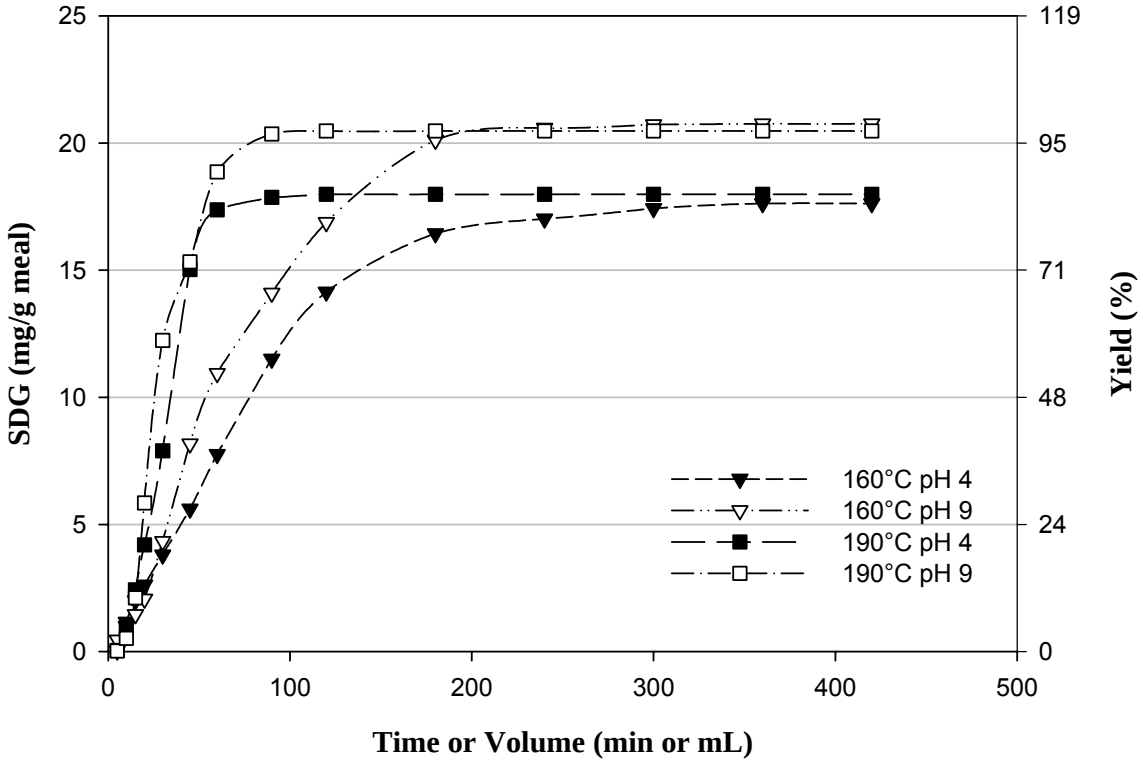


Figure 3.11. Effect of pH and temperature on extraction of SDG with 1 mL/min water from 2 g of flaxseed meal with 3 g co-packing glass beads

The pH was the major factor affecting extraction of proteins ($p<0.05$) (Tables 3.3; Figure 3.5A), and optimum recovery of proteins (225 mg/g meal) was obtained at pH 9 and 160°C using 210 S/S (mL/g) (Figures 3.4 and 3.5A). Figure 3.5A shows that proteins had higher recovery in alkaline water (pH 9) than in acidified water (pH 4), resulting in overall yields of about 225 mg/g meal and 25 mg/g meal, respectively. These results reflect the higher solubility of flaxseed proteins in alkaline buffered water, and are in agreement with published reports on the solubility of flaxseed meal protein at various pHs (Wanasundara and Shahidi, 1994a; Krause et al., 2002). Flaxseed meal contains a high level of non-protein nitrogen (Wanasundara and Shahidi, 1994b; Wanasundara and Shahidi, 1996), thus, in this report only the protein results determined by the Bradford BSA method, and not those determined by the combustion method (Table 3.3) have been discussed.

The effect of pH on yield of total carbohydrates at 160°C is shown in Figure 3.5B. The graph illustrates that more carbohydrates were extracted in neutral and slightly acidic water. There was about 10% increase in carbohydrate yields as pH decreased from 9 to 4 for runs with co-packing material (Figure 3.5B). These results are in agreement with the enhanced yield of flaxseed mucilage at weak acidic to neutral pH values reported by Cui et al. (1994). Mazza and Biliaderis (1989) also reported that temperature was the major factor affecting the extraction yields of flaxseed gum irrespective of the changes in pH or water to seed ratio.

3.3.5 Effect of solvent to solid ratio

The solvent to solid ratio values shown in Table 3.1 define the end point for each extraction. However, since the extractions were dynamic and hence sequential volumes of extracts (various S/S) can be collected and analyzed. The effect of solvent to solid ratio was shown to be significant ($p<0.05$) for lignan, protein and carbohydrate (Table 3.4). Temperature is the variable related to the solvent to solid ratio. As mentioned in previous sections, high temperature reduces the extraction time to reach equilibrium, hence reduces the solvent volume.

Figure 3.8 shows that solvent to solid ratio (S/S) exhibited a linear effect whereas temperature showed a quadratic effect on the SDG yields. This is consistent with data in Table 3.4 where it is shown that there is a linear effect of solvent volume and quadratic effect of temperature which were both significant ($p<0.05$). Besides, the temperature-solvent volume interaction was significant ($p<0.05$) for SDG (Table 3.4), and their interaction is shown in Figure 3.8. The amount of SDG extracted increased linearly with solvent to solid ratio until the temperature-S/S interaction became dominant at about 160°C to 190°C. Solvent to solid ratio did not have much effect on the SDG yield when it was changed from 100 to 210 mL/g at 160°C.

For protein, extraction yield also increased with solvent volume as shown in Figure 3.4. Again, increasing the temperature from 130 to 190°C decreased the extraction volume from 420 mL to 150 mL. At 130°C maximum recovery was not reached even after 420 mL of pH 9 water was used (Figure 3.4). Other drawbacks of low temperature extractions (130°C) were longer extraction time and the need for more

solvent which would have to be removed as the result of the large volume (>420 mL) generated.

For carbohydrates, using larger S/S resulted in an increase in carbohydrate recovery for temperature in the lower range between 130 to 160°C (Figure 3.10). This is consistent with the findings of Cui et al. (1994) who reported that higher water to solid ratios favored the extraction of flaxseed carbohydrates by increasing the concentration gradient and hence rate of diffusion of compounds from the solid matrix to the solvent at temperatures near boiling water. However, the positive effect of larger solvent volume became negligible at > 160°C in our study. Therefore, using large solvent volume does not always provide enhanced solute yield as long as an equilibrium concentration in the extract is reached and the respective equilibrium time can be reduced by increasing temperature up to an optimal value.

CHAPTER 4

Pressurized low polarity water extraction of lignans, proteins and carbohydrates from flaxseed meal: optimization of flow rate, bed depth and solvent to solid ratio

4.1 Introduction

Functional foods are foods that are able to provide additional physiological benefits beyond the basic nutritional and energetic requirements (Health Canada, 1998). Flaxseed can be considered a functional food as it is rich in fat, protein, carbohydrate and phenolics, namely lignan which possesses properties capable of reducing the risk of chronic disease (Oomah and Mazza, 1997). Flaxseed meal has long been perceived as an industrial by-product from linseed oil extraction, which is used in paint and linoleum (Tadros, 1992). After the recovery of oil, the residual meal is primarily used as protein-rich livestock feed (Bell and Keith, 1993). Recently, there has been considerable interest in the inclusion of flaxseed in Western diets. Part of the interest comes from studies that report significant health benefits associated with consumption of flaxseed lignans (Sano et al., 2003). Thus, the development of isolated lignans from flaxseed for the improvement of human health is driven by growing health-conscious customers' demands (Hyvarinen et al., 2006). Flaxseed meal was shown to contain about twice as much lignan as that in whole flaxseed (Eliasson et al., 2003).

In compliance with good manufacturing practices (GMP), solvents (ethanol, acetone) used to extract lignans and other phenolics are generally regarded as safe (GRAS). However, organic solvents are usually volatile, flammable, toxic and hazardous which could contaminate the environment upon discharge in streams. The search for

replacements for classical organic solvents is therefore one of the active fields of "green" chemistry. Use of water as an extraction solvent could circumvent these problems. Water is a non-hazardous, chemically and thermally stable, non-flammable, consumer acceptable and inexpensive solvent. However, its application as a solvent is limited because the low solubility of a variety of target hydrophobic bioactives at "low" temperature (<100°C), as water temperature cannot go beyond 100°C at ambient pressure. Pressurized low polarity water (PLPW) is based on the use of water as extractant, at high temperature but below 374°C (critical point of water, 22.4 MPa and 374°C) and pressure high enough to maintain the liquid state. Increasing the water temperature from 25°C to 250°C causes similar changes in solvent polarity as those achieved by conventional mixing of methanol or acetonitrile with water (Yang et al., 1998). Physical properties such as surface tension and viscosity are also affected by the state of water (Lewicki, 2004). Elevated extraction temperatures also facilitate the liberation of phenolics from plant matrices, increase the solubilization rate of phenolics in solvents, and increase diffusion coefficients (Cacace and Mazza, 2005). Hence, PLPW can shorten the extraction time and improve the selectivity of extracting both slightly polar and relatively non-polar aromatic compounds like flaxseed lignans.

In Chapter 3, the results of an investigation on the application of PLPW extraction of lignans secoisolariciresinol diglucoside (SDG) and other flaxseed meal bioactives at varying temperature, pH, solvent to solid ratio (S/S) and co-packing material was presented (Ho et al., 2006). The results of that work have shown that the optimal conditions for the extractions of lignans were temperatures from 170°C to 180°C, pH 9, S/S 100 mL/g and a 1:1.5 meal to co-packing material ratio. Therefore, the objective of

this study was to further examine the feasibility of optimizing the PLPW extraction process by manipulating flow rates and the geometric variable. Different combinations of flow rate, bed depth and S/S ratio were investigated by response surface methodology.

4.2 Materials and methods

Flaxseed meal was produced as described in Chapter 3. Proximate composition of defatted meal was: 6% moisture, 33% protein, 43% carbohydrate, 12% lipid and 6% ash. Particle size was approximately 0.22 mm in diameter. Pressurized low polarity water extraction was performed in a laboratory-built apparatus as described in Chapter 3. All extractions were carried out in one of three stainless steel cylindrical extraction cells of lengths 16, 27 and 30 cm (depending on the bed depth with minimum void volume) with 10.6 mm inside diameter (1.27 cm outside diameter). The cell length was chosen to minimize dead volume in the cell depending on the bed depth. The extraction cell was loaded with flaxseed meal and mounted vertically in the oven with solvent flowing from bottom to top. An appropriate amount of co-packing glass beads (3 mm in diameter) were mixed with the meal to achieve a 1:1.5 ratio (w/w) of meal to glass beads. The collection vessel was changed periodically to provide a plurality of collection volumes, thereby separating and individually collecting multiple eluant fractions. Buffered deionized ultrapure water used for extraction was made using 0.25 M sodium carbonate and 0.25 M sodium bicarbonate and was adjusted to pH 9 as shown in Appendix 1.

4.2.1 Analysis of lignan, protein and carbohydrate

Lignan content in extracts and residues was measured by the direct hydrolysis method of Eliasson et al. (2003) as modified and described by Cacace and Mazza (2005). Analysis of lignan SDG was performed using a Waters HPLC system (Waters Corp., Milford, MA) equipped with a Waters Model 600 pump, a 717 plus autosampler, an Agilent 1100 degasser, and a 996 photodiode array detector. The procedure of Muir and Westcott (2000) was followed with minor modifications as previously discussed in Chapter 3. Protein analysis was performed by the Bradford BSA dye-binding assay for the liquid extracts with bovine serum albumin as protein standard (Bradford, 1976). BSA standard was diluted to 10 concentrations from 0 to 1000 µg/mL. Ten µL of sample and 200µL of reagent filled each well in the plate and were shaken for 30 sec, then incubated for 30min before being read at 595 nm with a micro plate reader (Spectra max Plus, Molecular Devices Corporation, Sunnyvale, CA). Protein content of freeze-dried solid residue samples and flaxseed meal was determined by a nitrogen combustion method (FP-428, LECO Instruments Ltd., Mississauga, ON Canada) (Horwitz, 2003; Sweeney and Rexroad, 1987). Total carbohydrates were determined by the phenol-sulphuric acid colorimetric assay method (Southgate, 1991; Dubois, 1956). The absorbance was read at 490nm in a spectrophotometer (DU 640, Beckman Instruments Inc., Fullerton, CA). Analysis of protein in samples was replicated 4 times and analyses for carbohydrate were conducted in duplicate.

4.2.2 Experimental design

A central composite design with 3 factors and 5 levels was selected to study the optimization of extractions for lignan, protein and carbohydrate (Haaland, 1989) (Table

4.1). The experimental design consisted of 18 runs, including 4 replicates of the centre point. Variables studied were flow rate, bed depth and solvent to solid ratio. Fixed conditions employed in the experiments included a uniform temperature of 180°C, constant flaxseed meal to co-packing material ratio (1:1.5) and pH 9 buffered water. The actual and coded (inside bracket) values of the factors of the experimental design are given in Table 4.1. One or two runs were carried out in randomized order daily.

Data were analyzed using the response surface regression (RSREG) procedure of SAS (SAS Institute Inc.) to fit the following second order quadratic polynomial regression model.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^{\,2} + \sum \sum_{i<j=1}^{3} \beta_{ij} X_i X_j$$

where Y are dependent variables (lignan, protein and carbohydrate yields), $\beta_0, \beta_i, \beta_{ii}, \beta_{ij}$ are constant and regression coefficients of the model. X_i and X_j are the independent variables in the model (flow rate, bed depth and solvent to solid ratio). RSREG provides the analysis of variance (ANOVA) and is able to estimate the coefficient parameters of the model, the contribution of each type of effect (linear, quadratic, and cross-product), and the shape of the curve. A goodness of fit test of the model was performed with regression (REG) procedure by backward elimination to keep variables significant at the 0.1% level. Response surface plots were generated using the predicted value from the fitted model by holding the least significant independent variable at a constant value, and changing the other two variables. Plots were obtained using Sigma Plot software (SPSS Inc., Chicago, IL).

The three variables investigated in this study were flow rate, bed depth and solvent to solid ratio. Other extraction parameters related to flow rate were superficial

velocity and residence time. The flow rate was used to determine the superficial velocity calculated as the ratio of flow rate to cross section surface area of the extraction cell. The residence time is the time that water is in contact with the flaxseed meal calculated as the ratio of bed depth to superficial velocity. The bulk density of the packed flaxseed meal bed was kept constant to achieve consistent porosity of the bed. This was achieved by loading the extractor with a constant weight of flaxseed meal and at constant bed depth. For experiments using different initial sample mass, bed depth was adjusted according to the amount of material added in order to keep the density of the packed bed constant. As the inside diameter (10.6 mm ID) of the extraction cell was kept constant, this geometric parameter (bed depth) could also be interpreted as bed depth to internal diameter ratio (Depth/ID).

Table 4.1. Central composite experimental design with 3 variables for extraction of lignans and other bioactives at 180°C with pH 9 buffered water in a 10.6 mm internal diameter cell

Run	Flow rate mL/min	Bed depth cm	Solvent to solid ratio mL/g
1	2 (-1) [a]	7 (-1)	39 (-1)
2	2 (-1)	7 (-1)	115 (+1)
3	2 (-1)	21 (+1)	39 (-1)
4	2 (-1)	21 (+1)	115 (+1)
5	6 (+1)	7 (-1)	39 (-1)
6	6 (+1)	7 (-1)	115 (+1)
7	6 (+1)	21 (+1)	39 (-1)
8	6 (+1)	21 (+1)	115 (+1)
9	0.6 (-1.68)	14 (0)	77 (0)
10	7.4 (+1.68)	14 (0)	77 (0)
11	4 (0)	2.2 (-1.68)	77 (0)
12	4 (0)	25.8 (+1.68)	77 (0)
13	4 (0)	14 (0)	12 (-1.68)
14	4 (0)	14 (0)	142 (+1.68)
15	4 (0)	14 (0)	77 (0)
16	4 (0)	14 (0)	77 (0)
17	4 (0)	14 (0)	77 (0)
18	4 (0)	14 (0)	77 (0)

[a] Numbers in parentheses are coded values of variables in the experimental design.

4.3 Results and discussion

The models developed by surface response analysis for yields of lignans ($p<0.01$), proteins ($p<0.01$) and carbohydrates ($p<0.1$) were significant (Table 4.2). Experimental values for lignans, proteins and carbohydrates in the extracts were analyzed by RSREG and the corresponding analysis of variance (ANOVA) was provided (Table 4.2). The coefficients of determination, R^2, indicated the degree of fit of the second order regression models were high for SDG ($R^2= 0.89$) and protein ($R^2=0.94$) but carbohydrate yield exhibited larger variability ($R^2=0.76$). Regression coefficients and analysis of variance of the adjusted polynomial second-order models for lignans, proteins and carbohydrate yields were analyzed by REG and are presented in Table 4.3. Effects of independent variables varied depending on which response variable was analyzed. Values for the independent process variables studied (flow rate X_1; bed depth X_2; solvent to solid ratio X_3) and their contributions towards the concentrations of the extracted lignans, proteins and carbohydrates are presented in Table 4.3. The coefficients of solvent to solid ratio and bed depth were positive for lignan and protein implying that higher levels of solvent to solid ratio and bed depth would result in higher recovery of the target compounds in the extracts. Therefore, solvent to solid ratio and bed depth were found to have significant positive effects on the extraction of lignan and protein from flaxseed meal. The order of significance of process variables affecting flaxseed meal extraction can be ranked as follows: solvent to solid ratio > bed depth > flow rate.

Table 4.2. Surface response and ANOVA for lignans, proteins and total carbohydrates yields in extracts

Run	SDG		Proteins		Total carbohydrates	
	Amount [a] mg/g	Yield [b] %	Amount [c] mg/g	Yield [b] %	Amount [d] mg/g	Yield [b] %
1	6.6	31.6	32.1	9.6	48.3	11.2
2	17.4	83.0	132.5	39.8	144.5	33.5
3	19.1	91.2	212.0	63.7	152.2	35.3
4	20.9	100.0	227.1	68.2	199.3	46.3
5	5.4	25.9	35.8	10.7	59.0	13.7
6	8.7	41.5	61.3	18.4	102.0	23.7
7	4.8	22.8	30.6	9.2	53.9	12.5
8	17.1	81.7	139.0	41.7	164.3	38.1
9	20.7	99.2	211.9	63.6	166.5	38.6
10	7.7	37.0	51.1	15.3	84.3	19.6
11	2.8	13.5	13.7	4.1	33.5	7.8
12	19.4	93.0	207.1	62.2	341.2	78.9
13	2.5	12.0	17.6	5.3	26.4	6.1
14	17.5	86.9	162.5	48.8	137.4	31.9
15	13.4	64.2	111.4	33.4	123.3	28.6
16	12.4	59.4	89.1	26.8	110.9	25.7
17	8.1	38.6	70.4	21.2	74.6	17.3
18	10.9	52.3	86.3	25.9	101.9	23.7
Model		*** [e]		***		*
Linear		***		***		**
Quadratic		NS		NS		NS
Cross-product		NS		NS		NS
R^2		0.8927		0.9388		0.7598
Effects						
Flow rate		**		***		NS
Bed depth		**		***		**
Solvent to solid ratio		**		**		NS

[a] Lignan yields in mg per g of flaxmeal expressed as SDG equivalents

[b] Compound yields in weight percentage of total content in flaxseed meal

[c] Protein yields in mg/g of flaxmeal measured by Bradford BSA dye-binding assay

[d] Total carbohydrate yield in mg/g flaxmeal expressed as glucose equivalents

[e] ***Significant at 0.01 level, **significant at 0.05 level, *significant at 0.1 level, NS non significant (p>0.1)

Table 4.3. Regression coefficients and analysis of variance of the second order polynomial model for lignans, proteins and total carbohydrates of flaxseed meal extracts

	Lignan Coefficients	Protein Coefficients	Total Carbohydrate Coefficients
Variables[a]	**Yield[c]**	**Yield [e]**	**Yield[f]**
Intercept	19.29 NS [d]	-10.88 NS	-2.08 NS
X_1 [b]	-15.95 *	-6.81 NS	
X_2	3.99 **	4.28 ***	
X_3	0.47 ***	0.28 ***	0.21 **
X_1^2	1.52 *	0.97 *	
X_2^2			0.11 ***
X_3^2			
X_1*X_2	-0.35 NS	-0.54 ***	-0.22 *
X_1*X_3			
X_2*X_3			
Model	***	***	***
R^2	0.8867	0.9314	0.7075

[a] Polynomial model $$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

adjusted by backward elimination with the goodness-of-fit test at the level of 0.1%

[b] X_1=Flow rate, X_2=Bed depth, X_3=Solvent to solid ratio

[c] Lignan yields in mg/g of flaxmeal as SDG equivalents determined by HPLC (Eliasson et al., 2003)

[d] ***Significant at 0.01 level, **significant at 0.05 level, *significant at 0.1 level, NS non significant (p>0.1)

[e] Protein yields in mg/g of flaxmeal measured by Bradford BSA dye-binding assay (Bradford, 1976)

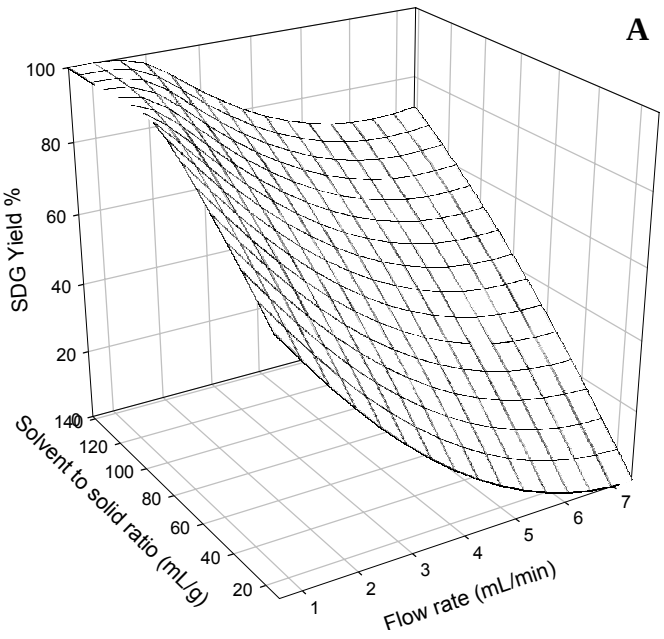
[f] Carbohydrate yields in mg/g flaxmeal measured by phenol-sulphuric colorimetric assay (Southgate, 1991)

4.3.1 Effect of solvent to solid ratio

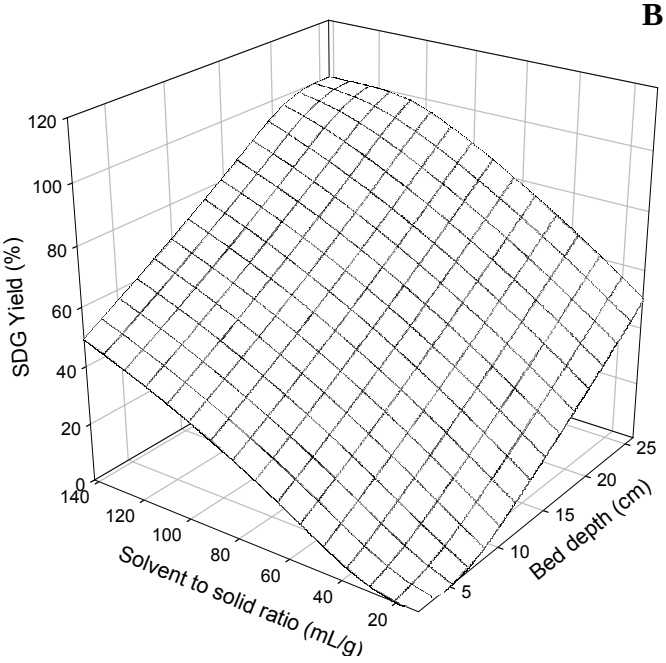
In order to evaluate the combined effect of two variables at a time, three-dimensional response surface curves were generated and plotted using the second order quadratic equations. Figure 4.1A and B show that SDG extraction yield increased with S/S ratio regardless of flow rate and bed depth, respectively. The optimum S/S ratio to reach the maximum SDG yield was about 110 mL/g with 2 mL/min flow at a 14 cm bed depth or 120 mL/g for a bed depth of 20 cm at 4 mL/min (Figure 4.1B). A larger S/S (>140 mL/g) ratio was required to improve recovery of SDG until equilibrium was reached when flow rate was higher than 4 mL/min at 14 cm bed depth (Figure 4.1A). Similarly, at a uniform flow of 4 mL/min, a large S/S ratio was necessary to expedite recovery when the bed depth was less than 20 cm (Figure 4.1B). However, the S/S ratio could be reduced with a lower flow rate and higher bed depth. Figure 4.2 shows that S/S decreased from 110 mL/g to 40 mL/g when flow rate was lowered from 6 mL/min to 2 mL/min at 21 cm. A similar degree of reduction occurred when bed depth was increased from 7 cm to 21 cm at a constant flow rate 2 mL/min (Figure 4.2).

The response surface of protein and carbohydrate yields also increased with S/S irrespective of flow rate or bed depth (Figure 4.3 and 4.4). Protein yield increased from 60% to 76% when the S/S ratio was increased from 100 to 140mL/g at low flow rate (Figure 4.3A) and high bed depth (Figure 4.3B). Carbohydrate yields reached equilibrium concentration at 120 mL/g for all flow rates and bed depths examined, with the highest yield being obtained at low flow rate (1-2 mL/min) and high bed depth (20-25cm). An increased S/S ratio increased carbohydrate recovery but began to reach a plateau after 120 mL/g (Figure 4.4A, B). Lignan, protein and carbohydrate yields were

all affected by solvent to solid ratio. A higher ratio reduced the concentration of solute at the interface. Results in Table 4.3 showed that the linear effect of S/S was significant for SDG ($p<0.01$), protein ($p<0.01$) and carbohydrates ($p<0.05$). Neither quadratic nor cross-product interaction effects with S/S were detected. This was consistent with response surface plots of SDG, protein and carbohydrate which demonstrated yields increased almost linearly with S/S (Figure 4.1, 4.3, 4.4). As fresh fluid was supplied during the dynamic extraction in PLPW, mass transfer and hence extraction rate were improved. Therefore, an increase in S/S would favor extraction by modifying the concentration gradient between the solution in the extraction cell and the surface of the sample matrix. Yields were also higher by increasing the solvent to powder ratio in the extraction of anthocyanins from sunflower husks (Pifferi and Vaccari, 1983). Use of high solvent to solid ratios, however, results in dilute solutions. The total volume of water used was equivalent to the weight of flaxseed meal multiplied by the solvent to solid ratio (S/S). The solvent to solid ratio (S/S) determines the volume of water consumed at a given flow rate and sample mass until the extraction reaches equilibrium (maximum recovery). Time to reach maximum recovery is taken as equilibrium time. Alternatively, it may be stated that the S/S effect is controlled by time at a constant flow rate.



constant bed depth 14 cm



constant flow rate 4 mL/min

Figure 4.1. Effect of flow rate (A) and bed depth (B) and solvent to solid ratio on extractions of SDG from flaxseed meal at 180°C using pH 9 buffered water with 1:1.5 meal to glass beads ratio.

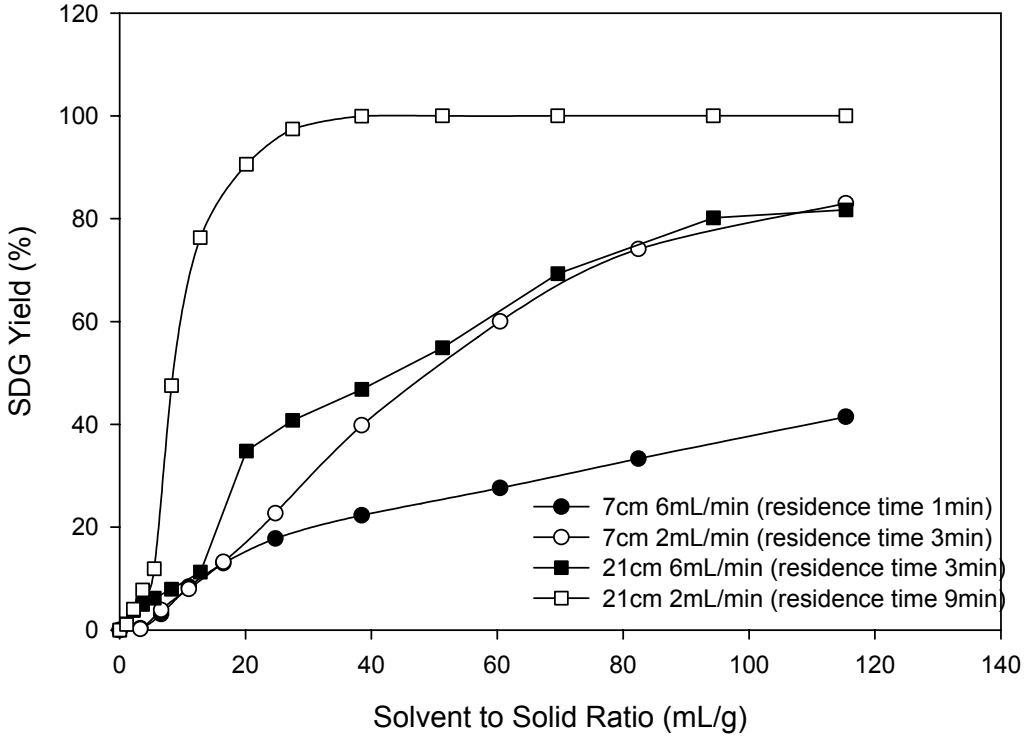
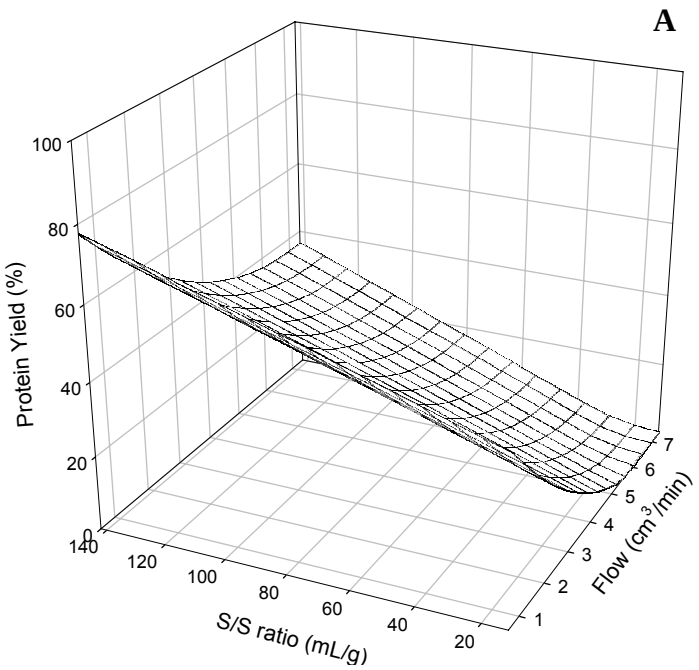
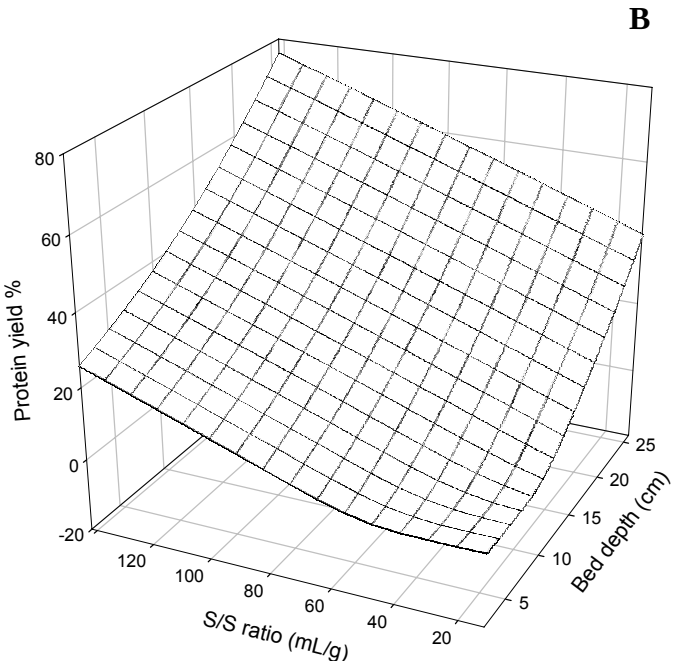


Figure 4.2. SDG yield as a function of solvent to solid ratio for extraction at 180°C, pH 9 for two bed depths and two flow rates. Bed depth 7 cm (1.8 g meal + 2.7 g glass beads); 21 cm (5.5 g meal + 8.2 g glass beads)

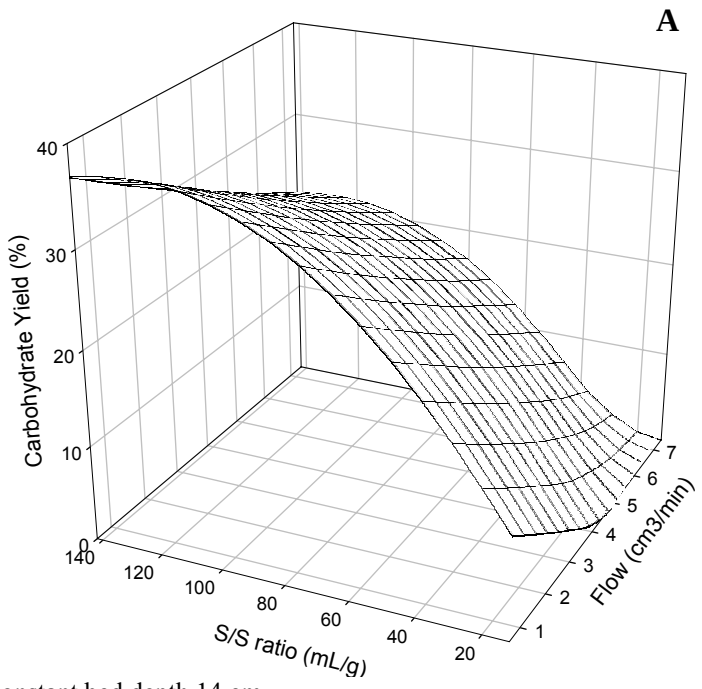


constant bed depth 14 cm

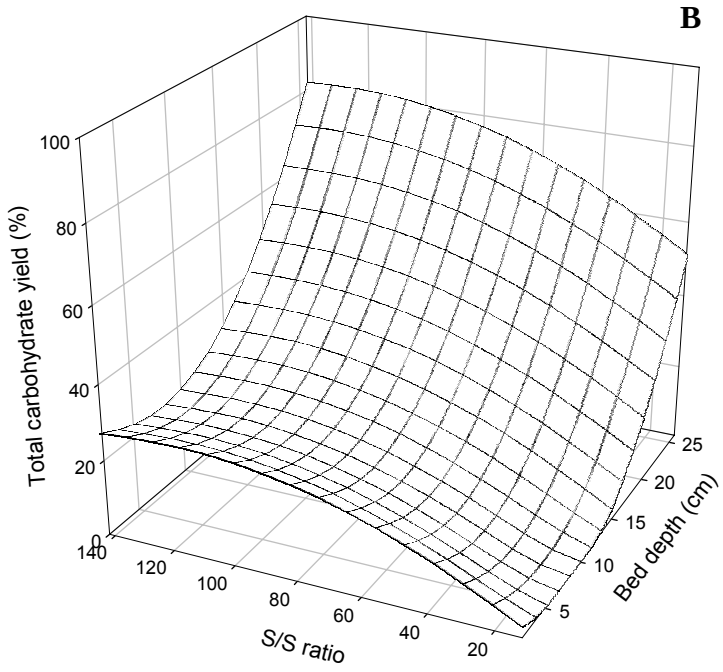


constant flow rate 4 mL/min

Figure 4.3. Effect of flow rate (A) and bed depth (B) and solvent to solid ratio on extractions of proteins from flaxseed meal at 180°C using pH 9 buffered water with 1:1.5 meal to glass beads ratio.



constant bed depth 14 cm



constant flow rate 4 mL/min

Figure 4.4. Effect of flow rate (A) and bed depth (B) and solvent to solid ratio on extractions of carbohydrates from flaxseed meal at 180°C using pH 9 buffered water with 1:1.5 meal to glass beads ratio.

4.3.2 Effect of flow rate

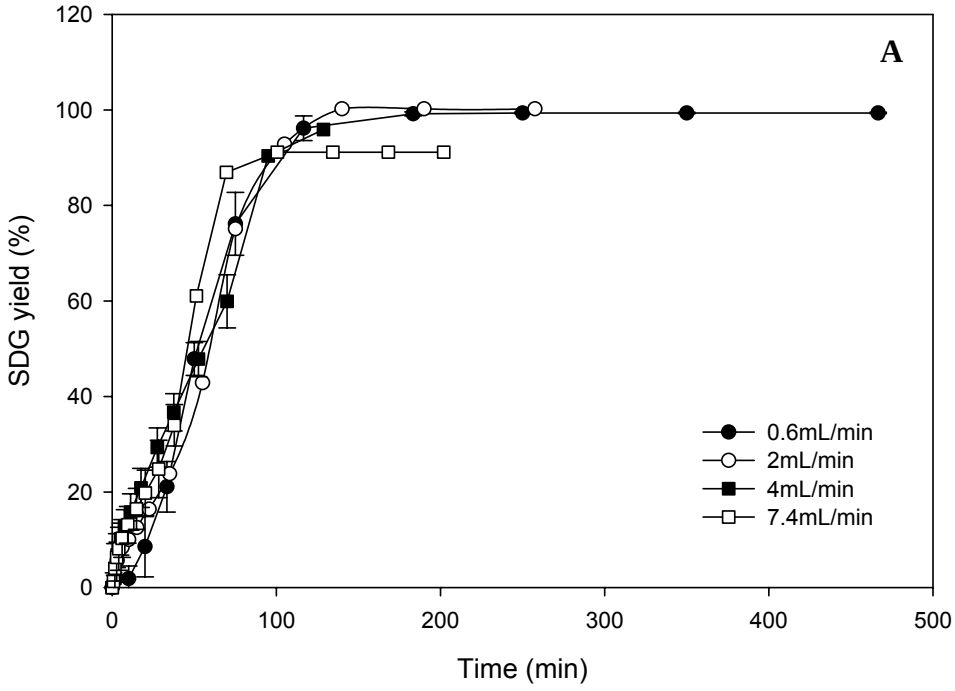
The effect of a wider range of flow rate (0.6 - 7.4 mL/min) and variable solvent to solid ratio (12 – 140 S/S) on lignan, protein and carbohydrate yields at a constant bed depth of 14cm is illustrated in surface response diagrams projected in Figures 4.1A, 4.3A and 4.4A, respectively. Figures 4.1A, 4.3A and 4.4A show enhanced yields of extracted solutes with declining solvent flow rate. Flow rate was significant for SDG ($p<0.05$) and proteins ($p<0.01$) but not for carbohydrates (Table 4.2). SDG and proteins showed larger percentage increase in yields (40%) at any given S/S in Figures 4.1A, 4.3A than percentage gain in total carbohydrates in Figure 4.4A. This is consistent with the results in Table 4.2 which show that flow rate was not a significant factor for carbohydrates. Figure 4.1A, 4.3A and 4.4A show that the combination of low flow rate (1-2 mL/min) and low S/S provides SDG yields higher than those at high flow (6-7 mL/min) rate and low S/S. High flow rate coupled with high S/S (>100) could produce similar yields to those at low S/S; however, large S/S leads to huge solvent consumption (diluted extract) which is undesirable, because it must be removed. Therefore, a flow rate between 1 to 2 mL/min may be preferred.

To further investigate the effect of flow rate on extraction rate and to evaluate the extraction mechanism model, experimental runs using a "one-factor-at-a-time" method were carried out at different flow rates. For these experiments, flaxseed meal (3.6 g + 5.5 g glass beads at 14 cm bed depth) was sequentially extracted with pH 9 water at 180°C at four different flow rates (0.6, 2, 4, 7.4 mL/min). Kinetics of extraction were similar for the four flow rates studied (0.6, 2, 4, 7.4 mL/min) as shown by the yield of lignan SDG against time (Figure 4.5A). The extraction mechanism can be determined by simply

comparing the effect of changing flow rate on the extraction rate of SDG. The amount of SDG extracted did not increase proportionally with the flow rate. Thus, mass transfer is controlled by the internal diffusion in the flaxseed meal which suggests a negligible effect of increased flow rate (Kubatova et al., 2002). If the diffusion through plant material is the rate-limiting step, the rate of extraction will be independent of the flow rate and the amount of extract should be higher for the same volume of solvent used when extraction occurs at lower flow rates. On the other hand, if solvent partitioning is the rate-determining step, the rate of extraction should be proportional to flow rate, and if the amount extracted is plotted against the amount of solvent used, the results for different rates should fall on the same curve, as is found in Figure 4.5A.

Increased flow rate resulted in increases in superficial velocity which hypothetically should in turn increase the mass transfer. However, there was little mass transfer difference among the extractions performed at 0.6, 2, 4 and 7.4 mL/min (Figure 4.5A). Thus, increasing superficial velocity is not the major mass transport resisting factor. In light of this evidence, runs at high flow rate required an equivalent amount of time compared to runs at low flow rate for extraction to reach equilibrium. Figure 4.5B supports this observation as the amount of lignan extracted with the same volume of water was higher for low flow rate (0.6mL/min). At 0.6 mL/min, extraction was completed at 100mL but runs at 4 and 7.4 mL/min required 500mL and 600mL of water respectively. Therefore, Figure 4.5A indicates that low flow rate could produce a comparable extraction as high flow rate, and Figure 4.5B indicates that higher flow rates required a larger amount of solvent to reach extraction equilibrium, resulting in more dilute extracts, and this is consistent with the obervations in Figure 4.1A, 4.3A and 4.4A.

The recoveries of SDG from flaxseed meal extracts at high flow rate (7.4mL/min) did not reach 100% (Figure 4.5A). Although decomposition of SDG cannot be completely excluded, it is more likely that SDG was bound to the meal matrix and was not easily extracted, or the SDG concentration reached the limit of detection of the HPLC method employed (small quantities of solutes dissolved in a large volume of extract, especially in the last few fractions collected). The detection limit is defined as the minimum concentration capable of giving a chromatographic signal. Another reason could be the reduced residence time observed at high flow rate. Residence time is defined as the time required for solvent to enter and travel the full bed depth height. It is calculated as bed depth divided by flow rate. There were significant differences in yields observed for extractions with different residence times, suggesting that high extraction efficiency is presumably related to residence time (Table 4.4). The connection between superficial velocity, residence time and improved extraction yields are further elaborated in Figure 4.2. Figure 4.2 shows two curves at a constant bed depth of 7 cm with different flow rates. The curve at 2 mL/min gave > 60% more SDG than a flow of 6 mL/min at the same S/S ratio. Extraction at 2 mL/min, again, enabled higher SDG recovery than 6 mL/min at the same bed depth of 21 cm (Figure 4.2).



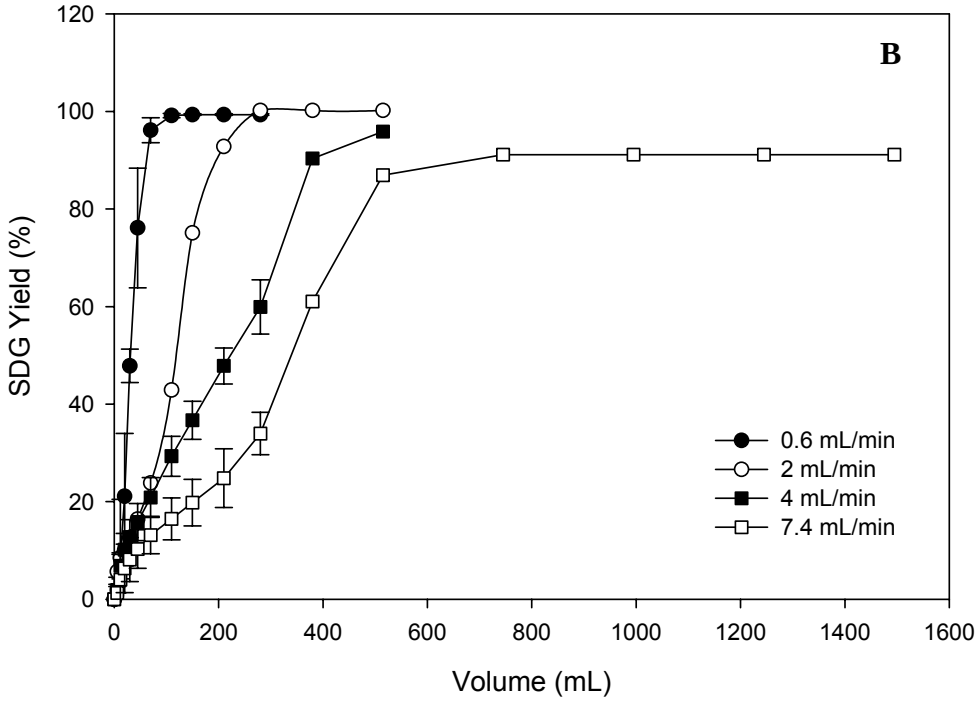


Figure 4.5. Effect of flow rate on extraction of SDG with time (A) and volume (B) from flaxseed meal at a fixed bed depth 14 cm (3.64 g meal + 5.46 g glass beads) with pH 9 buffered water at 180°C with 1:1.5 meal to glass beads ratio.

Table 4.4. Experimental conditions for extraction of lignans and other bioactives from flaxseed meal in a 10.5 mm ID cell at 180°C using pH 9 buffered water

Run	Residence time[a] (min)	Superficial velocity[b] (cm/min)	Depth to ID ratio	Mass meal (g)	Mass glass beads (g)	Extraction time (min)	Volume (mL)
1	3.0	2.3	6.7	1.82	2.73	35	70
2	3.0	2.3	6.7	1.82	2.73	105	210
3	9.1	2.3	20.0	5.46	8.18	105	210
4	9.1	2.3	20.0	5.46	8.18	315	630
5	1.0	6.9	6.7	1.82	2.73	11.7	70
6	1.0	6.9	6.7	1.82	2.73	35	210
7	3.0	6.9	20.0	5.46	8.18	35	210
8	3.0	6.9	20.0	5.46	8.18	105	630
9	20.3	0.7	13.3	3.64	5.46	466.7	280
10	1.6	8.5	13.3	3.64	5.46	37.8	280
11	0.5	4.6	2.1	0.57	0.86	11.25	45
12	5.6	4.6	24.6	6.70	10.05	129	516
13	3.0	4.6	13.3	3.64	5.46	11.2	44.8
14	3.0	4.6	13.3	3.64	5.46	128.8	515.2
15	3.0	4.6	13.3	3.64	5.46	70	280
16	3.0	4.6	13.3	3.64	5.46	70	280
17	3.0	4.6	13.3	3.64	5.46	70	280
18	3.0	4.6	13.3	3.64	5.46	70	280

[a] Calculated as ratio of bed depth to superficial velocity

[b] Calculated as ratio of volumetric flow rate to cross sectional area

In general, high flow rate implies a higher solvent volume which dilutes the concentration of the dissolved compounds at the surface of the particle, thus providing a higher concentration gradient between the concentrations inside and at the surface of the particles. The high concentration gradients thus, in turn, gave a faster extraction rate minimizing the extraction time as well as amount of solvent (Shi and Le Maguer, 2003; Gertenbach, 2002). High flow rate should also reduce the thickness of a liquid film surrounding a biomass particle, thereby facilitating the removal of solutes from the particle surface (Liu and Wyman, 2003). However, this was not the case for flaxseed meal in the packed bed tubular extractor used in the present experiments. Deviations of extraction system performance from experimental results and changes in controlling steps have been reported in the literature (Dibert et al., 1989). Ozel et al. (2003) and Jimenez-Carmona et al. (1999) both mentioned that PLPW exhibited the highest efficiency at a medium flow rate of 2 mL/min for extracting essential oil over a range of 0.5 to 3 mL/min, but Ayala and Luque de Castro (2001) reported that 1 mL/min was the optimal flow rate for isolating essential oil from a different biomass. More recently, Cacace and Mazza (2005) reported that permeability within flaxseed (diffusion) exhibited greater resistance than external mass transfer, and the optimal flow rate for the extraction of lignans from whole flaxseed with PLPW was 0.5 mL/min in the tested range of 0.3 to 4 mL/min.

4.3.3 Effect of bed depth

The effect of bed depth was significant for yields of lignan, protein and carbohydrate ($p<0.05$) (Table 4.2). Yield percentages for SDG (Figure 4.1B), proteins

(Figure 4.3B) and carbohydrates (Figure 4.4B) all increased when raising the bed depth from 2 cm to 26 cm regardless of the S/S selected. The highest bed depth studied (26cm) (Depth/ID 25) gave higher yields for SDG (100%), protein (72%) and carbohydrate (83%) extraction. The gain in recovery for bed depth was higher (steeper slope) (>50%) than the gain obtained by S/S (shallow slope) (<40%). This confirms that the use of a greater bed depth (large sample mass) can reduce the use of water due to the need for a lower S/S ratio. Table 4.3 shows that bed depth had a significant linear effect on SDG ($p<0.05$) and protein ($p<0.01$), but exerted a quadratic effect on carbohydrate ($p<0.01$). The results presented in Figure 4.1B and 4.3B show that solute yield increased with bed depth. Carbohydrate yields shown in Figure 4.4B, however, increased exponentially with bed depth thus producing a quadratic effect as seen from Table 4.3.

Extractions were carried out to compare bed depth effect on extractions at a single volumetric flow rate of 4 mL/min (Figure 4.6). Increasing bed depth from 2.2 to 25.8 cm increased SDG yields from 10% to 95% (Figure 4.6). Sample weights of 0.6, 3.6 and 6.7 grams of flaxseed meal were used to achieve corresponding bed depths of 2.2, 14 and 25.8 cm, respectively (Table 4.4). It seems that the low SDG yield (10%) for a bed depth of 2 cm observed in Figure 4.1B was probably due to short extraction time (11 min), compared to 129 min for a bed depth of 25.8 cm. Higher bed depth meant that more sample mass filled the column such that a longer residence time would result (given a uniform superficial velocity). Longer residence time implies that flowing water takes more time to travel from the bottom to the top of the cell. Hence, there was a slight delay (Figure 4.6) in the extraction rate for the first 10 min for runs performed at the higher bed depth of 25.8 cm. Figure 4.2 shows the effect of increasing the bed depth from 7 to 21

cm at two separate flow rates of 2 and 6 mL/min. Raising the bed depth from 7 to 21 cm gave additional SDG yields (20-40%) under both flow rate circumstances. This phenomenon, again, could also be the consequence of higher residence time. The highest SDG yield (100%) was obtained at a residence time of 9 min while the lowest yield (40%) was observed at 1 min residence time (Figure 4.2). In addition, runs at 21 cm and 2 mL/min (9 min residence time) required the least S/S ratio to reach extraction equilibrium, whereas runs with the same 3 min residence time resulted in almost identical yields when the same S/S ratio was used (Figure 4.2). These results support points made in the discussion on the effect of residence time presented earlier, and clearly show that in order to obtain the maximum concentration of SDG-enriched water extract, both larger bed depth and longer residence times are essential for enhanced SDG recovery. A possible drawback of high bed depth is that water channeling could occur and possibly impede solute transfer from the sample matrix.

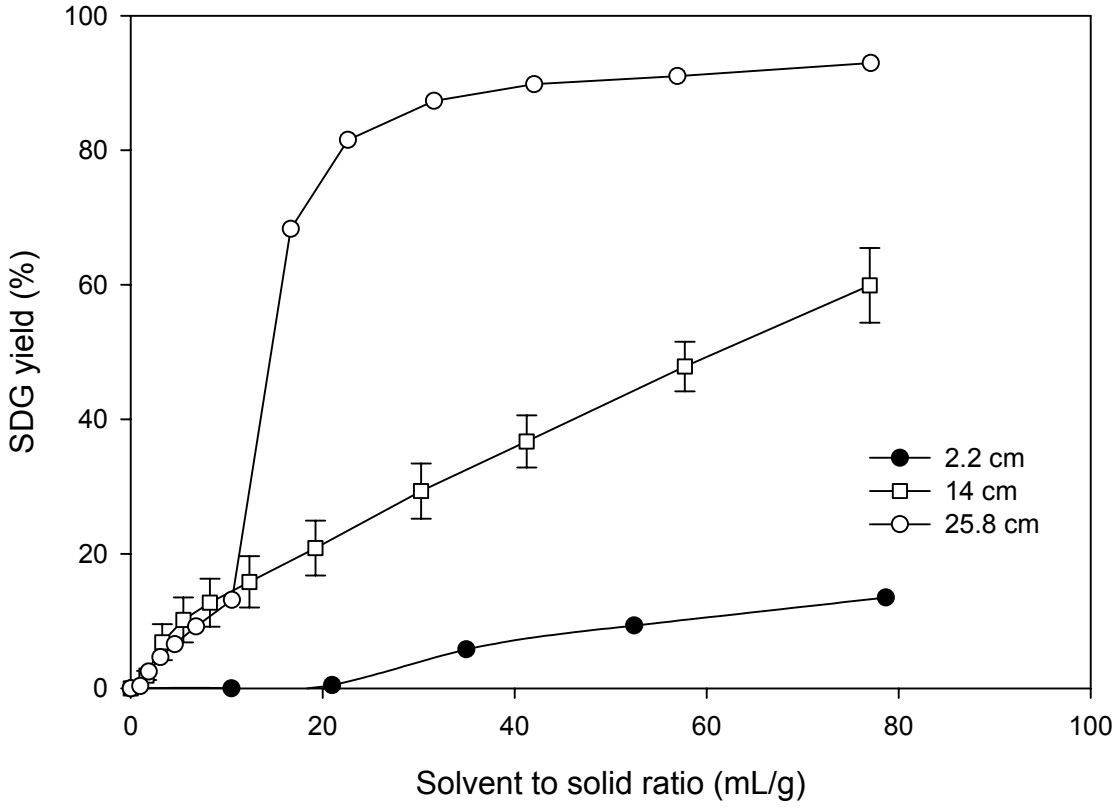


Figure 4.6. Effect of bed depth on extraction of SDG from flaxseed meal at constant flow rates 4 mL/min with pH 9 buffered water at 180°C against solvent to solid ratio. Bed depth 2.2 cm (0.6 g meal + 8.6 g glass beads); 14 cm (3.6 g meal + 5.5 g glass beads); 25.8 cm (6.7 g meal + 10.1 g glass beads)

Table 4.3 showed that interaction of bed depth with flow rate was highly significant for protein ($p<0.01$). The effects of bed depth and flow rate on protein yields are illustrated in Figure 4.7. The extraction yields for protein reached 99% for flow rates between 0.6 to 2 mL/min at bed depths of 20 to 25.8 cm (Fig 4.7). The protein extractions for flow rate above 2 mL /min at bed depths below 20cm, however, did not reach maximum yields. The slope was steeper at low flow rate, increasing more than 60% yields on average. Thus, the extractions performed at 2 mL/min at 7 and 21cm bed depths, for instance, showed a significant difference in terms of protein recovery, since residence time at 21 cm (9 min) was three-fold larger when compared to the extraction at 7 cm (3 min) (Figure 4.7). These observations support the idea that the degree of increase in yields changed in response according to specific combinations of bed depth and flow rate, which would in turn affect residence time.

In summary, high bed depth allowed maximum recovery of SDG, protein and carbohydrate. High bed depth coupled with low superficial velocity also allowed the use of a lower S/S ratio but still resulted in high recovery due to extended residence time. Hence, it is apparent that a high recovery extraction could be obtained with a higher bed depth at low flow rate with increased residence time.

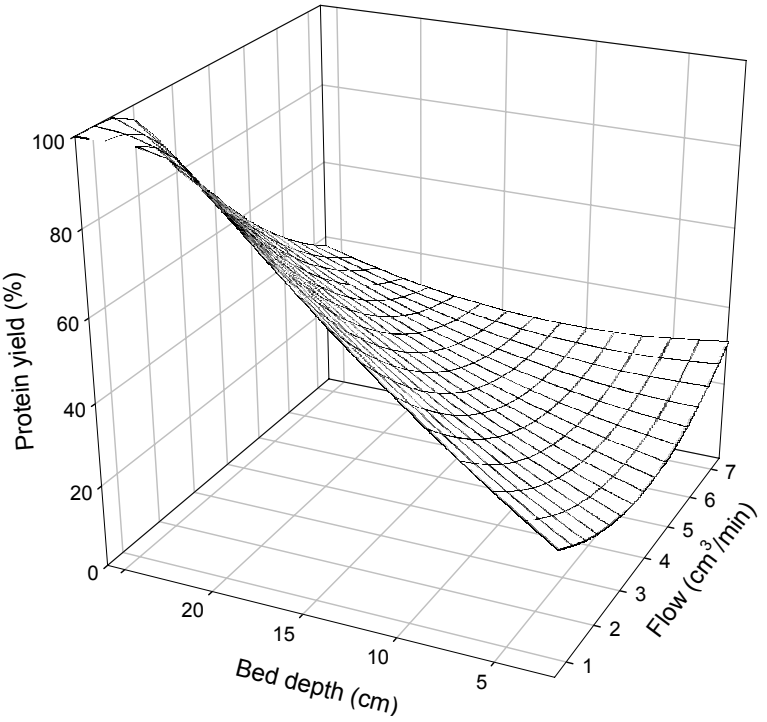


Figure 4.7. Effect of bed depth and flow rate on extraction of protein from flaxseed meal at 180°C with pH 9 buffered water with 1: 1.5 meal to glass beads ratio at a constant S/S ratio 77 mL/g.

CHAPTER 5

Mass Transfer during pressurized low polarity water extraction of lignans from flaxseed meal[1]

5.1 Introduction

Safe, environmentally acceptable and technologically promising bioseparation has become a key research need for industrial and chemical engineering industries in the 21st century (Noble and Agrawal, 2005). Water, an inorganic solvent, exerts high affinity for hydrophilic compounds. As a result, its application in extraction of low polarity compounds is limited (Wongkittipong et al., 2004). Pressurized low polarity water (PLPW) (also referred to as superheated water; subcritical water; pressurized hot water), has been widely promoted as an environmentally benign alternative to orthodox organic solvents (methanol, acetone, ethanol, hexane) due to its ability to manipulate the water polarity beyond the normal boiling point while maintaining water at liquid state. PLPW defines a working temperature between 100 and 374°C (Poliakoff and Licence, 2002; Cacace and Mazza, 2005). Advancement in PLPW extraction is necessary to improve process efficiency and economic potential. Mathematical modeling involving mass transfer parameters (flow rate, temperature, pressure) in food systems is gaining more attention (Welti-Chanes et al., 2005). It is important to develop models for the extraction process where different extraction operating parameters are optimized for process economics (Varzakas et al., 2005). However, such predictions require an understanding of mass transfer mechanisms.

[1]Part of this chapter has been submitted for publication: Ho, C. H. L., Cacace, J. E., and Mazza, G. (2006). Mass transfer during pressurized low polarity water extraction of lignans from flaxseed meal. *Journal of Food Engineering*. (submitted)

The PLPW extraction process usually begins by putting the biomass in a fixed/packed bed inside a cylindrical/tubular extraction cell. The packed sample is in contact with the flowing liquid and the separation process kinetics requires the collection and analysis of solute-enriched extracts in a time-dependent manner. PLPW is effective in altering sample matrices and displacing analytes from their original binding sites. However, few attempts have been documented to differentiate the relative influence of partitioning thermodynamics and desorption kinetics from sample matrices on extraction rates and recoveries of phytochemicals from food samples (Kubatova et al., 2002). Despite the increased interest in PLPW for its application in the separation of bioactives from food, the transport mechanisms of PLPW extraction of plant components are not yet well understood.

The typical solid-liquid extraction process depends on how fast the target compound will leach from the solid matrix, dissolve in the solvent and reach an equilibrium concentration in the liquid. Four major mass transfer steps are generally involved: 1. diffusion of solute through a stagnant liquid film around the solid plant particles; 2. diffusion of solvent into solid particles through the pores; 3. diffusion of the dissolved solute from within particles to the particle surface through the pores; 4. removal by partition from the particle surface into the bulk solvent (Shotipruk et al., 2004; Gertenbach, 2002). The effect of step 1 is typically small and often neglected. Although the diffusion of the dissolved solute within the solid is usually the rate limiting step for most botanicals (Schwartzberg and Chao, 1982; Gertenbach, 2002), partitioning of solute between the solid matrix and solvent have been reported as the rate-limiting mechanism for subcritical water extraction of essential oil from savory (Kubatova et al., 2002).

Therefore, the prevailing mechanism during mass transfer depends on the solute to be extracted and properties of the extracting solvent.

In the present work, the extraction of lignans and other bioactives from flaxseed meal using PLPW was studied. The extraction curves at different water flow rates, temperatures and pHs were used to determine whether the extractions were limited primarily by diffusion of the analyte within the solid matrix, or by analyte partitioning between the sample matrix and surrounding solvent (external mass transfer resistance). Two models were employed to describe the data. The first model is based on Fick's second law. This model is usually used to study solid-liquid extraction kinetics. Fick's model attempts to predict the extraction rates based on the effective diffusion coefficient, D_e. The second model is a two-site exponential kinetic model which attempts to predict the extraction rates using a fast and a slow kinetic rate constant. This model can describe both the rapid and prolonged stages of extraction (Kubatova et al., 2002).

The purpose of this paper is to elucidate the mechanisms controlling the extraction rates achieved with PLPW under different conditions (temperature, pH, co-packing materials, flow rate, bed depth and S/S ratio). The relative importance of the diffusion step and the external mass transfer step were determined during PLPW by varying the extraction flow rate.

5.2 Materials and methods

The extraction apparatus and analytical techniques used to investigate the mechanism of mass transfer have been presented in Chapter 3 and 4. Two mass transfer models, the diffusion model and the two site kinetic model, were used to describe the extraction of lignans, proteins and carbohydrates from flaxseed meal by PLPW.

5.2.1 Mass transfer models

(a) Diffusion model

Mass transfer can be defined as the migration of a substance through a mixture under the influence of a concentration gradient in order to reach chemical equilibrium. The diffusion coefficient (D_e) is the main parameter in Fick's law, and application of this mathematical model to solid foods during solid-liquid extraction is a common way to calculate the effective diffusion coefficient (Crank, 1975). However, Gekas (1992) noted, values of D_e can vary by several orders of magnitude for the same material which may be due to structural changes in the food material during different stages of the process. Therefore, it is important to keep a constant particle size as breakage of cell wall or grinding can reduce the particle size and hence decrease the distance for solute to travel from inside to surface of particle.

Fick derived a general conservation equation for one-dimensional non-steady state diffusion when the concentration within the diffusion volume changes with respect to time, known as Fick's second law (Cussler, 1984; Mantell et al., 2002):

$$\frac{\partial C}{\partial t} = D_e\left(\frac{\partial^2 C}{\partial r^2}\right) \qquad (5.1)$$

with the initial condition:

$$C_{(t=0)} = C_i$$

And boundary conditions:

$$\frac{\partial C}{\partial r}_{(r=r_c)} = 0$$

$$C_{(r=r_b)} = 0$$

where C is the solute concentration (mg/mL) at any location in the particle at time t (s); C_i is the initial solute concentration (mg/mL); D_e is the effective diffusion coefficient (m^2/s) assuming that D_e is constant with the concentration; t is extraction time (s); r is the radial distance from the centre of a spherical particle (m); r_c is the centre of the spherical particle (r=0); r_b is radius of spherical particle (m).

Various solutions of Fick's second law have been presented for the diffusion of a compound during solid-liquid extraction depending on the shape of the particle (Crank, 1975; Schwartzberg and Chao, 1982; Cussler, 1984). An approximate numerical solution to Fick's second law (Eq. 1) for a spherical particle was given by Crank (1975) and Cussler (1984):

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left[\frac{-D_e n^2 \pi^2 t}{r^2}\right] \qquad (5.2)$$

where: M_t = total amount of solute (mg/g) removed from flaxseed meal after time t, M_∞ = maximum amount (mg/g) of solute extracted after infinite time. M_t/M_∞ = ratio of

total migration to the maximum migration concentration, r = average radius of an extractable (flaxseed meal) particle.

When time becomes large, the limiting form of Eq (5.2) becomes:

$$1-\frac{M_t}{M_\infty}=\frac{6}{\pi^2}\exp\left(-\frac{D_e\pi^2 t}{r^2}\right) \qquad (5.3)$$

To determine the effective diffusion coefficient values two methods were used. The first method was a linear (graphical) solution in which D_e was determined from the slope of the ln ($1-M_t/M_\infty$) vs time plot (Dibert et al., 1989). Thus**,** equation (5.3) can be solved by taking the natural logarithm of both sides. It shows that the time to reach a given solute content will be directly proportional to the square of the particle radius and inversely proportional to D_e

$$\ln\left(1-\frac{M_t}{M_\infty}\right)=\ln\frac{6}{\pi^2}-\frac{D_e\pi^2 t}{r^2} \qquad (5.4)$$

where $Slope=\frac{\pi^2 D_e}{r^2}$

The second method of solution used involved nonlinear regression with effective diffusivity (D_e) as a fitting parameter. In this method, the effective diffusivity D_e was estimated from Eq (5.2) using a Microsoft Excel Solver program. The program minimizes the mean square of deviations between the experimental and predicted ln ($1-M_t/M_\infty$) values (Tutuncu and Labuza, 1996). The first 10 terms of the series solution are taken into consideration by the program as the solution to the series becomes stable after 10 terms (n=10).

(b) Two site kinetic model

The diffusion model described above may not be able to predict the kinetics of mass transfer during the initial period of extraction. The mathematical model proposed by So and MacDonald (1986) and Kubatova et al. (2002) used two steps to define an extraction curve: a certain fraction (F) of the analytes desorb at a faster rate defined by k_1, and the remaining fraction (1-F) desorbs at a slower rate defined by k_2. The two-site kinetic model consists of two first order exponential terms:

$$\frac{S_t}{S_0} = 1 - \left[Fe^{-k_1 t}\right] - \left[(1-F)e^{-k_2 t}\right] \qquad (5.5)$$

where t is extraction time (min); S_t is the mass of the analyte removed by the extraction fluid after time t (mg solute/g meal); S_o is the total initial mass of analyte in the matrix (mg solute/g meal). S_t/S_o is the fraction of the analyte extracted after time t, F is the fraction of the analyte released quickly, (1-F) is the fraction of the analyte released slowly; k_1 is the first order rate constant describing the fast-released fraction (min^{-1}); and k_2 is the first order rate constant describing the slow-released fraction (min^{-1}); e is exponential function. The Excel solver regression routine was used to fit data to Eq (5.5).

5.3 Results and discussion

The lignan SDG content of flaxseed meal used was 20 mg/g meal; protein content was 330 mg/g; carbohydrate content was 430 mg/g, lipid content was 124 mg/g, and moisture content was 5.8% (w/w) (Ho et al., 2006). Previously described work on PLPW extraction of lignans, proteins and carbohydrates from flaxseed meal (experiment 1 section 3.3.3) has shown that their yields increased with temperature to a maximum at

about 170-180°C and then decreased with further increases in temperature, irrespective of the solvent to solid ratio (Ho et al., 2006). Lignan, protein and carbohydrate yields also increased with the addition of co-packing materials in a 1:1.5 meal to co-packing glass beads ratio. The increase in yield was observed for lignans and proteins extracted at pH 9, but carbohydrates were slightly more soluble at neutral pH 6.5. Results from experiment 2 section 4.3.3 showed that lignan, protein and carbohydrate extraction yields increased with increased bed depth at an internal diameter ratio (L/ID) of 20-25, the range of flow rates tested (0.6-7.4 mL/min), on the other hand, did not significantly alter the extraction rate.

5.3.1 Mass transfer coefficients

The mass transfer coefficient (K_s) can be calculated from empirical correlations that relate mass transfer with dimensionless numbers. Equations have been proposed to represent the process of mass transfer from a sphere-shaped solid to a fluid. For particles in a fixed bed, the empirical mass transfer correlation for 0.01<Re<50 was described by Perry et al. (1997) as:

$$Sh = 0.828 Sc^{1/3} \mathrm{Re}^{0.5} \qquad (5.6)$$

where Re, Sh and Sc are Reynold, Sherwood and Schmidt numbers, respectively,

$$\mathrm{Re} = \frac{\rho u d_p}{\mu},\ Sh = \frac{K_s d_p}{D_e},\ Sc = \frac{\mu}{\rho D_e} \qquad (5.7)$$

where p and μ are density and viscosity of the fluid, respectively, u is superficial velocity, d_p is the particle diameter and D_e is the effective diffusion coefficient. K_s can then be obtained from the following equation:

$$K_s = \frac{ShD_e}{d_p} \quad (5.8)$$

The calculated values of K_s were then used with the diffusion coefficient to evaluate the mass transfer Biot number:

$$B_i = \frac{K_s r}{D_e} \quad (5.9)$$

where K_s is the mass transfer coefficient in the extract (m/s); r is the particle radius; and D_e is the diffusivity in the solid. The Biot number is used to evaluate whether the mass flow in the system is dominated by internal diffusion or external diffusion. If Biot numbers are greater than 10, diffusion is the controlling stage of the extraction process (Perez-Galindo et al., 2000).

5.3.2 Effect of temperature

Figure 3.8 in Chapter 3 shows the effect of temperature on the extraction of lignan, with maximum recoveries at around 160-190°C. Diffusion coefficient can be strongly affected by temperature as established by the Einstein equation:

$$D\alpha\frac{T}{\eta} \quad (5.10)$$

where T is the temperature and η, the dynamic viscosity. Fick's second law diffusion model can be used to calculate the diffusion coefficients from the experimental data obtained. The increase in extraction rate with temperature was reflected in the increase of diffusivity values (Table 5.1). Both linear (graphical method) and non-linear (non-linear regression) diffusion coefficients of lignan and protein increased with temperatures from 130 to 190°C. When comparing the solution methods, the linear

method produced higher effective diffusion coefficients in all cases. This is due to the differences between the calculations of the two methods to obtain the solutions (Tutuncu and Labuza 1996). The values of diffusion coefficients (both linear and non-linear) ranged from 1.4 to 15.7x10^{-13} m^2s^{-1} for lignan and from 0.7 to 3.5 x10^{-13} m^2s^{-1} for protein (Table 5.1). The effective diffusivities are comparable to values reported in the literature for other solid-liquid extractions. For instance, Wongkittipong et al. (2004) found D_e values ranging from 0.7 to 5.2 ×10^{-13} m^2s^{-1} when extracting andrographolide from leaves using ethanol-water as solvent. Effective diffusivities ranging from 13.26 x 10^{-14} to 105.49 x 10^{-14} m^2s^{-1} were reported for phenolic extraction from grapes using ethanol (Pinelo et al., 2005). Variations in effective diffusivity may depend on several factors, including (but not limited to), particle size, ionic strength, number of polar functions of target molecules, pretreatment method, temperature and concentration of solvent (Cacace and Mazza, 2003).

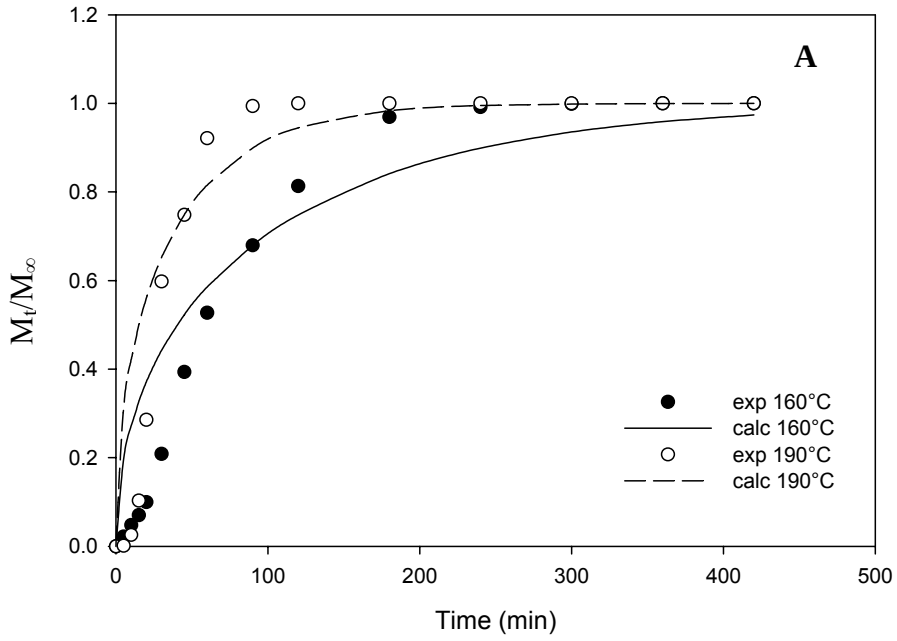
Using Eq (5.2) and the obtained diffusion coefficient, the ratio of lignan and protein concentration removed from the flaxseed meal were calculated. The lowest diffusivity values were obtained for both lignan and protein at low temperatures and pH (Table 5.1), which further indicates that the longer times required for extractions at low temperature and pH were due to reduced diffusivities. Extraction time increased as temperature decreased and the change was more noticeable at pH 4. At pH 9, the favorable effect of pH on extraction yield might have compensated for the reduction of extraction rate caused by lower temperature, preventing a larger increase in extraction time. Figure 5.1 shows a plot of M_t/M_∞ vs extraction time for extraction of lignan and protein using pH 9 buffered PLPW at 160 and 190°C from 2g flaxseed meal with 3g co-

packing glass beads. The effective diffusion coefficient was obtained by substituting M_t/M_∞ with 0.5 and measuring the half time ($t_{1/2}$), which is the time required to reach half of the migrant concentration. Favetto et al. (1981) used the same approach in determining diffusivity and estimating the diffusion profile of salt during immersion cooking of meat. It is worthy of note that at the beginning of the extraction process shown in Figure 5.1, the values predicted by the model were much bigger than the experimental values, so that the actual extraction rate was smaller than that calculated from Fick's second law with constant equivalent diffusivity. A possible explanation for this observation could be either a rapid decrease in concentration of solute remaining in the sample or a reduction of mass transport resulting from the development of a hydrocolloid gum film from material present in the flaxseed meal. The viscous film may retard the dissolution of macromolecules such as protein. The discrepancy between the theoretical curve and the experimental points decreased when temperature increased from 160 to 190°C (Figure 5.1).

An increase in temperature causes a decrease in water viscosity, dielectric constant and therefore an increase in diffusion (Ong et al., 2006; Herrero et al., 2006). The increase in diffusivity with a rise in temperature may also be caused by an increase of the internal energy of the molecules and thus their mobility (Schwartzberg and Chao, 1982). Also, at high temperature, tissue softening may occur and result in cell disruption which leads to increased cell membrane permeability and hence accelerated extraction of solute (Turker and Erdogdu, 2006).

Table 5.1. Values of effective diffusion coefficients for lignans and proteins at different temperature and pH with a fixed meal to co-packing glass beads ratio of 1:1.5 using 420mL solvent volume (S/S 210 mL/g).

		Lignan		Protein	
		Linear	Non-linear	Linear	Non-linear
Temp (°C)	pH	$Dx10^{13}$ (m^2s^{-1})	$Dx10^{13}$ (m^2s^{-1})	$Dx10^{13}$ (m^2s^{-1})	$Dx10^{13}$ (m^2s^{-1})
130	9	2	1.4	1.2	0.7
160	9	5.3	3.5	2.1	1.7
190	9	15.7	10.8	3.5	3.3
130	4	1.4	1.1	1.5	0.9
160	4	3.2	2.6	1.9	1.3
190	4	14.3	10.1	2.8	2.6



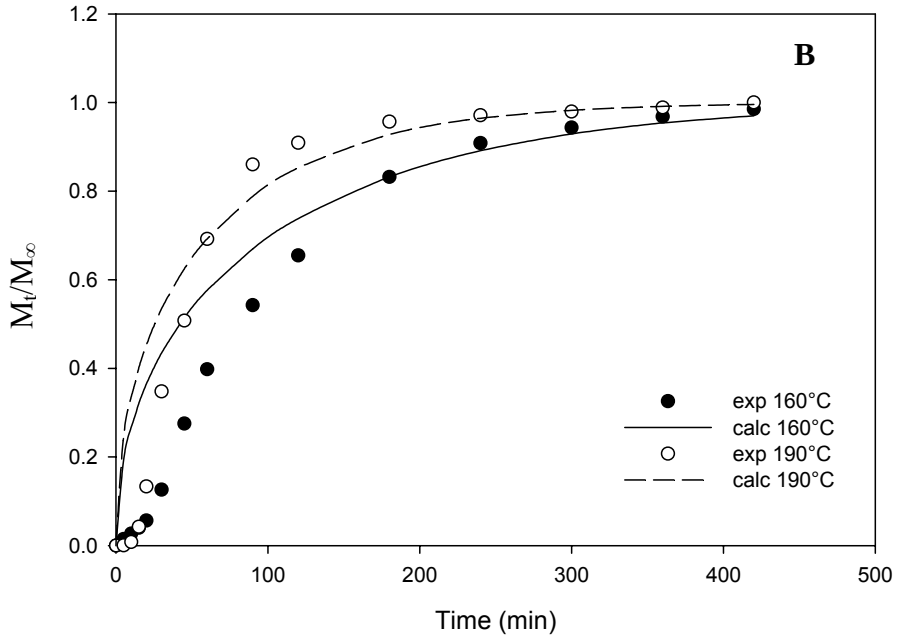
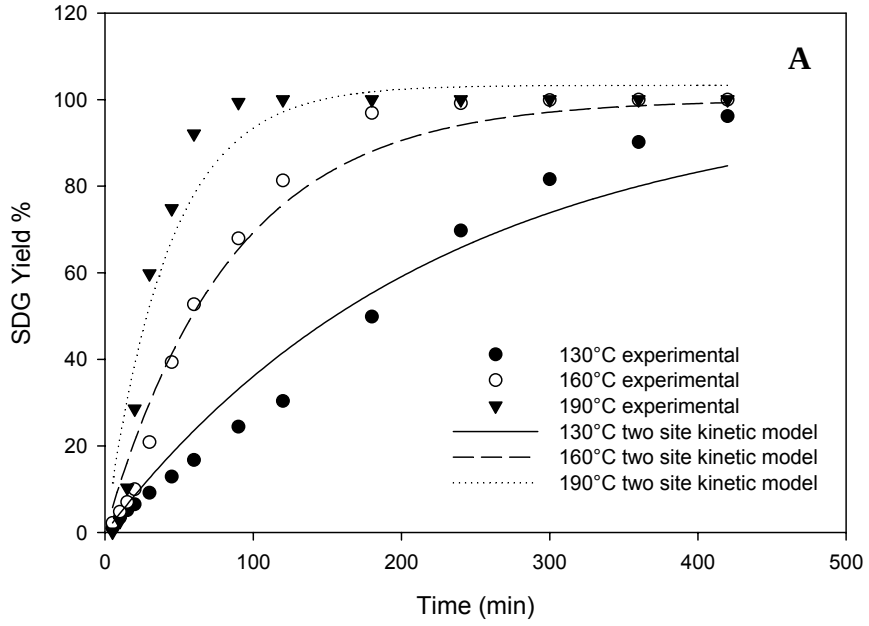


Figure 5.1. Representation of calculated data (line) and experimental data (symbols) using Fick's second law for the extraction of SDG (A) and protein (B) from 2 g flaxseed meal with 3 g co-packing glass beads using pH 9 buffered water at 160 and 190°C.

Apart from diffusion model, the extraction kinetic can be estimated by two site kinetic model. The curves for lignan SDG at three different temperatures were fitted to two site kinetic models (Figure 5.2A). The plots show that the values calculated from the model adequately match the experimental values (R^2>0.96). Elevated temperatures were found to enhance extraction rates and reduce extraction time from 420 min at 130°C to 100 min at 190°C. About 80 to 90% of lignans were extracted in less than 60 min at 190°C (Figure 5.2A). The effect of temperature was higher on the diffusivity of SDG than on the diffusivity of protein; and an increase from 160 to 190°C decreased extraction time from 250 min to 100 min for SDG (Figure 5.2A). However, the problem with the use of very high temperature is that all reactions are accelerated, including unwanted side reactions and degradations (Wongkittipong, 2004). Therefore, the optimum temperature selected for extracting lignans from flaxseed meal was 180°C.

At 190°C, the equilibrium fraction of the fast extracting stage (F) was higher than the equilibrium fraction of the slow stage, (1-F) (Table 5.2). This means that at this temperature, the majority of solute was extracted rapidly (F=0.9, 190°C). The pH and co-packing glass beads had no apparent effect on F values at 190°C. In addition, k_1 and k_2 increased sharply with temperature (Table 5.2). At 160°C, both fast and slow fractions were equally important. The kinetic constants in Table 5.2 at 160°C demonstrated moderate extraction rates. Extraction rate was improved by either increasing pH or addition of glass beads. At low temperature (130°C), the fast extracting stage was not important as the F values were small (Table 5.2).



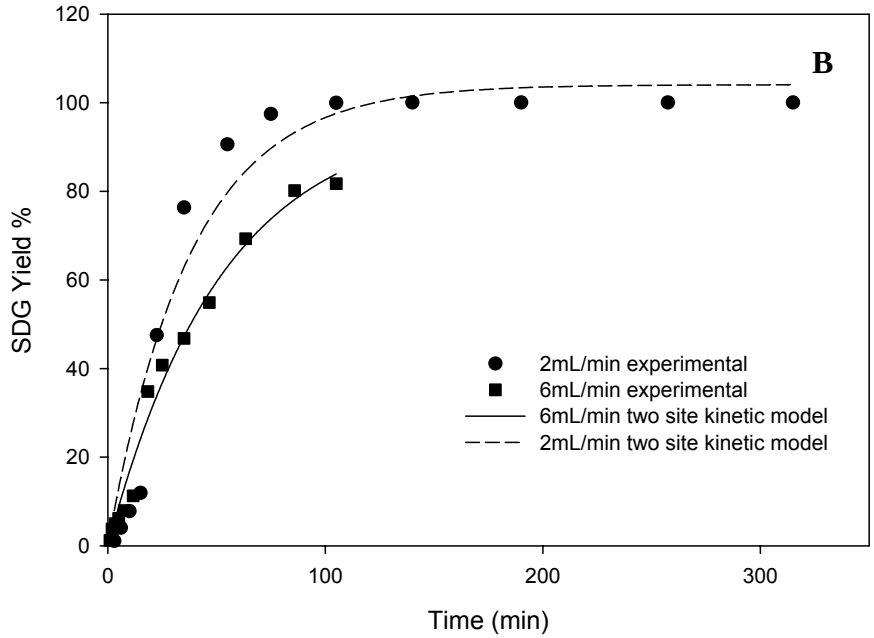


Figure 5.2. Experimental fitting of two site kinetic model to SDG recovery data obtained at various temperatures (A) and at two different flow rates at fixed bed depth 21cm (B) with meal to co-packing ratio 1:1.5 using pH 9 buffered water.

Table 5.2. Values of predicted equilibrium concentrations and kinetic coefficients obtained by fitting a two site kinetic model to SDG extraction data at 1mL/min from 2 g flaxseed meal.

pH	Packing (g)	Equilibrium conc stage 1 F* [a]	Equilibrium conc stage 2 1-F* [b]	Kinetic coefficient min-1 stage 1 $k_1 \times 10^4$ [c]	Kinetic coefficient min-1 stage 2 $k_2 \times 10^4$ [d]
Temp 130°C, 420mL solvent volume (210mL/g S/S)					
4 (-1)	0 (-1)	0.2	0.8	10	43
4 (-1)	3.0 (+1)	0.3	0.7	52	58
9 (+1)	0 (-1)	0.1	0.9	4	28
9 (+1)	3.0 (+1)	0.2	0.8	7	36
Temp 160°C, 420mL solvent volume (210 mL/g S/S)					
4 (-1)	0 (-1)	0.3	0.7	34	61
4 (-1)	3.0 (+1)	0.4	0.6	114	84
9 (+1)	0 (-1)	0.4	0.6	114	84
9 (+1)	3.0 (+1)	0.5	0.5	118	133
Temp 190°C, 420mL solvent volume (210mL/g S/S)					
4 (-1)	0 (-1)	0.9	0.1	505	165
4 (-1)	3.0 (+1)	1.0	0.0	564	822
9 (+1)	0 (-1)	1.0	0.0	503	737
9 (+1)	3.0 (+1)	0.9	0.1	498	145

[a] F (dimensionless) fraction of solute released quickly

[b] 1-F is the fraction of the solute released slowly

[c] $k_1(min^{-1})$ is the first order rate constant for the quickly released fraction

[d] $k_2(min^{-1})$ is the first order rate constant for the slowly released fraction

The values of k_1 and k_2 reflect the shape of the extraction curve as they dictate the inverse of the exponential rise to maximum term in Eq (5.5) controlling the rate of the extraction. When k is small, the curve rises slowly against time due to a slow extraction. On the other hand, when k is large, the extraction rate is high and the corresponding curve showed a sharper increase in yield in a short period of time (higher slope for curve in yield vs time). The increase in k value at high extraction temperature is likely caused by an increase in the diffusion coefficient as mentioned earlier. It is also speculated that such an effect can be the consequence of a polarity barrier and a less permeable solute matrix at low water temperature.

The effective diffusivity values were correlated with the reciprocal of the absolute temperature, according to the Arrhenius equation:

$$D_e = D_0 \exp\left(-\frac{E_a}{RT}\right) \tag{5.11}$$

where D_e is the effective diffusion coefficient (m^2/s); D_o is the temperature-independent pre-exponential or Arrhenius factor (m^2/s); E_a is the energy of activation (kJ/mol) for diffusion, R is the universal gas constant (8.314 J/mol K), and T is the absolute temperature (K). The natural logarithms of lignan and protein diffusivities obtained at 130, 160, 180 and 190°C were plotted as a function of the reciprocal of absolute temperature (1/T) and the energies of activation for lignans and proteins were calculated from the slope (E_a/R) of the straight lines (Figure 5.3). Energies of activation for SDG were 51 and 56 kJ/mol at pH and 9 and pH 4, respectively. Activation energy values for protein were between 20 and 38 kJ/mol at pH 4 and pH 9 respectively. Cacace and Mazza (2003) reported energy of activation values of 70-97 kJ/mol for diffusion of

phenolics from berries. Spiro and Selwood (1984) reported energy of activation for caffeine diffusion through coffee beans and tea leaves to be 32 kJ/mol and 23 kJ/mol, respectively. When diffusivity increased at both pH 4 and pH 9, activation energy was reduced accordingly due to a lower energy barrier for initiation of diffusion. Lower diffusivities are associated with higher activation energy since thermal energy can overcome cohensive (solute-solute) and adhesive (solute-matrix) interactions by decreasing activation energy required in the extraction process (Richter et al., 1996).

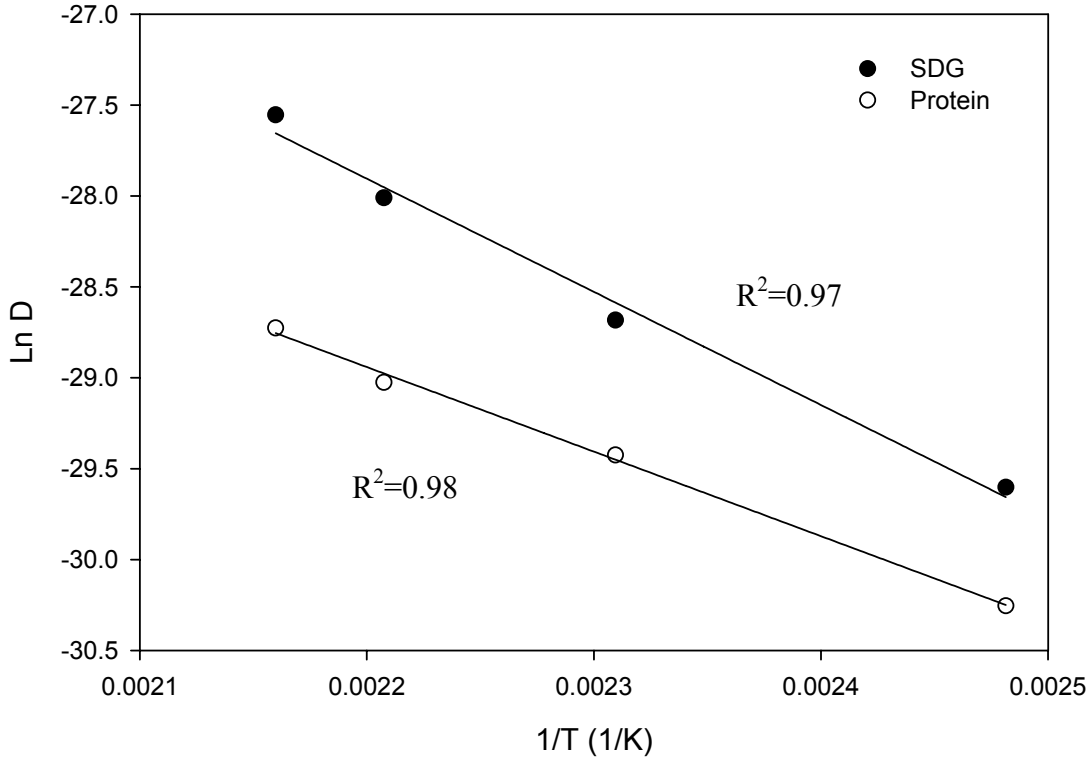


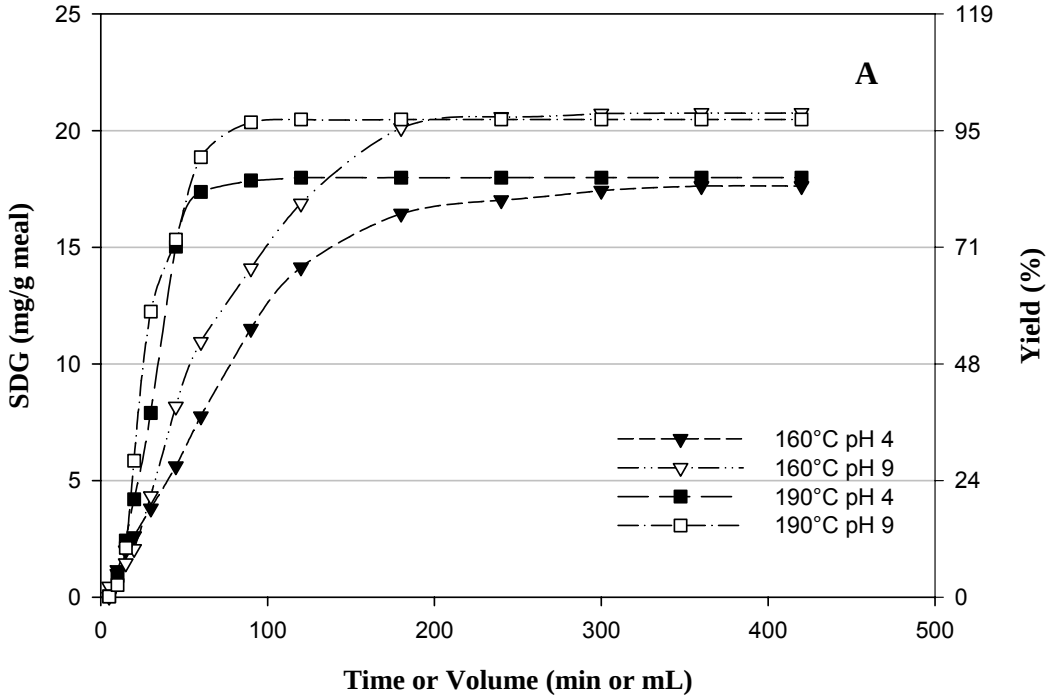
Figure 5.3. Arrhenius-type relationship between effective diffusivity and temperature for SDG and protein using 1 mL/min pH 9 buffered water with 1: 1.5 meal to co-packing material ratio and 420 mL solvent volume (210 mL/g S/S).

5.3.3 Effect of pH

The yields of lignans at 160 and 190°C increased by 10-20% with the increase of pH from 4 to 9 (Figure 5.4A). The pH was the factor that defined the equilibrium yields of SDG irrespective of the temperature. Extraction of SDG from flaxmeal at both 160°C and 190°C reached the same equilibrium yield at pH 4, and similarly at pH 9 (Figure 5.4A). These results suggest that either flaxseed lignans are readily soluble in alkaline buffered water or that they are bound to a macromolecule in which acidic groups are prevalent. The macromolecule is probably a protein, and since flaxseed proteins are known to be highly soluble at pH 9 (Figure 5.4B) (Oomah and Mazza, 1993a), the likely reason for the higher yields of lignans at pH 9 is that alkaline water helps liberate protein-bound lignans from the matrix and make them more accessible for the bulk solvent to carry away the solutes. Alkaline pH may have also hydrolyzed complex polymeric phenolics, reducing them to a more available and easily extracted form (Lee, 2004).

The effect of pH on effective diffusion coefficients for lignans and proteins is shown in Table 5.1. Diffusion coefficients for both lignans and proteins tended to increase with pH at all temperatures tested. This can be attributed to the increased rate of extraction (Figure 5.4) at pH 9 at a given temperature. At 130, 160°C and 190°C, the increase in pH resulted in higher lignan and protein diffusion values (Table 5.1) even though the increase was minor compared to the effect of temperature. The change in pH can enhance the ionic strength of solvents to dissolve target molecules and improve their solubility during extraction (Balke and Diosady, 2000; Xu and Diosady, 2003). Since SDG contains phenol structure, it behaves like other phenolics in term of solubility. SDG is a fairly acidic compound and is essentially insoluble in water. However, in alkaline

media, with the addition of OH^-, the salts of phenol would become soluble in water. Therefore, SDG would be at stable state at alkaline pH and high pH might increase cell permeability leading to higher SDG recovery.



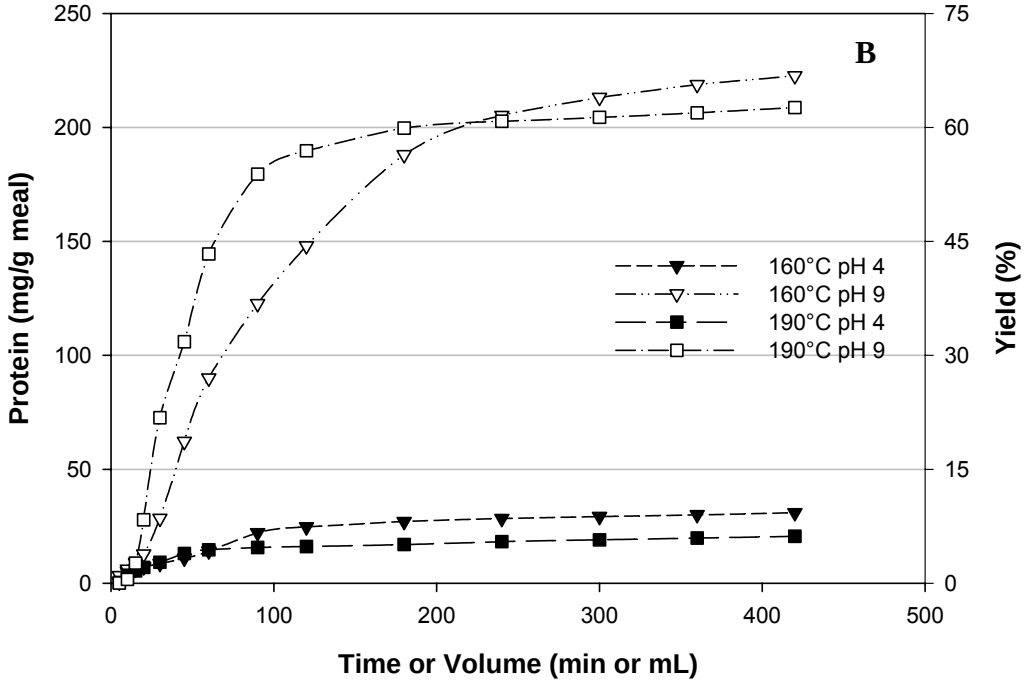


Figure 5.4. Effect of pH and temperature on extraction of SDG (A) and protein (B) with 1mL/min water from 2 g of flaxseed meal with 3 g co-packing glass beads

5.3.4 Effect of bed depth

Lignan yields in the extracts increased with bed depth as briefly mentioned in Chapter 4 (Figure 4.2). The correlation between bed depth and extraction yield was related to longer residence time. Residence time was calculated as a function of bed depth to superficial velocity t=L/*u* where L is bed depth and *u* is the superficial velocity. Therefore, changing the bed depth from 7 cm to 21 cm (Figure 4.2) had a significant effect on both the extraction rate and total recovery of SDG from flaxseed meal. Raising the bed depth from 7 to 21 cm increased SDG yields (20-40%) and extraction time was reduced by about 50-80% at both flow rate conditions. The highest SDG yield (100%) was obtained at a residence time of 9 min while the lowest yield (40%) was observed at 1 min residence time (Figure 4.2; Figure 5.5). In addition, the run at 21 cm and 2 mL/min (9 min residence time) required the lowest S/S ratio to reach extraction equilibrium; whereas runs with 3 min residence time obtained by combination of 2 bed depths and 2 velocities produced almost identical yields utilizing the same S/S ratio (Figure 4.2; Figure 5.5). These results showed that in order to obtain maximum yields in SDG enriched water, the combined effect of bed depth and flow rate are essential for maximum SDG recovery due to variations in residence time. Longer residence time thus allows the solvent adequate time to penetrate through the solid matrix. Liu and Wyman (2003) proposed that longer residence time was partly responsible for enhanced removal of solutes from biomass using PLPW.

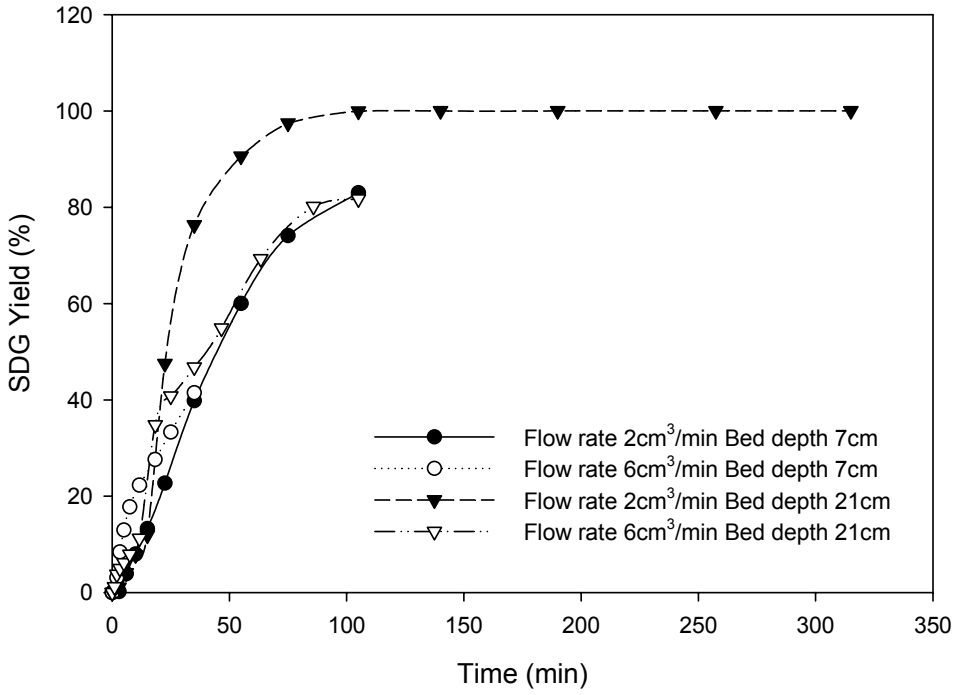


Figure 5.5. Effect of bed depth vs time on extractions of SDG from flaxseed meal at 180°C using pH 9 buffered water with 1:1.5 meal to glass beads ratio. Bed depth 7 cm flow 2mL/min (residence time 3 min, 1.8g meal + 2.7g glass beads); 21 cm 2 mL/min (residence time 9 min, 5.5g meal + 8.2 g glass beads); Bed depth 7 cm flow 6 mL/min (residence time 1 min, 1.8g meal + 2.7g glass beads); 21 cm 6 mL/min (residence time 3 min, 5.5 g meal + 8.2 g glass beads).

5.3.5 Effect of flow rate

The effect of flow rate on lignan yield at a constant bed depth is shown in Chapter 4 (Figure 4.5A). The amount of SDG extracted did not increase proportionally with the flow rate. There were small differences in extraction rate during the initial portion of the curve (first 100 min). Although higher flow rates favor higher concentration gradients between the sample and the liquid, the results indicate that the residence time had a greater impact than the concentration gradient on the mass transfer kinetics of the process. At low flow rates, the solvent had high residence times and had sufficient time to penetrate into particles and dissolve solutes. On the other hand, the residence time of the solvent in the extractor decreased at higher flow rate. The observed phenomenon suggests that extraction kinetics may be more dependent on diffusion coefficients with less dependency on external mass transfer between the solid surface and bulk liquid phase. In fact, high flow rate required larger solvent volumes at any given time implying that high flow rate was not efficient in extracting flaxseed meal components under these extraction conditions. Shotipruk et al. (2004) found that extracted anthraquinone concentration was higher for lower flow rates when plotted against volume of water used. Raising the flow rate of water had little effect on the mass of lignans recovered per unit of time. It is worth noting that the lowest flow rate used (0.6 mL/min) resulted in very high lignan recoveries and low solvent consumption. Thus, high flow rate does not always speed up mass transfer and overall effects depend on internal structure of the food matrix and the extractive capacity of the solvent (Pinelo, 2005). Plots of the ln (1-M_t/M_∞) function against time using Eq 5.4 for the flow rates 2 and 6 mL/min resulted in a straight line with slope $= \frac{\pi^2 D_e}{r^2}$ (Figure 5.6).

Mass transfer coefficients K_s for lignan SDG at various flow rates and bed depths ranged from 4.5×10^{-8} to 2.3×10^{-7} m/s (Table 5.3). The values obtained for the different dimensionless numbers were also presented in Table 5.3. Sherwood numbers ranged from 22 to 93 (Table 5.3). When Sh >20, diffusion is the main controlling step of the extraction process and external mass transfer resistance is negligible (Dibert et al., 1989; Mantell et al., 2002; Simeonov et al., 1999). In other words, the error in D_e due to neglected external resistance will be less than 1% (Schwartzberg and Chao, 1982). Thus, the values were higher than the minimum Sherwood number when assuming negligible external resistance (Table 5.3). Calculations of the Biot number resulted in values from 11 to 47 (Table 5.3). These values are higher than 10 and hence diffusion within the solid controls the process, which agrees with our findings from the flow rate experiments discussed earlier.

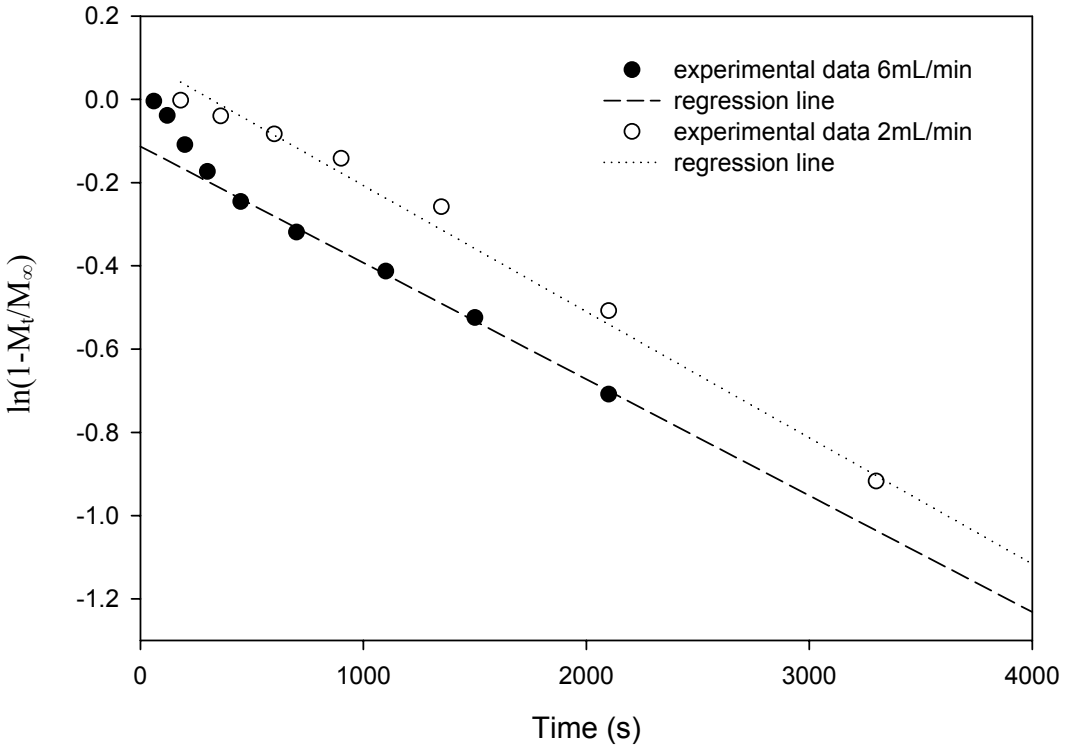


Figure 5.6. Application of linear solution for SDG extraction at flow rate 2 and 6 mL/min respectively with pH 9 PLPW at bed depth 7 cm at 180°C.

Table 5.3. Dimensionless numbers and mass transfer coefficients obtained for PLPW extraction of SDG for conditions studied at 180°C, 5.2 MPa, pH 9 buffered water with 1:1.5 meal to glass beads ratio

Flow (mL/min)	Bed depth (cm)	S/S ratio (mL/g)	Residence time (min)	Depth/ID	u[a] (m/s)	Sh[b]	Sc[c]	Re[d]	Bi[e]	K_S[f] (m/s)
2	7	115	3	6.7	3.8E-04	48.6	6.9E+05	0.5	24.3	5.6E-08
2	21	115	9.1	20	3.8E-04	32.2	2.0E+05	0.5	16.1	1.3E-07
6	7	115	1	6.7	1.1E-03	93.3	9.5E+05	1.4	46.6	7.8E-08
6	21	115	3	20	1.1E-03	56.6	2.1E+05	1.4	28.3	2.2E-07
0.6	14	77	20.3	13.3	1.1E-04	22.1	3.9E+05	0.1	11.0	4.5E-08
7.4	14	77	1.6	13.3	1.4E-03	64.8	2.3E+05	1.8	32.4	2.3E-07
4	25.8	77	5.6	24.6	7.7E-04	45.4	2.0E+05	1.0	22.7	1.8E-07
4	14	77	3	13.3	7.7E-04	69.5	7.2E+05	1.0	34.7	7.7E-08

[a] Superficial velocity
[b] Sherwood number
[c] Schmidt number
[d] Reynolds numbers were calculated using data from NBC/NRC Steam Tables data (Haar et al., 1984):
[e] Biot number
[f] Mass transfer coefficients
p density of water at 180°C, 50 bar = 889720 g/m^3
μ viscosity of water at 180°C, 50 bar = 0.15885 g/ms
dp measured particle diameter of flaxseed meal = 0.000224 m

CHAPTER 6

Conclusions

At present, no satisfactory commercial process has been developed for the extraction of SDG from defatted flaxseed meal. Therefore, the main objective of the present study was to provide an improved process based on the use of an eco-friendly solvent. This study has shown for the first time that lignans, proteins and carbohydrates can be successfully extracted from flaxseed meal with PLPW. In experiment 1, yields of lignans, proteins and carbohydrates were improved using a 1:1.5 ratio of meal to co-packing glass beads. Optimal conditions for extraction of lignans were pH 9 buffered water at 170-180°C and 5.2 MPa (750psi), and 100 mL/g solvent to solid ratio (S/S). Optimal protein yield was obtained at pH 9, 160°C and 210 mL/g S/S. For carbohydrates, a temperature of 150°C, 210 mL/g S/S and pH 4 or 6.5 is recommended. Protein degradation and carbohydrate hydrolysis were lower at 160 than at 190°C.

Based on the results from experiment 1, experiment 2 was performed to investigate the effect of flow rate, bed depth and solvent to solid ratio. Defatted flaxseed meal in experiment 2 was then extracted with pH 9 buffered water and a meal to co-packing glass beads ratio of 1:1.5 at 180°C. The optimal extraction yields for lignan, protein and carbohydrate were found at flow rates of 1 to 2 mL/min with bed depth between 20 and 26 cm and a S/S ratio of 40 to 100 mL/g. The combination of low flow rate and high bed depth (longer residence time) allowed the use of lower S/S ratio with minimum total solvent volume consumption.

The experimental kinetic data of PLPW extractions of lignans, proteins and carbohydrates from flaxseed meal subjected to various processing conditions (temperature, pH, solvent to solid ratio, co-packing materials, flow rate, bed depth) were described by two models. The first model was a two-site kinetic model which successfully described the fast and slow stage extraction kinetics. The second model was derived from Fick's second law and used to calculate the effective diffusion coefficients. High temperature (180°), high pH (9) with 1:1.5 meal to co-packing material allowed a more rapid extraction by increasing diffusivity and reducing activation energy. Further increase in temperature was not recommended due to SDG and protein degradation. Raising the flow rate of water had little effect on the mass of lignans recovered per unit of time since low flow rate (1-2 mL/min) required extraction times similar to high flow rate (6-7.4 mL/min).

Based on the results of this study, PLPW has the potential to develop into a commercially viable technology for the extraction of lignans, proteins and carbohydrates from flaxseed meal without the use of hazardous and expensive organic solvents. Despite all the desirable attributes, PLPW is not without its deficiencies. Other problems to be solved are the separation of desired components at the end of the process both from the extracting water and from co-extracted highly water-soluble plant material in the water extract. For example, the aqueous extract was often coloured like brown tea and frequently contained a precipitate in which the oxygenated compounds are absorbed. From the results of the present studies, areas for further research include:

1. Quantification of other minor components of flaxmeal meal as well as elimination of other interfering factors that may alter the extraction efficiency.
2. Improvements in energy recycling by pre-heating room temperature water from heat generated by post extraction hot water using a heat exchanger as suggested in Appendix 4.
3. Scaling-up the process to pilot plant scale by using larger quantity of biomass and adjusting related process dimensions. This could be achieved by varying variables such as superficial velocity and residence time.
4. Determination of energy and operation costs, as well as product quality and production capacity.

Although PLPW is still at an embryonic stage with respect to full commercial application, it will inevitably find further favour as a revolutionary and environmentally friendly, non-invasive extraction tool that enables production of biobased products from a variety of plant biomass materials including flaxseed meal.

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Appendix 1. Preparation of buffered water for extractions

1. Preparation of pH 4 buffer with 0.1M buffer strength:

Biological buffer selected: citric acid/disodium hydrogen phosphate buffer

- Citric acid monohydrate formula weight: 210.14 g/mol
- Disodium hydrogen phosphate Na_2HPO_4 formula weight: 142.0 g/mol
- Make 100mM (0.1M) citric acid = 0.1mol/L x 210.14g/mol = 21.014g/L or 10.507g/500mL
- Make 100mM (0.1M) Na_2HPO_4 = 0.1mol/L x 142g/mol = 14.20g/L or 7.10g/500mL
- Put 100mL 0.1M citric acid monohydrate in beaker with stir bar (pH 2.31)
- Add 130.2mL 0.1M Na_2HPO_4

2. Preparation of pH 6.5 buffer with 0.1M buffer strength:

Biological buffer selected: citric acid/disodium hydrogen phosphate buffer

- Put 100mL 0.1M Na_2HPO_4 in beaker with stir bar (pH 9.24)
- Add 21mL 0.1M citric acid

3. Preparation of pH 9 buffer with 0.25M buffer strength:

Biological buffer selected: sodium bicarbonate/sodium carbonate buffer

- Sodium bicarbonate $NaHCO_3$: 84.01 g/mol
- Sodium carbonate Na_2CO_3 formula weight: 106.0 g/mol
- Make 250mM (0.25M) $NaHCO_3$ = 0.25mol/L x 84.01g/mol = 21.0g/L or 10.501g/500mL
- Make 250mM (0.25M) Na_2CO_3 = 0.25mol/L x 106.0g/mol = 26.5g/L or

13.25g/500mL

- Put 450mL 0.25M $NaHCO_3$in beaker with stir bar (pH 8.30)
- Add 35mL 0.25M Na_2CO_3

Appendix 2. Preparation of stock solution for direct hydrolysis of SDG

1. Calculations on preparation of solvent 2N sodium hydroxide NaOH

   - M.W. of NaOH = 40.0g/mol

   - 2mol/L x 40.0 g/mol = 80g/L or 80g/1000mL or 40g/500mL

2. Calculations on preparation of solvent 2N sulphuric acid H_2SO_4

   - M.W. of H_2SO_4= 98.0g/mol

   - 1mol/L x 98.0 g/mol = $\frac{98g/L}{1.84g/mL}$ = 53.26mL/L

   - Assume 97% purity $\frac{53.26mL/L}{0.97}$ = 54.91 mL/L

   - For 500mL, we need 27.5mL/500mL

Appendix 3. Moisture content calculation and particle size distribution of flaxmeal

Calculations of moisture content of flaxseed meal were performed using vacuum oven drying and moisture analyzer.

Moisture content was determined using the following formula:

$$\frac{\text{(initial weight of flaxmeal – final weight of flaxmeal)}}{\text{initial weight of flaxmeal}}$$

The moisture content of flaxmeal was 5.31% calculated from the conventional vacuum oven determination method. The moisture content obtained from moisture analyzer (Mettler Toledo) was about 5.06% on average.

Particle size distribution of flaxmeal

Initial wt = 0.99266g

Sieve no. & size	Initial wt	Final wt	Wt on sieve	sample retained
	g	g	g	%
20 (850µm)	45.2373	45.2828	0.0455	4.5836
40 (420µm)	39.7021	39.7564	0.0543	5.4702
60 (250µm)	37.8688	38.1589	0.2901	29.225
80 (180µm)	36.0255	36.2984	0.2729	27.492
100 (150µm)	35.6839	35.8981	0.2142	21.578
400 (38µm)	33.3663	33.4703	0.1040	10.477

Weighed-average of particle size = (850×4.5836+420×5.4702+250×29.225+180×27.492
+ 150×21.578+38×10.477)/98.8258
= 22083.18/98.8258
= 223.4556µm
= 0.2235 mm

Appendix 4. Energy considerations

Because of the bench-top scale of the PLPW extractor used in this research, no real attempt was made to carry out an energy analysis of the process. However, published reports on the comparison of energy requirement for PLPW extraction, steam distillation, soxhlet extraction and supercritical carbon dioxide extraction indicate that PLPW requires less energy than the other three processes (Jimenez-Carmona et al. 1999; Hawthorne et al., 2000a). For PLPW extraction, it is relatively easy to recycle the heat with a heat exchanger that can capture the heat leaving the extraction vessel and use that energy to reheat the water flowing to the extractor. Also, for PLPW extraction, the water used for the extraction is kept in the liquid phase and thus no latent heat of vaporization is required.