University of Alberta

SUBCRITICAL WATER EXTRACTION OF BIO-MOLECULES FROM LENTIL BIOMASS

by

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ABSTRACT

Solubility of glucose and lactose in subcritical water (SCW) was first determined at different pressures and temperatures. Sugar solubility increased with temperature but decreased with an increase in pressure. Then, carbohydrates and phenolics were extracted from lentil husk and cotyledon using SCW. Effect of temperature (120-180°C), pressure (15-120 bar), flow rate (2-5 mL/min), pH (4-10) and ethanol content (0-80%, v/v) in pressurized ethanol+water mixtures were evaluated. For lentil husk, the optimum yield of carbohydrates (60.54±1.32 g/100 g husk), pentosans (18.26±1.47 g/100 g husk) and phenolics (4.78±0.15 g/100 g husk) were obtained at 200°C, pH of 4, 22.8% ethanol (v/v) and 65 bar. For lentil cotyledon, the optimum yield of carbohydrates (61.66±0.72 g/100 g cotyledon) and phenolics (2.05±0.06 g/100 g cotyledon) were obtained at 172.9°C, 80.2 bar and pH of 6.2. The findings demonstrate that SCW extraction is a potential alternative to conventional extraction to obtain carbohydrates and phenolics from lentil.

Keywords: carbohydrates, glucose, lactose, lentil, phenolics, solubility, subcritical water.

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NOMENCLATURE

Symbols

T_b	boiling point temperature
T_c	critical temperature
P_c	critical pressure
ρ	Density
pK_w	ionic strength
ε	dielectric constant
R_m^E	excess molar refractivity
R_m	molar refraction of the solution
R_{mi}	molar refraction of a pure component 'i' in the solution
n_D	refractive index
x_i	molar fraction of a pure component 'i'
M_i	molar mass of a component 'i' of the mixture

LIST OF ABBREVIATIONS

SCW	subcritical water
LC	lignocellulosic
NLC	non-lignocellulosic
ANOVA	analysis of variance
RI	refractive index
SEM	scanning electron microscopy
RSM	response surface methodology
CCD	central composite design

1 Introduction and thesis objectives

Biomass is composed of parts of plants and plant-derived materials. It is the fourth largest energy source in the world with a total production of approximately 146 billion tons/year (Demirbas, 2001). In Canada, biomass production is approximately 278 million tons/year, excluding current forestry operations (Bradley, 2006). The biomass generated is composed of both lignocellulosic (LC) and non-lignocellulosic (NLC) materials (Wayman and Parekh, 1990).

Lentils are composed of both LC and NLC materials with high carbohydrates and protein contents. Cellulose, hemicellulose and lignin are the main organic compounds of LC materials along with phenolics. The lignin present in the LC prevents the biodegradation of cellulose and hemicellulose (Kayhanian and Tchobanoglous, 1992), restricting its use as animal feed. Therefore, it is crucial to understand the structural composition of the LC materials to explore its potential uses.

Extraction of carbohydrates and phenolics from LC and NLC had been conducted using conventional methods that use organic/inorganic solvents and water (Lee et al., 2007, Ronzio et al., 1996, Fan et al., 1987). The main disadvantage of the conventional solvent extraction is the emission of volatile compounds to the atmosphere along with long processing times, ranging from minutes to several hours (Dueñas et al., 2002). Although conventional organic solvent extraction is good to remove phenolics, it is not good to extract lignocellulose (Harmsen et al., 2010). The use of hot water at 100°C has been attempted to remove starch, but it requires the use of hazardous acids or alkali mixtures. The use of steam explosion

has also been attempted but this extraction method is not energy efficient (Zimbardi et al., 2002). Therefore, the treatment of these materials requires developing new processing methods, such as hydrothermal processing, pyrolysis, microwave processing, etc (Singh and Saldaña, 2011, Rexen and Munck, 1984). Recently, the use of subcritical water (SCW) has shown high extraction rates for both LC and NLC materials. Water at high temperature (100 to 375°C) and pressure (5 to 221 bar) is known as the SCW. Its performance as a good extraction solvent is well documented in recent studies (Singh and Saldaña, 2011, Teo et al., 2010). Therefore, the use of SCW shows great potential for efficient extraction of phenolics and carbohydrates from LC and NLC materials of lentils.

1.1 Objectives

The main objective of this thesis was to extract bio-molecules, such as carbohydrates and phenolics from lentil husk (LC material) and lentil cotyledon (NLC material) using SCW technology with(out) ethanol as a co-solvent.

The specific objectives were to:

- Determine the solubility of pure sugars, such as glucose and lactose in SCW.
- Identify and optimize process parameters affecting the extraction of total carbohydrates and phenolics from lentil husk or cotyledon in SCW and pressurized ethanol+water mixtures.
- Compare yields of total carbohydrates and phenolics obtained using SCW and pressurized ethanol+water mixtures with the yields obtained by using conventional extraction.

2 Literature review

2.1 Lentil

Lentil, a food legume, belongs to the *Leguminosae* family with the scientific name of *Lens culinaris* Medic. It is an annual bushy herb, slender almost erect or sub-erect and branched to about 15-75 cm of height (Muehlbauer et al., 1985). Currently, lentils are used almost exclusively for human consumption. Lentils are considered one of the major staple foods in human diet in Southeast Asia and Africa (Clancey, 2009). They are often used as a meat extender or as a meat substitute (Heinz and Hautzinger, 2007) because of the high protein content (25.3-26.9%) (García-Alonso, 1998). Lentil flour is added to cereal flour to make breads, cakes and noodles (Ismail, 2008). In southern Asia, split red lentils are used in curries. Only small amounts (1.5-2.5% of total production) of low-grade lentils are used for feed (Demirbas, 2001).

2.1.1 Lentil production

Most lentil varieties (*Lens culinaris*) grown in Canada have green husks and yellow cotyledons (Goodwin, 2003). Green lentils are divided into three market classes in Canada: large green, medium green and small green. Large green lentils include Laird, Glamis, Sovereign, and Grandora varieties. Medium green lentils include Richlea and Vandage varieties and small green lentils include Eston and Milestone varieties.

Canada has recently become an important producer and exporter of red lentils. Red lentil varieties typically have brown to pale green husks with red cotyledons.

Red lentil production mainly consists of the varieties Crimson and Redwig as well as Blaze, Redcap, and Robin (Goodwin, 2003).

In 2009, 3.92 million tons of lentils were grown worldwide (Fig. 2.1). Canada was the largest producer of lentil with a total production of 1.51 million tons. Saskatchewan is the most important producing province in Canada, followed by southern Alberta. India was the second largest producer of lentils with almost one-third of the total production (0.95 million tons). Canada was also the largest exporter of lentils with revenues accounting to USD 588.2 million in 2009.

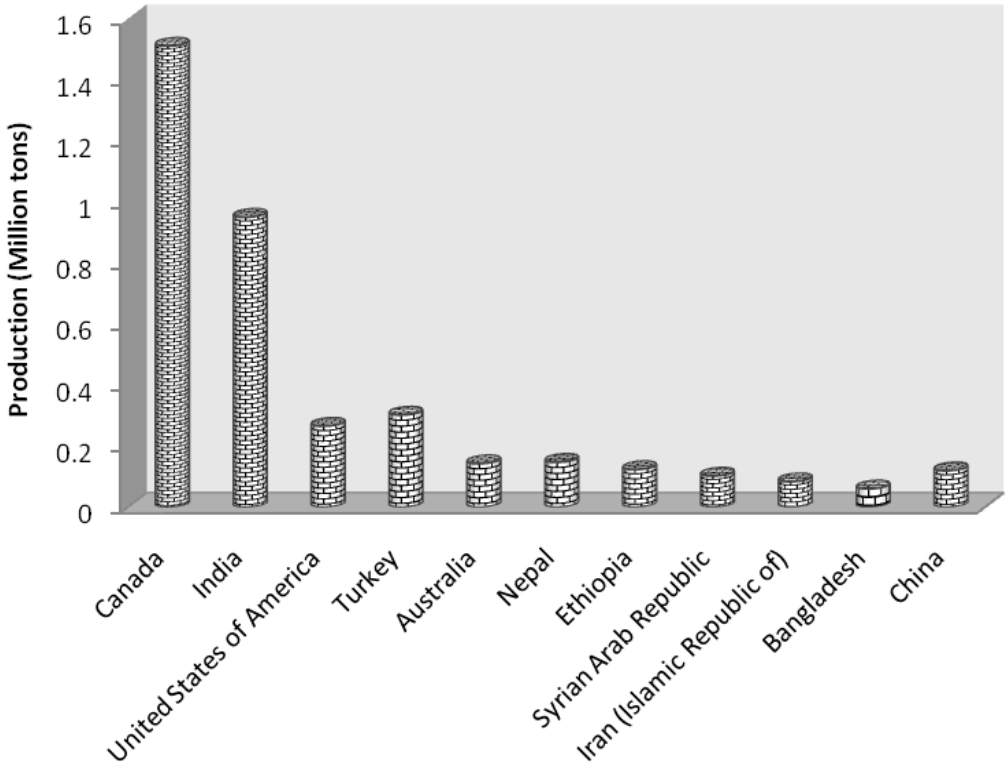


Figure 2.1 Lentil production in 2009 (adapted from FAO, 2009)

2.1.2 Lentil seed composition

Lentils are traditionally known to be rich in proteins and carbohydrates. A 100 g of dried lentil seeds contain 340-346 calories, 12 g moisture, 20.2 g protein, 0.6 g fat, 65 g total carbohydrates, about 4 g fiber, 2.1 g ash, 68 mg Ca, 325 mg P, 7 mg Fe, 29 mg Na, 780 mg K, 0.46 mg thiamine, 0.33 mg riboflavin and 1.3 mg niacin (Muehlbauer et al., 1995). Among pulses, lentil is considered a rich source of amino acids such as, lysine, arginine, leucine, and sulphur containing amino acids (Duranti, 1997). About 90% of lentil protein is found in the cotyledons with albumins and globulins being the major fractions. The starch content ranges from 35 to 53% in the seed and 42% in dry matter. In which amylose varies from 20.7 to 38.5% and amylopectin from 62 to 79% of the seed starch (Sotomayor et al., 1999). Phenolics in lentils are more concentrated in the lentil husk as compared to the lentil cotyledon.

2.1.3 Lentil husk (LC material)

Over 80% of the terrestrial biomass produced annually on earth comes from LC materials, such as crop by-products (straw, chaff and stover) and forest logging activities (Table 2.1). These LC materials represent an underutilized and vastly available renewable resource (>3,720 million tons/year) (Wayman and Parekh, 1990).

Table 2.1 World production of biomass

(adapted from Wayman and Parekh, 1990)

	Production (million tons/year)
LC material	
Crop by-products (straw, stover, etc)	3300
Forest logging residues	360
Plantation forests	60
Cassava tops	45
Sugarcane bagasse	24
Sub-total	3789
NLC material	
Municipal waste	250
Starch	116
Grain, low grade	103
Cane and beet molasses	38
Cassava and potato peel	14
Sub-total	521
Total	4310

Each lentil seed contains almost 10% of lentil husk which is considered inedible for human consumption (Dueñas, 2002). So it is mostly used in the animal feed industry. Canada being the largest exporter of lentils in the world (FAO, 2009) produces almost 0.15 million tons of lentil husk as a byproduct. This husk is rich in cellulose (62 g/100 g, dry basis, db), hemicelluloses (31 g/100 g, db), lignin (2.5 g/100 g, db) and pectin (1.28 g/100 g, db) (Rani and Kawatra, 1994) (Table 2.2).

Table 2.2 Comparison of various biomass sources on basis of cellulose, hemicellulose and lignin (dry basis, db)

(Adapted from NREL, 2011)

Source	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Bamboo	41-49	24-48	24-36
Barley straw	44	27	7
Corn stover	35	28	16-21
Hardwood	45	30	20
Lentil husk	62	31	4
Maize straw	53	15	16.2
Miscanthus	44	24	17
Oat straw	41	16	11
Paddy straw	33	26	7
Poplar	42-56	18-25	21-23
Softwood	42	21	26
Sugarcane	32-48	19-24	22-32
Sweet sorghum	27	25	11
Switch grass	44-51	42-50	13-20
Wheat straw	42	32	10

Cellulose has been traditionally used in the paper pulping industry (Woodings, 1995). Derived products from cellulose like cellulose composites are used as packaging materials (Nishino et al., 2004) and hydrolyzed cellulose is used in fermentation and in pharmaceutical industries to grow microbial cultures (Saeman, 1945, Stemberg and Vuyayakumar, 1977). The high amount of hemicelluloses are converted to biodegradable films or coatings for industrial purposes (Kayserilioğlu et al., 2003) and also used for ethanol production.

Moreover, lentil husk also contains phenolics (4.05 g/100 g, db), which are rich in proanthocyanins (2.22 g/100 g, db) and catechins (1.49 g/100 g, db) (Dueñas et al., 2003). These compounds exhibit antioxidant properties and are regarded as anti-inflammatory, antiviral, antibacterial and antiparasitic (Franco et al., 2008). So they are used extensively in pharmaceutical industries.

2.1.3.1 Cellulose

Cellulose is a polysaccharide consisting of linear long chain carbohydrates (Fig. 2.2a). This linear polymer has glucose units that are connected with α (1,4) glycosidic linkages. These linear polymers in turn aggregate to form fibrils where secondary cell wall fibrils are considerably thick due to aggregation of primary fibrils. Each cellulose molecule contains almost 4500-14000 glucose units. Biomass has about 40-55% of cellulose, depending on the source (Table 2.2).

The degree of polymerization can also vary between primary (<4500) and secondary celluloses (<14000) (Hessler et al., 1984). The degree of hydrogen bonding is unique in carbohydrates structures and is the main reason for the strength of the cellulose molecules. The extent of association between individual cellulose chains confers their degree of parallelism or crystallinity index (Cowling and Kirk, 1976). Both crystalline (highly oriented) and amorphous cellulose is present in plants, with the former structure being more commonly observed. This highly ordered three-dimensional structure confers the mechanical strength of cellulose and also results in its low susceptibility to chemical and enzymatic attack. The disruption of this structure requires severe treatment conditions, which can result in a substantial improvement of cellulose accessibility. But the

existence of external components, e.g. lignin-hemicellulose structures, which are closely associated with cellulose, can also determine the extent of cellulose utilization by, for example, enzymes and microorganisms (Cowling, 1975).

Physical treatment, e.g. ball milling, which affect native characteristics of cellulose (e.g. degree of polymerization and crystallinity index) improve its bio-utilization but limiting chemical barriers remain (e.g. lignin bonding). Chemical treatments (e.g. alkali, acidic and oxidative processes) can disrupt both the native cellulose structure and the associated lignin barriers, resulting in enhanced bio-utilization (Millett et al., 1975).

2.1.3.2 Hemicellulose

Hemicellulose is a branched polymer, primarily composed of pentose sugar joined by β (1,4) glycosidic bonds (Fig. 2.2b). In general, hemicelluloses are polymers of xylose (Fig. 2.2b) and arabinose, forming arabino-xylan molecules. Hemicelluloses are formed of shorter chains of sugar units, ranging from 500-3000 monomer units.

Although considerable differences in sugar composition and degree of substitution can be observed amongst various hemicellulose sources, there are still similarities with respect to their physico-chemical properties and response to a particular treatment. In general, hemicellulose, in particular xylans, can be efficiently solubilized by mild acid-hydrolysis (Saska and Ozer, 1995). To be able to hydrolyze cellulose to a similar extent, there is a need to use considerably harsher acid-hydrolysis or, alternatively, a combination of acid-hydrolysis with

enzymatic treatment. The relative easy extraction of hemicellulosic sugars by chemical methods has led scientists to consider hemicelluloses as a possible source of fuel, feed and chemicals (Overend and Chornet, 1987).

2.1.3.3 Lignin

Lignin is a major component of wood biomass, constituting one-fourth to one-third of the total dry weight of trees. It is the main non-carbohydrate component present in mature plant cell walls. It is an amorphous, high molecular weight condensed polymer of phenylpropane units linked by carbon-carbon (C-C) and ether (C-O-C) bonds. Lignins have two main functions providing: (i) resistance to microbial attack, and (ii) mechanical strength to the cell wall (Summerell and Burgess, 1989). Lignin is a cross-linked racemic macromolecule with molecular masses in excess of 10,000 monomer units. The strong covalent bonds in the complex structure of lignin, for e.g. C-C and ether bonds, makes lignin highly resistant to hydrolytic action of acid and alkali (Vance et al., 1980). These linkages can, however, be disrupted by mild oxidation, e.g. nitrobenzene oxidation, which is very often used for analytical measurements (Wayman and Chua, 1979).

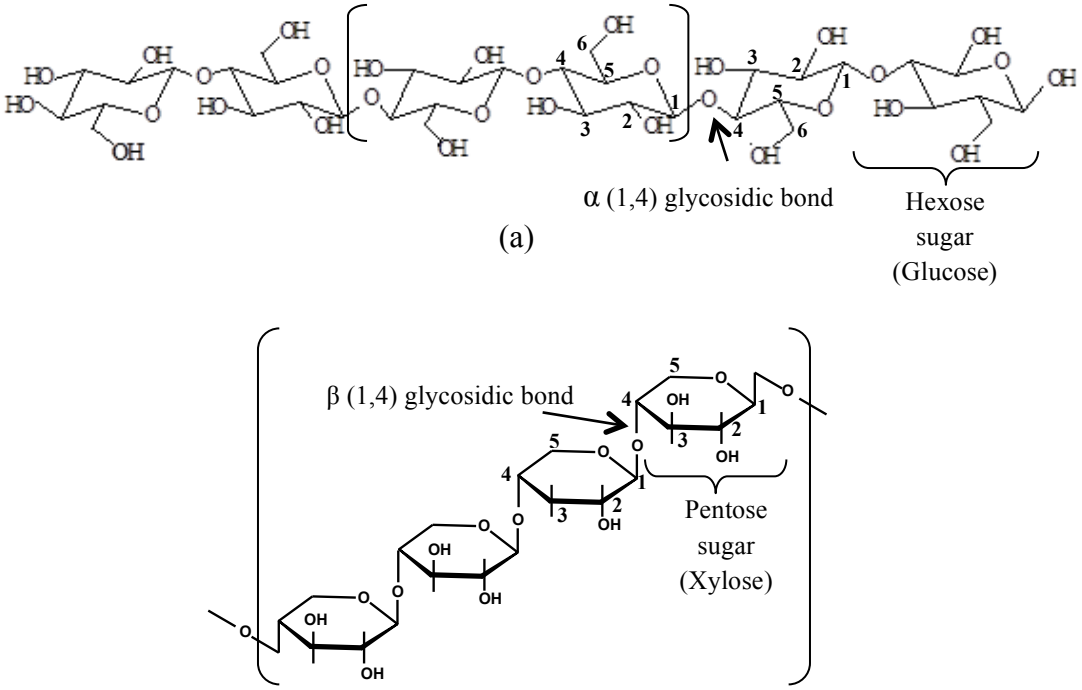


Figure 2.2 Structure of: (a) cellulose and (b) hemicellulose.

2.1.3.4 Phenolics

Phenolics are secondary plant metabolites widely spread throughout the plant kingdom, including more than 9,000 different compounds (Schieber and Saldaña, 2009). Phenolics can be categorized into two groups, flavonoids and non-flavonoids. Phenolics present in the lentils are mostly composed of flavonoids (94-96%) and has a very low amount of non-flavanoids (4-6%) (Dueñas et al., 2002). The functional properties of phenolics are discussed below.

2.1.3.4.1 Phenolics as colorants

The main phenolic colorant present in red lentil (*Lens culinaris* Medic.) is anthocyanin (54% of the total phenolics) (Dueñas et al., 2002). It covers a broad range of colors, including blue, purple, violet, magenta, red and orange. Anthocyanin, a subgroup of flavonoids, contains $C_3C_6C_3$ carbon skeleton. As shown in Figure 2.3, anthocyanins differ in the number of hydroxyl and/or methoxy groups present and sugars, such as glucose, galactose, arabinose and xylose are attached to position R (3 and 5) in the C ring.

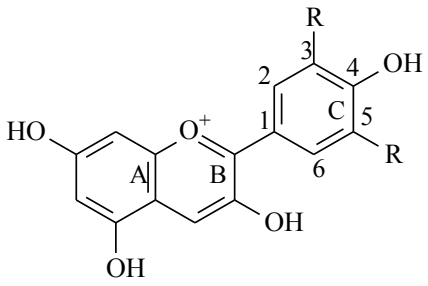


Figure 2.3 Structure of anthocyanin (R-sugars).

When the attached sugars are hydrolyzed, producing the aglycone and sugar, the aglycone is referred as an anthocyanidin. The color of anthocyanins and anthocyanidins in aqueous solution come from excitation of the molecule by light. The strength of color is determined by the relative electron mobility in the structures (Hurwitz, 2007). Since the two colorants have many double bonds, they are readily excited in aqueous solution, those compounds can release color readily in the presence of light.

2.1.3.4.2 Antioxidant properties

Phenolics bind to free radicals to transform them into non-damaging compounds or repairing cellular damage. Flavonoids (94-96% of total phenolics present in lentils) are regarded as better antioxidants than non-flavonoids due to its hydrogen accepting/electron donating (reduction) potential, breaking chain reactions of lipid peroxidation (electron donation) and chelating transition metal ion properties. The reduction potential of flavonoids starts from o-dihydroxyl catechol structure in the B ring, which contains several OH groups (hydrogen and/or single electron donating) and additional gallate group (e.g. epigallocatechingallate) with OH groups.

The antioxidant activity of phenolics depends on their stability, which is related to the number of hydroxyl groups present in it. The increase in hydroxyl groups increases the chemical stability of the phenolic compounds by intermolecular hydrogen bonding through the hydroxyl group (Baum and Perun, 1962).

It is known that consumption of flavonoid-rich foods, especially fruits and vegetables, benefits human health. Epidemiological studies have found associations between lower incidence of heart disease, cancer, gastrointestinal and neurological diseases, liver diseases, atherosclerosis, obesity and allergies (Ramos, 2007, Fresco et al., 2006). Phenolics have positive effects on preventing cancer, cardiovascular diseases and immune disorders (Bao et al., 2004). Duthie et al. (2003) demonstrated that a diet rich in phenolics (phenolic acids, flavonols, catechin monomers, proanthocyanidins, flavones, flavanones and anthocyanins)

decreases the risk of premature mortality from major clinical conditions, like cancer and heart disease.

2.1.4 Lentil cotyledon (NLC material)

Starch from lentil cotyledons (34.7-52.8%) (Ofuya and Akhude, 2005) is the main source of non-lignocellulosic (NLC) biomass (Table 2.1). Starch, a polymer of glucose, is composed of two polymers (amylose and amylopectin). The two polymers are naturally packed into granules with sizes ranging from 2 to 100 μm. The gelatinization temperature of starch is between 52-85°C, depending on the source. The starch granules are dense and insoluble in cold water. In order to dissolve the starch granules in excess water, heat has to be applied. At 80°C, unmodified starch granules form a paste in presence of water with very high viscosity (Belitz et al., 2009).

Cotyledons form almost 90% of the whole lentils (Dueñas et al., 2002). Lentil cotyledons are rich in starch (52 g/100 g, db) and proteins (23 g/100 g, db). It also has soluble carbohydrates (9.5 g/100 g, db), ash (2.6 g/100 g, db) and fat (1.8 g/100 g, db) (El-Nahry and Mourad., 1980). Humans have traditionally used lentil cotyledons as food for a long time as they are rich in starch, but the functionality of proteins is still undetermined. Lentil protein contains lysine and arginine, which are lacking in most other cereal based diets (Duranti, 1997) and can be used in fortification. Lentil starch could be directly used as food thickeners, while; starch-derived products find multidimensional use in the food industry such as, fat replacers (used in low fat yoghurt, cheese and mayonnaise), texture improvers (used in bread, snacks and frying batter), resistant starches (slow digestible

cookies and resistant starch muffin), encapsulating material (used for flavor oils and spice oleoresins), etc. Starches are also modified for stability to high shear and temperature conditions like high temperature gelling starch and starch based dairy desserts (Abbas et al., 2010). Thus starch extraction and processing is economically attractive.

Lentil cotyledons also have soluble sugars like raffinose (13-13.5% of total soluble sugars) and stachyose (28-28.5% of total soluble sugars), which have functional attributes and could be used as value added products (Solanki et al., 1999, Sánchez-Mata et al., 1998). Raffinose has been used as an osmotic agent (Kohan et al., 1998), while stachyose is known to delay consenescence, regulate microeubiosis in the gastrointestinal tract, enhance immunity, eliminate toxins and lower blood fat and pressure (Singh et al., 2008).

2.1.4.1 Amylose

Amylose is a linear chain of linked α-D-glucopyranosyl units. Many amylose molecules also have very few α-D-glucopyranosyl branches (Belitz et al., 2009). Amylose has a right-hand helix linear structure and the inside of this helix is lipophilic. On the outside, there are hydrophilic hydroxyl groups. α-D (1,4) branches may occur once in every 180-320 units, or 0.3-0.5% of the linkages (Belitz et al., 2009). The molecular weight of amylose is about 10^6 kDa. Most starches contain about 25% amylose, but some can have up to 70% amylose (Belitz et al., 2009) like Hi-Maize™ developed by Penford Ingredients (Denver, USA). Lentil cotyledon has approximately 20.7-38.5% amylose content which is common in most leguminous starches (Sotomayor et al., 1999).

2.1.4.2 Amylopectin

Amylopectin is a highly branched polymer. Its molecular weight ranges from 107 to $5x10^8$ kDa. It is considered as one of the largest polymers in nature. It is present in all starches and makes up 75% of the most common starches with 62-79% in lentils (Sotomayor et al., 1999). It has (1-4) and (1-6) α-D-glucopyranosyl units. The structure has double helices (Belitz et al., 2009).

2.2 Extraction of lentils

2.2.1 Conventional extraction

Both LC and NLC lentil biomass can be treated by methods classified as mechanical, chemical and biological treatments. Dilute acid hydrolysis has been investigated using a wide range of catalysts, such as hydrogen fluoride (Hawley et al., 1986), sulfuric acid, nitric acid, and hydrochloric acid (Parisi, 1989) for LC materials. However, when dilute acid hydrolysis was evaluated at a commercial scale, sugar degradation to furfurals and hydroxylmethylfurfurals (HMF) was high (52%) (Parisi, 1989). Humic substances such as furfurals and HMF inhibitory to fermentation were produced and other operating problems, such as acid corrosion and the need of extensive effluent treatment had a negative impact on the overall process and were discouraging (Parisi, 1989).

The use of concentrated acid processes has been usually based on the solubilization of plant polysaccharides in 72% (v/v) sulfuric acid or 41% (v/v) hydrochloric acid at low temperatures (22-32°C), followed by dilution to a 3-6% (v/v) acid concentration and heating at 100 to 120°C for 30 to 360 min (Hawley et al., 1986). Although high yields (85%) can be achieved through this technology,

the process involves high capital investment, high acid consumption and high acid recovery costs (Parisi, 1989).

Conventional extraction of lentil starch has been conducted using alkaline solutions with pH ranging from 8 to 9.5 and temperatures ranging from 22 to 40°C (Lee et al., 2007). This conventional extraction showed that lentil starch and protein could be extracted at high pH (9.5). But, starch still remains mostly in its stable form because it does not reach the gelatinization temperature (65-66°C) and so the amylose or amylopectin chains could not be broken. Thus starch finds less utilization than soluble sugars (glucose, sucrose or maltose). Moreover, the process is cumbersome and time consuming (4 h of extraction) and not suitable for high productivity (lee et al., 2007).

Saccharification of cellulosic residues has also been accomplished using highly specific enzymes (cellulase, endoglucanases, cellobiohydrolases, β-glucosidase) (Fan et al., 1987, Wood and Saddler, 1988, Wood and Garcia-Campayo, 1990). However, efficient enzymatic hydrolysis requires a pretreatment to break the structure of lignocellulosics (Parisi, 1989, Fan et al., 1987, Wood and Saddler, 1988). Even starch requires some form of pretreatment such as, dry and wet milling, alkaline extraction, etc. (Lee et al., 2007) to enhance its rate and efficiency of hydrolysis (70-90%) (Maher, 1983b). The ease with which starch substrates are hydrolyzed can be increased by milling, which enhances swelling and increases surface area of the substrate. Lignocellulosics, however, require more drastic measures to increase accessibility because they have been primarily designed by nature to act as structural materials. In order to make pretreatment an

economically competitive process, the method must also result in high recovery yields of hemicelluloses and lignin for further utilization (Nguyen and Saddler, 1991).

Considerably more research has been directed into chemical treatments, as these are frequently found more efficient in treating biomass and more feasible than other types of treatments. The main disadvantage of chemical treatments is the large requirement for chemicals, which cannot always be recycled and often creates environmental problems. Additionally, when considering that one of the important reasons for utilizing biomass is that these materials are a source of renewable energy and that the use of chemicals, which are directly produced from fossil fuels, necessary for its treatment is often seen as a serious disadvantage.

To overcome this dilemma, the use of subcritical water (SCW) for performing hydrolysis of these materials is growing. SCW treatment affects cell wall structure as a result of an acid-hydrolysis type of reaction (Baugh and McCarty, 1988). Production of organic acids from acetyl and formyl groups present in hemicellulose leads to acidolysis of cell wall components. Solubilization of hemicellulose and depolymerization of lignin are the main results of this reaction. The water-soluble fraction containing easily fermentable carbohydrates (hemicellulosic sugars) could be used for many biological processes, such as alcohol and single cell protein production or as an animal feed. Production of chemicals, such as furfural and 5-hydroxymethylfurfural is an alternative use for SCW treatment (Sproull et al., 1985). The solid fraction, rich in cellulose, can be

used as a raw material for the pulp industry, for alcohol production or as a ruminant feed.

Some researchers (Jensen et al., 2007, Franco et al., 2008) have shown that ethanol is a good solvent for phenolics extraction from plant sources, such as grapes, rose and hazelnut. Moreover, ethanol is less toxic than methanol (Hufferd, 1932) or acetone (LeBlanc and Surprenant, 1983) and is still used for conventional extraction processes (Franco et al., 2008).

2.2.2 Subcritical water (SCW) extraction

2.2.2.1 SCW

In the phase diagram of water (Fig. 2.4), when the temperature of water is brought above its boiling point (T_b) and sufficient pressure is applied to prevent its transition to the gaseous state, the water is said to exist in a subcritical state up to its critical temperature (T_c) and pressure (P_c). Supercritical water is obtained when the temperature of water exceeds its critical temperature (T_c = 374°C) and critical pressure (P_c = 221 bar). Under these conditions, supercritical water behaves both as a liquid and as a gas. In subcritical state, water undergoes important changes in its physical and electrochemical properties, such as density, viscosity, diffusivity, specific heat capacity, static dielectric constant and ion product.

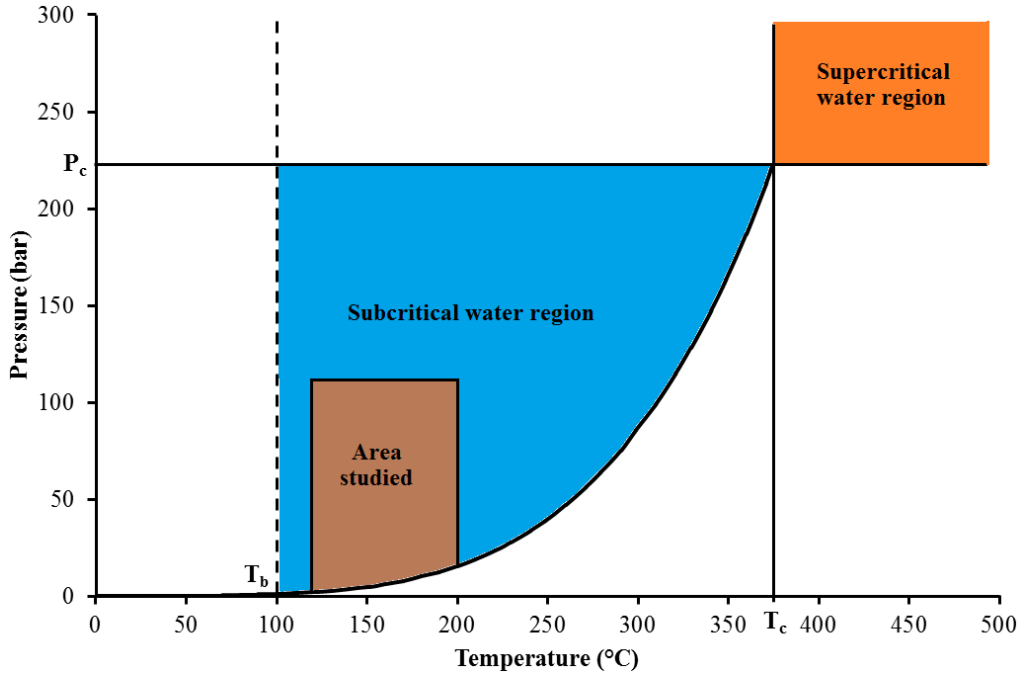
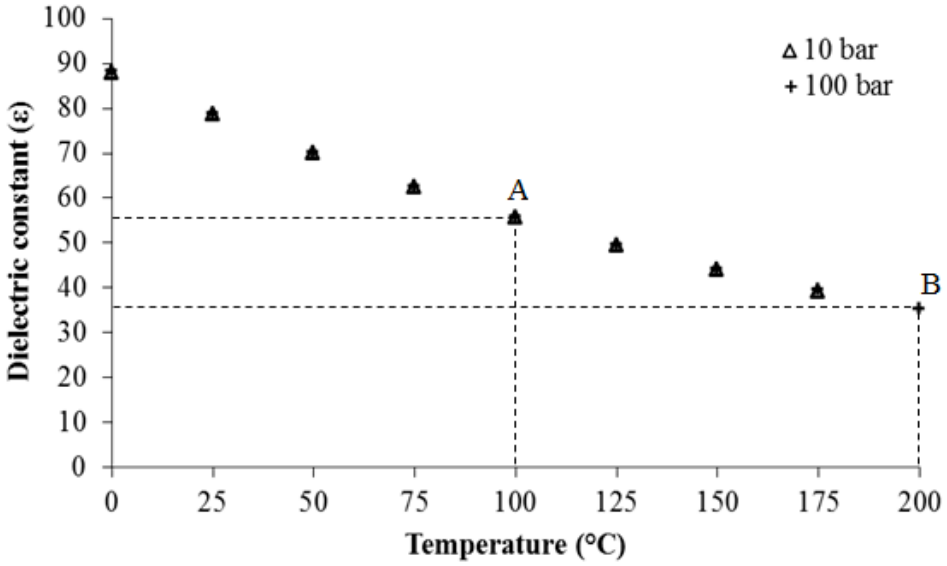


Figure 2.4 SCW region in the phase diagram of water (T_c – critical temperature, 374°C, P_c – critical pressure, 221 bar, T_b – boiling point, 100°C).

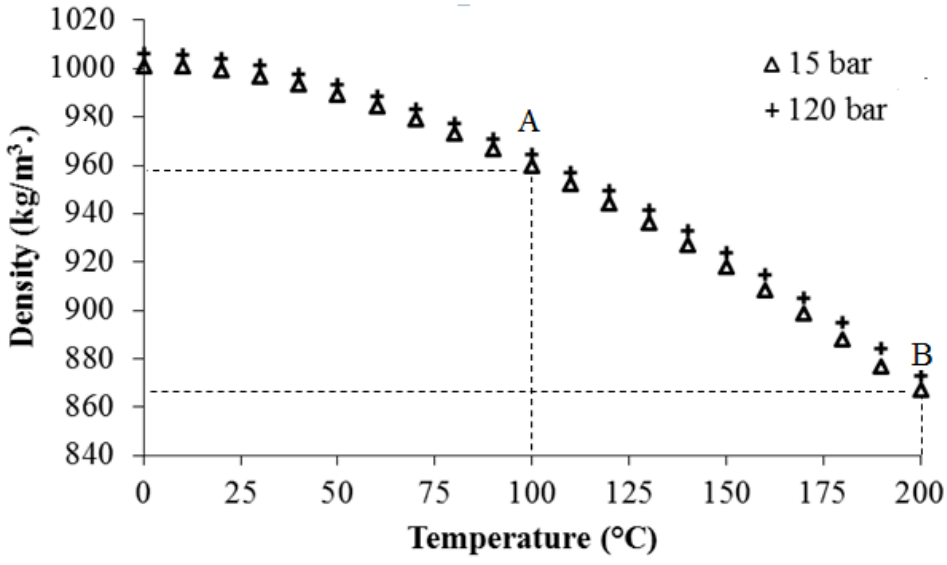
The solvent property of water changes significantly in the subcritical region (Fig. 2.4). The organic solvents become partially soluble in the SCW due to the low polarity of the water at the subcritical state. The decrease in the polarity is due to the decrease in the dielectric constant of water, which decreases with increase in temperature (from point A→B) (Fig. 2.5a). For example, water at 25°C and atmospheric pressure has a dielectric constant (ε) of 80, mostly due to hydrogen bonds (Yesodharan, 2002), but at 100°C, the dielectric constant (ε) reduces to 55 (point A), which is near to formic acid (ε = 58), and as the temperature reaches 200°C, the dielectric constant (ε) becomes 35 (point B), similar to that of methanol at 25°C.

High temperature and high pressure reduce the surface tension and viscosity of water, which results in enhanced solubility, allowing better penetration into the

matrix and resulting in better extraction. Increasing the temperature breaks the hydrogen bonds and the density and viscosity of water decreases rapidly. As the density decreased from 960 kg/m^3 at 100°C (point A) to 870 kg/m^3 at 200°C (point B) (Fig. 2.5b), the diffusivity of the water increased, enhancing its penetrating power.



(a)



(b)

Figure 2.5 Properties of SCW as a function of temperature.
(a) dielectric constant at 10 bar and 100 bar, (b) density at 15 bar and 120 bar
(adapted from Bröll et al., 1999 and Yesodharan, 2002)

The ionic strength (pK_w) of water varies with the density and temperature. At $pK_w < 14$, water is more suitable for heterolytic reaction while at $pK_w > 14$, it is suitable for hemolytic reaction (Bröll et al., 1999). The physicochemical properties of subcritical and supercritical water are summarized in Table 2.3. Here, the SCW was selected due to its relatively low density, dielectric constant and ionic strength that could facilitate the extraction of carbohydrates and phenolics at much lower temperature conditions compared to supercritical water (over 374°C) or superheated steam (>400°C), preventing degradation of the extracts.

Table 2.3 Properties of water (adapted from Bröll et al., 1999)

Parameters	Normal water	SCW	Supercritical water	Superheated steam
Temperature (°C)	25	100-374	374-400	400
Pressure (bar)	1	2-250	250-500	1
Density (kg/m^3)	1000	170-800	58-170	3
Dielectric constant (ε)	79	6-58	6-11	1
Ionic strength (pK_w)	14	11-12	11-19	-

2.2.2.2 Parameters affecting SCW extraction

Temperature is regarded as the most important parameter in SCW extraction processes for extraction of carbohydrates (Ho et al., 2007, Bobleter, 1994) and phenolics (Singh and Saldaña, 2011, Shalmashi et al., 2007, Rangsriwong et al., 2009). The change in the temperature of water changes its dielectric constant,

thus altering its solvent properties as discussed in the previous section. When the temperature of water is increased, the surface tension and viscosity of the water are reduced, while diffusion and thermal desorption of the analytes are increased (Andersson, 2007), improving the mass transfer of compounds of interest during the extraction.

The effect of temperature on the extraction of carbohydrates from flaxseed meal was studied by Ho et al. (2007) with temperatures of 130-190°C, flow rates of 0.5-1 mL/min and an unknown pressure. They found that the increase in temperature accelerated the extraction while reducing the time to reach equilibrium. The positive effect of temperature by using SCW on pure biopolymers (starch and cellulose) was reported by Rogalinski et al. (2008a). They hydrolyzed biopolymers at 190-310°C with a flow rate of 0.5 mL/min, residence time of 1-8 min and an unknown pressure. They proposed that at high temperatures (above 200°C), the glycosidic bond present in the hexose monomers of cellulose break, releasing bioactive compounds that are trapped in the polymer matrix. Similarly, Bobleter (1994) found that the hemicellulose from softwood plants solubilize completely at moderate residence time (17 min) and a high temperature (180°C).

An increase in temperature disrupts the solute-matrix interactions and increases the capacity of solvents to solubilise the analytes. These factors and the improved mass transfer due to the decrease in water density and viscosity enhance the recoveries. Relatively low temperatures are sufficient for extraction of polar compounds, but high temperatures (250-300ºC) are required when hydrophobic

organic compounds, such as polycyclic aromatic hydrocarbons (PAHs) are extracted from soil and air particulates (Alvarez and Saldaña, 2011, Hawthorne et al., 1994). It is not practical, however, to apply very high extraction temperatures (over 374ºC) as the supercritical water can cause corrosion of the high pressure vessel due to surface oxidation (Hayward et al., 2003). The analytes may also degrade or otherwise react at high temperatures. The selectivity in the extraction is often lost at high temperatures, and substantial amounts of matrix compounds are extracted along with the target analytes, leading to post-processing of the extract. Degradation of the analytes and co-elution of matrix compounds is a particular problem when food and plant materials are extracted at high temperatures.

Unlike in supercritical water extraction, pressure does not have a marked effect on recovery organic pollutants from solid environmental samples using SCW extraction (Hawthorne et al., 1994). As long as the physical state of the water is not changed, the effect of pressure on the recovery is small. In SCW extraction, the pressure needs to be high enough that water exists in liquid state. As the relative permittivity of water increases with pressure, a large pressure increase leads to high recoveries of non-polar compounds (Kubátová et al., 2012).

Steam has proved to be more effective than liquid water in the extraction of non-polar organic compounds for two reasons (Hartonen et al., 2009, Yang et al., 1995). First, the relative permittivity of steam is lower than that of liquid water at the same temperature, and lower relative permittivity favors the extraction of non-polar compounds. Secondly, steam spreads more uniformly through the sample in

the extraction vessel because of its lower viscosity, and it diffuses more effectively than liquid water, and thus undesired channeling, leading to lower recoveries is avoided. On the other hand, the capacity of steam to dissolve analytes is lower due to the low density. Thermal desorption is dominant with steam.

Flow rate affects the recovery in SCW. An increase in the flow-rate increases the amount of solvent available to dissolve the target compound, thus improving recoveries. This hypothesis has also been supported by the study of Liu and Wyman (2003) who found that there is significant increase in the recovery of xylose from woody biomass when the flow rate of water increased from 1 to 10 mL/min at subcritical conditions (200°C and an undisclosed pressure).

2.2.2.3 SCW reactions

For treatment of biomass in SCW, the primary reaction that occurs is the depolymerization of biomass by breaking the glycosidic linkages. This is mainly dependent on acids that are released from the biomass (e.g. acetic acid from acetylated hemicelluloses) (Ramos, 2003). During SCW and supercritical water treatment, the sugar degradation occurs due to four processes: pyrolysis, oxidation, dehydration and Maillard reaction. Pyrolysis, oxidation and dehydration commonly occur at high temperatures (above 220°C). Pyrolysis occurs in the absence of oxygen, resulting in thermal decomposition of organic matter (Brownell and Saddler, 1984). Oxidation promotes degradation of organic matter to carbon dioxide and water and also contributes to a partial conversion of pentoses to carboxylic acids (Springer and Harris, 1982). Dehydration occurs with

high temperature treatments, producing furfural and hydroxymethylfurfural from pentoses and hexoses, respectively (Springer and Harris, 1982, Brownell and Saddler, 1984).

The most common reaction occurring in food materials during SCW extraction at temperatures of 120-200°C is the Maillard reaction (McEvily et al., 1992, Willits et al., 1958). It is the most common type of non-enzymatic browning reaction of food. First reported in 1912, Maillard browning involves a series of chemical reactions that occur when amino acids and reducing sugars are heated together (McEvily et al., 1992). The sugar interacts with amino acids, producing various aromas and imparting color to foods. The type of amine and reducing sugar influences the reaction rate as well as the products formed, which ultimately are brown melanoidin pigments (Willits et al., 1958). There are three basic phases of Maillard reaction: (i) initial reactions between a reducing sugar (glucose) and an amino acid (lysine), involving generation of glycosyl-amino products by losing one water molecule, producing an imine that is able to cyclise. Instead of glycosylation, it can undergo Shiff's base rearrangement to yield the Amadori product, (ii) intermediate reactions involving the fragmentation of these Amadori products to 1- and 3-deoxydicarbonyl compounds (deoxyosones). The deoxyosones undergo Stecker dehydration with amino acids to form aldehydes and condensation to aldols (Fig. 2.6); and (iii) final reactions involving aldol condensation, polymerization, and the production of heterocyclic nitrogen compounds and melanoidins (O'Brien et al., 1998).

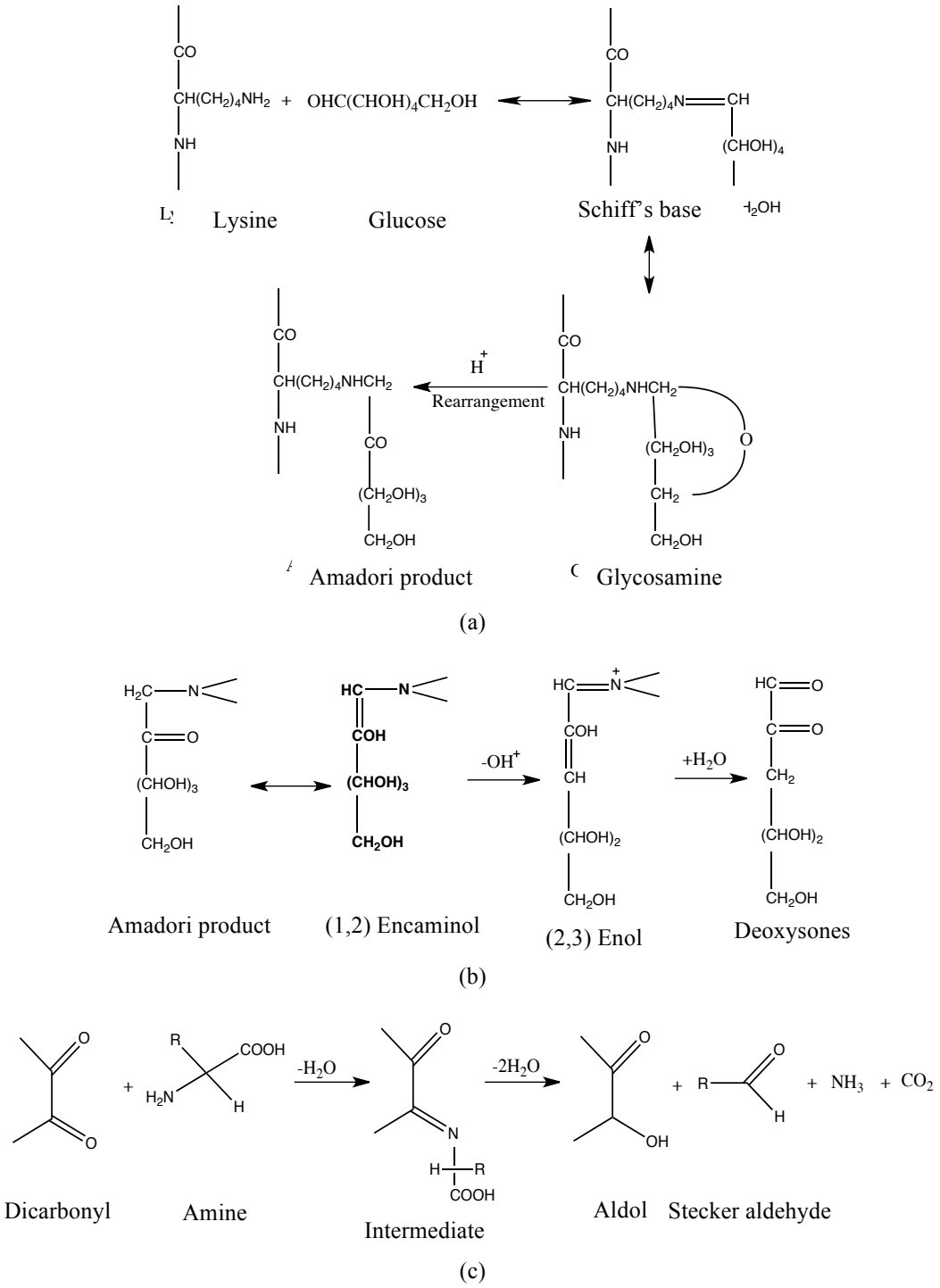


Figure 2.6 Maillard reaction: (a) formation of Amadori products, (b) Amadori rearrangement, (c) Stecker dehydration (Adapted from Laroque et al., 2008)

2.2.2.4 Solubility of sugars

Physicochemical data of sugars, particularly, their solubility in water or binary solvents, such as ethanol + water (Peres and Macedo, 1997) plays an important role in designing and optimizing unit operations, such as extraction, reaction and crystallization. Thermodynamic properties of sugars, such as melting point and density act as critical factors in determining their solubility and stability at high temperatures and pressures. The need for solubility data is of particular importance in the food industry, where sugars are used as sweeteners, in the pharmaceutical industry, where they are used as drug delivery systems (Rogers, 1999, Cummings, 1995, Davis and Robinson, 2002) and in biomass conversion processes, where solubility data near or above 100°C are crucial to select process parameters like temperature and pressure (Kruse and Gawlik, 2003, Yu and Wu, 2009, Liu and Wyman, 2003).

Due to the vast number and nature of monosaccharides and polysaccharides, there is interest in the determination of solubility of these compounds in water. These mixtures, containing low and high molecular weight, can easily be found as ingredients in some industries, including bio-ethanol and food processing industries. The aqueous solubility is the maximum quantity of solid dissolved in water, an important property to develop a subcritical process. Refraction index is measured to determine particle size of polymers and crystals (Cariou et al., 1986, Lai et al., 2005, Singh, 2001) and solid concentration of phytoplanktons (Aas, 1996) and corn syrup (Kurtz and Eliason, 1979). It has been successfully applied because of its accuracy and simplicity.

Currently, solubility data available as a function of temperature is limited to boiling point temperature of water at atmospheric pressure. Due to the above-mentioned uses of sugars in industrial and manufacturing processes, it is important to obtain solubility of sugars in the subcritical region. A search for solubility data of sugars in the SCW region yielded very few results particularly for two reasons: i) experimental difficulties for the measurements at high temperatures and pressures, and ii) possible sugar degradation to aldehydes or furans at temperatures above 280°C (Usuki et al., 2008, Haghighat Khajavi et al., 2005, Haghighat Khajavi et al., 2006, Qi and Xiuyang, 2007). Most sugar solubility data in SCW have been reported at temperatures below 100°C (Koivistoinen et al., 1980, Taylor, 1957, Bates, 1942, Stephen and Stephen, 1963, Young, 1957) or in binary solvent mixtures, such as ethanol+water. Detailed study of sugars (glucose, lactose, leucrose, maltose, raffinose, sucrose, and trehalose), polyols (maltitol, mannitol, sorbitol, and xylitol), and polysaccharides (β-cyclodextrin, dextrans, and inulin) was carried out by Bouchard et al. (2007) at atmospheric pressures up to 100°C. Solubility of D-glucose was studied by Peres and Macedo (1997) for alcohol/alcohol systems, such as methanol+ethanol at various concentrations. Solubility of D-xylose and D-mannose in ethanol+water binary solvents, below 100°C and at atmospheric pressures was reported by Gabas et al. (1988) and Martínez et al. (2011).

A compilation of solubility data in SCW obtained from different sources is presented in Table 2.4. Solubility data reported up to 83°C is not consistent. This could be attributed to the different experimental techniques used to determine the

solubility and the water quality used for the experiments and analysis. The values reported by the U.S. National Bureau of Standards (Bates, 1942) are not in agreement with the ones reported in the literature (Yalkowsky and He, 2003, Young, 1957, Peres and Macedo, 1997). Young (1957) and Dehn (1917) reported higher solubility of D-glucose in distilled water compared to data reported by Yalkowsky and He (2003) and Zhang et al. (2010), who used ultrapure water. Moreover, most of the researchers with the exception of Zhang et al. (2010) used a batch process to determine the solubility of sugars at different temperatures. Zhang et al. (2010) reported solubility of D-glucose, D-mannose and D-xylose at temperatures above the boiling point (30-183°C). They found unexpectedly low solubility and char formation in the high pressure vessel, which indicated degradation of sugar molecules at temperatures above 140°C, when the flow rate of water was below 0.5 mL/min. This means that an increase in the residence time of the sugar+water solution from 1s to 5s in the high pressure vessel increases its degradation.

Table 2.4 Solubility data of sugars in water as a function of temperature

Temperature (°C)	Density (g/mL)	Glucose (g/g water)	Xylose (g/g water)	Sucrose (g/g water)	Lactose (g/g water)
25	0.997[a]	0.480[1], 1.036[4], 0.608[5], 0.820[6], 0.467[7]	0.433[1], 0.122[2], 0.672[3], 0.464[7], 0.701[9]	0.682[1], 2.120[8]	0.291[9], 0.217[10]
42	0.991[a]	0.620[1], 1.644[4], 0.679[5], 0.569[7]	0.739[3], 0.655[7]	0.711[1], 2.401[8]	0.338[10]
62	0.982[a]	0.750[1], 2.932[4], 0.711[7]	0.930[3], 0.868[7]	0.761[1], 2.925[8]	0.660[10]
83	0.970[a]	0.840[1], 4.413[4], 0.758[7]	1.175[7]	3.892[8]	
104	0.956[b]	1.214[7]	1.717[7]		
123	0.941[b]	2.296[7]	2.211[7]		
144	0.923[b]	3.186[7]	2.850[7]		
165	0.903[b]	3.778[7]	4.000[7]		
183	0.884[b]		4.798[7]		

[1]Yalkowsky and He (2003), [2]Gabbas et al. (1988), [3]Jonsdottir et al. (2002), [4]Bates (1942), [5]Young (1957), [6]Peres and Macedo (1997), [8]Browne (1912), [9]Bouchard et al. (2007), [10]Hudson (1985). Only [7]Zhang et al. (2010) performed dynamic solubility study. (Original data in g/L. Converted using density of water at: [a]atmospheric pressure and [b]pressure of 15 bar).

2.2.2.5 Extraction of lentil husk

Extraction of phenolics from lentil husks have been carried out with various solvent mixtures, such as methanol+water (1:1, v/v) (Ronzio et al., 1996) and acetone (80%) (Amarowicz et al., 2010) using material-to-solvent ratios of 1:10 (w/v). The extraction yielded 6.0 g and 6.8 g phenolics per 100 g lentil husk, respectively. But methanol and acetone are regarded as toxic solvents and therefore its use is not environmentally viable. Moreover, the extraction time

varies between 3 to 5h, which is considered long. Then, the use of acid (1% of HCl) in methanol+water (80:20, v/v) mixtures was also attempted to disintegrate the carbohydrate matrix and yielded 6.2 g phenolics/100 g lentil husk (Dueñas et al., 2002). Apart from organic solvents, pure water has also been used with different extraction temperatures (22 to 80°C). In these experiments, higher yields were obtained at 80°C (5.3 g/100 g lentils husk) than at 22°C (4.8 g/100 g lentils husk) (Oomah et al., 2011).

Extraction of carbohydrates, mainly lignin and hemicelluloses, from wood and agricultural residues has been under investigation in the last three decades (Dekker and Wallis, 1983, Hendriks and Zeeman, 2009, Makishima et al., 2009, Díaz et al., 2011, Saha et al., 2013). However, lentil husk has not been investigated for conventional extraction but similar lignocellulosic materials like wheat husk and corn stover have been extracted using acidic, alkaline and ammonia-based solutions (Hendriks and Zeeman, 2009). Of all the extraction methods, acidic solutions increased the effect on solubilisation of hemicelluloses and lignin compared to alkaline and ammonia solutions (Mosier et al., 2005). Even though the above mentioned extraction methods have been used for many years, the use of highly acidic and basic solutions or ammonia is hazardous to humans and to the environment. Therefore, recent advances in SCW extraction and its use in extraction of carbohydrates from lignocellulosic materials have been intensified. SCW technology uses water at temperatures between 100-374°C and enough pressure to maintain water in the liquid state. Table 2.5 summarizes SCW extraction conditions used for carbohydrates removal from agricultural residues,

such as corn stover, corn cob, flax shives, rice straw, sugarcane bagasse, sunflower stalks, switch grass and wheat straw. The results are compared based on the yield of xylose in the extract, quantified by using HPLC. From the reported work, it is evident that effective removal of xylose is obtained at above 150°C, while the effective time of extraction varies depending on the type of raw materials. Most of the experiments are performed at pressures high enough to keep the water in the liquid state, but use of high pressure for extraction had not been reported. Liu and Wyman (2003) demonstrated that flow rate only affects the extraction if it is above 10 mL/min, when they studied extraction of hemicellulose (as xylose equivalent) from corn stover between 160-240°C and flow rates from 1-40 mL/min. Liu and Wyman (2003) and Yang and Wyman (2004) found that addition of 0.1% sulphuric acid improved the xylose (quantified using HPLC) and lignin (Klason lignin) recovery by 60% at 160°C, a flow rate of 10 mL/min and unspecified pressure. They hypothesized that the acid helps to break the ester-ester bonds that binds the hemicellulose with the lignocellulosic matrix.

Table 2.5 Operational parameters for extraction of hemicellulose using SCW

Raw material	Temperature (°C)	Time (min)	Pressure (bar)	Yield of xylose (%)	Reference
Corn stover	120-200	5-15	15	72	Saha et al. (2013)
Corncob	200-210	10	15-18	82	Makishima et al. (2009)
Flax shives	180	117	52	90	Anvar et al. (2009)
Rice straw	150-190	8-20	24	96	Rodríguez (2009)
Sugarcane bagasse	150-190	100-300	n.a.	55	Boussarsar et al. (2009)
Sunflower stalks	180-230	5	n.a.	95	Díaz et al. (2011)
Switch grass	150-190	20	34	80	Kumar et al. (2011)
Wheat straw	170-180	7.5-15	10	69	Thomsen et al. (2008)

n.a. – not available

SCW treatment is used for the extraction of lignocellulose because: i) the method avoids the use of toxic chemicals like methanol, acetone, etc., making the process environment friendly; ii) hemicelluloses can be converted to hemicellulosic sugars (xylans, mannans and arabinans) with high yields of almost 85-99% (Mok and Antal, 1992), which could be used for production of xylitol and biodegradable films from xylose (Hartman, 2006) and cellulose could be partially hydrolyzed (Mok and Antal, 1992), making it suitable for fermentation; iii) its easy extraction procedure compared to acid/alkaline treatment, which generate hazardous waste (e.g. gypsum) (Hamelinck et al., 2005) or enzymatic treatment where the process is extremely temperature and environment sensitive makes SCW treatment suitable; v) it is economically advantageous over other lignocellulose

pretreatment methods. According to study undertaken by Kubikova et al. (1996) to develop a method for paper pulp production from wheat straw, they found that the SCW processing could be optimized cost effectively for lignocellulose pretreatment.

2.2.2.6 Extraction of lentil cotyledon

SCW extraction of lentil cotyledon has not been attempted to date, but SCW extraction of other starchy materials like ginger bagasse (Moreschi et al., 2004), turmeric (Moreschi et al., 2006) and rye (Rogalinski et al., 2008b) have been explored. The experiments done by Moreschi et al. (2004) showed that ginger bagasse starch underwent hydrolysis at 180-200°C and 150 bar. It also showed that higher degree of hydrolysis and higher yield of reducing sugar were generated at high temperatures (above 200°C) and longer processing times (>11 min). A similar study on turmeric starches (Moreschi et al., 2006) was conducted with the aid of CO_2 as a binary solvent with water. Their results showed that, at a pressure of 150 bar in CO_2+water binary media (1:1, CO_2:water) the degree of hydrolysis increased from 76 to 98%, when the temperature increased from 130°C to 200°C. High pressure facilitates in loosening the starch structure and temperature facilitates to solubilise it, as reported by Lee et al. (1994) using a potato starch. They found that the increase in pressure improved the enzymatic hydrolysis of starch by using amylase, increasing glucose and reducing sugar yield by 20% as pressure increased from 50 to 100 bar.

High availability of starches from lentil cotyledons could act as a prime source of starch-derived products after SCW treatment. But, this study has never been attempted.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

The chemicals used for compositional analysis were ethanol and methanol (99.9%, HPLC grade), hydrochloric acid (37%, ACS reagent), sulphuric acid (97%, ACS reagent), sodium hydroxide pellets (≥97%, ACS), phenol (99%), Folin-Ciocalteu reagent (2M), gallic acid standard (99.9%) and D-(+)-xylose standard (99%). All these chemicals and solvents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

For solubility experiments, sugar standards, such as D-(+)-glucose (99.9%), D-(+)-lactose (99%) and D-(+)-sucrose (99.9%) were obtained from Sigma-Aldrich (Oakville, ON, Canada).

For extraction experiments, glacial acetic acid (99.7%, ACS reagent) was obtained from Sigma-Aldrich (Oakville, ON, Canada). Glass beads (diameter ~5 mm) were obtained from Sigma-Aldrich (Oakville, ON, Canada). For all experiments, laboratory distilled water was used.

3.1.2 Sample preparation

Red lentil (*Lens esculenta*), variety Redberry, was kindly donated by Alberta Agriculture and Rural Development (Art Kruger, Brooks, AB, Canada). The

cotyledon was separated from the husk, following the method reported by Wang (2005). First, the oven drying method was used to measure the moisture content of the lentil seeds. The seeds were soaked in water for 12 h if the moisture content was below 13% (wet basis, wb). The amount of water needed to increase the moisture to 13% (wb) was calculated using the measured moisture content. The moisture content was adjusted to prevent breaking of the cotyledon due to excess of dryness. The seeds were dehulled in a laboratory dehuller and husk was separated from the cotyledon in an aspirator (model 3010-030, Udy Corp., Ft. Collins, CO, USA). The lightweight husk was collected at the top, leaving the heavier cotyledon at the bottom. The husk and cotyledon were then ground in a Retsch mill (ZM 200, Burlington, ON, Canada) and passed through sieves to obtain particles of 0.7 – 1.0 mm. These fractions were stored in vacuum-sealed packages at -17°C for further use in extraction experiments.

3.2 Analytical methods

3.2.1 Moisture content

The moisture content of lentil husk or cotyledon was determined gravimetrically following the AOAC methodology (AOAC, 2000). To remove any residual moisture, the aluminum dishes were first dried in an oven at 100°C for 6 h. Then, they were placed in a desiccator at ambient temperature. The aluminum dishes and lids were weighed to 4 decimal places using an analytical balance (Mettler Toledo, Mississauga, ON, Canada). Two grams of both husk and cotyledon were accurately weighed in the dish using the analytical balance. The samples were

spread in a uniform layer on the dish and the weights were recorded. Samples were then dried in the oven at 105°C for 2 h. Then, the samples were cooled in a desiccator for 1 h and weighed again. Moisture content was determined using the following equation:

$$\%\ Moisture\ \left(\frac{w}{w}\right) = \frac{W1-W2}{W1-d} \times 100 \quad \textbf{3.1}$$

where:

W1 = weight of sample + dish + lid before drying, in g

W2 = weight of sample + dish + lid after drying, in g

d = weight of dry dish + lid, in g

All determinations were carried out in duplicates.

3.2.2 Ash content

The ash content for lentil husk or cotyledon was determined according to the AOAC Method 923.03 (AOAC, 2000). One gram of powdered lentil husk/cotyledon was weighed into pre-weighed and dried porcelain crucibles using the analytical balance. Then, porcelain crucibles were transferred into the muffle furnace Model F-A1730 (Thermolyne Corporation, Dubuque, IA, USA) set at 550°C for 15 h. The crucibles were cooled in the desiccator for 1h and their weights were recorded. Total ash content of the lentil husk or cotyledon was determined using the equation:

$$\%\ Ash\ \left(\frac{w}{w}\right) = \frac{WCA-WC}{WCS-WC} \times 100 \quad \textbf{3.2}$$

where:

WCS = weight of crucible + sample, in g

WCA= weight of crucible + ash, in g

WC = weight of crucible, in g

All determinations were carried out in duplicates.

3.2.3 Lipid content

The lipid content was determined using the Goldfisch apparatus (Labconco Co., Kansas, MO, USA). Approximately four grams of powdered lentil husk/cotyledon was transferred onto a Whatman No. 4 filter paper and placed inside the extraction thimble (Whatman International Ltd., Maidstone, Kent, UK). The extraction beakers were pre-weighed and 40 mL of petroleum ether was poured into it for reflux. A blank sample was also run using the same amount of petroleum ether (40 mL). The extraction thimbles were clamped to the condenser, while the extraction beakers were clamped below to create a closed system for the reflux. The water condenser was turned on. The extraction was performed for 6 h at 60°C. After the extraction was completed, the apparatus was allowed to cool and the extraction beakers were removed and kept inside the fume hood for 30 min to remove any residual solvent. Then, the residual water was dried in a hot air oven (Despatch Oven Co., Minneapolis, MN, USA) at 110-120°C for 30 min and cooled at room temperature. The extraction beaker was weighed. The total lipid content was calculated using the following equation:

$$\%\ Lipid\ \left(\frac{w}{w}\right) = \frac{(WBE-WBR)-WB}{WS} \times 100 \qquad \textbf{3.3}$$

where:

WB= weight of beaker, in g

WBE= weight of beaker + extract, in g

WBR= weight of blank residue, in g

WS= weight of sample, in g

All determinations were carried out in duplicates.

3.2.4 Protein content

The determination of protein content of the lentil husk/cotyledon was carried out using a Leco nitrogen analyzer Model FP-428 (Leco instruments Ltd., Mississauga, ON, Canada). Dried ground lentil husk or cotyledon samples (0.16 g) were poured into an aluminum foil cone and then pressed to form pellets. Similarly, pellets were made with standards for protein content like ethylene di-amine tetra-acetic acid and cornstarch. The system was first calibrated with these standards and then the samples were inserted into the Leco analyzer sample port. The reported nitrogen (%) content is multiplied by a factor of 6.25 to obtain the protein content (Lee et al., 2007). The protein was calculated using the equation:

$$\%\ Protein\ \left(\frac{w}{w}\right) = \frac{N \times 6.25}{S} \times 100 \qquad \textbf{3.4}$$

where:

N = percentage of nitrogen in the sample, in %

S = weight of the dry sample, in g

3.2.5 Total carbohydrates content

The total carbohydrates content was determined using the phenol-sulphuric acid assay reported elsewhere (Current Protocols in Food Analytical Chemistry, 2001).

A liquid extract (1 mL) was placed in a 10 mL test tube and 500 µL of 4% (v/v) phenol reagent was added. The mixture was shaken for 1 min and 2.5 mL of 96% (v/v) sulphuric acid was added and shaken well. In the presence of acid, the carbohydrates are reduced to furfurals, which complexes with phenol, resulting in a yellow color. The absorbance was read at 490 nm. The calibration curve was made by plotting the absorbance of known sugar concentrations, ranging from 5-100 µg/g of solution (Appendix B.4.a). Extract dilutions were made if required.

3.2.6 Total phenolics content

The phenolics was measured using the Folin-Ciocalteau method proposed by Singleton and Rossi (1965). This colorimetric method is based on the chemical reduction of the Folin-Ciocalteu (F-C) reagent, which is a mixture of tungsten and molybdenum oxides. The extract (0.04 mL) containing the phenolics was mixed with 3.16 mL of milli-Q water. Then, 0.2 mL F-C reagent was added and and mixed while shaking. Sodium carbonate (20% w/v; 0.6 mL) was then added to the mixture with continuous shaking to neutralize the solution within 36s to 8 min of adding sodium carbonate. Then, the mixture was incubated for 90 min in dark. Total phenolics was calculated using a gallic acid standard calibration curve (Appendix B.4.a). The stock solution of 5 g/L of gallic acid was prepared by dissolving 0.25 g of gallic acid in 50 mL of distilled water. Different concentrations of gallic acid standards (20-250 µg /g solution) were prepared by diluting the stock solution with distilled water. The absorbance of all standards was measured at 765 nm using a spectrophotometer (Genova MK3, New Malden, Surrey, UK). The calibration curve was plotted with the absorbance of different

standards as a function of their concentrations. The concentration of phenolics in the samples were calculated using the calibration curve equation generated, which had a correlation coefficient greater than 0.995. All samples were analyzed in duplicate and final results were expressed as milligrams of gallic acid equivalents per 100 g lentil husk or cotyledon.

3.2.7 Total pentosan content

Total pentosan of lentil husk was determined using the methodology reported by Hashimoto et al. (1987) with some modifications. A sample of 30 mg was mixed with 2 mL of 2M HCl and incubated for 2.5 h at 100°C after vortexing. Then, 2 mL of 1M Na_2CO_3 and 2 mL of yeast solution was added and vortexed after cooling the solution to room temperature. The mixture was incubated for 2 h at 37°C, while vortexing every 20 – 30 min for 2 h. Then, 18 mL of milli-Q water was added to the mixture and centrifuged at 3500 rpm for 5 min. One mL of the supernatant was mixed with 2 mL milli-Q water, 0.3 mL of orcinol solution and 3 mL $FeCl_3$ in HCl solution. Simultaneously, D-(+) xylose standards were prepared with concentrations ranging from 40 to 200 µg/mL (Appendix B.4.a), along with one blank containing only milli-Q water. The mixtures were boiled for 45 min in a water bath and then cooled and filtered through Whatman#2 filter paper. The absorbance of the filtrate was read at 670 nm. The pentosans were quantified on basis of total xylose present. The amount of pentosan was determined using the following equation.

$$\%\ Pentosan = (A_s - A_b)\times 6\times (m)\times \frac{1}{W}\times 0.88\times \frac{100}{1000}\times 4 \qquad \textbf{3.5}$$

where:

A_s= absorbance of sample,

A_b = absorbance of blank,

m = slope of xylose standard curve,

W = weight of the sample analyzed, in mg

3.2.8 Starch content

To determine the starch content, the AOAC method 996.11 (AOAC, 2011) was used with the kit from Megazyme™ (Bray Co., Wicklow, Ireland). In the method, 100 mg of milled sample with 0.5 mm particle size was mixed with 0.2 mL of aqueous ethanol (80%, v/v) in a test tube. Then, 3 mL of thermostable α-amylase was added and incubated in a boiling water bath for 6 min. The tube was stirred for 6 min during incubation every 2 min to ensure mixing. The tube was then cooled to 50°C and maintained in a water bath at 50°C and 0.1 mL of amyloglucosidase was added and stirred for 30 min. The whole solution was transferred to a 100 mL volumetric flask and made up to 100 mL with distilled water. The solution was then centrifuged at 3000 rpm for 10 min. The filtrate (0.1 mL) was taken in a test tube and 3 mL of GOPOD reagent was added and incubated at 50°C for 20 min. D-Glucose controls consisting of 0.1 mL of D-glucose standard solution (1 mg/mL) and 3 mL of GOPOD reagent were prepared. The absorbance of the extracts and standards were measured at 510 nm.

$$\%\ Starch\ (\frac{w}{w}) = \frac{A_{510} \times F \times FV \times 0.9}{W} \quad \textbf{3.6}$$

where:

A_{510} = absorbance of the sample at 510 nm

$$F = \frac{100\ \mu g\ of\ D-glucose}{Absorbance\ of\ 100\ \mu g\ of\ D-glucose}$$

FV = final volume, 100mL

W = weight of the sample analyzed, in mg

Preparation of GOPOD reagent: GOPOD reagent is prepared by mixing freeze dried powder of glucose oxidase peroxidase and 4-aminoantipyrine (5 g) into 1 L of buffer made by dissolving 48 mL of *p*-hydroxybenzoic acid and sodium azide (0.4%, w/v) in distilled water up to a volume of 1 L.

3.2.9 Lignin content

The experiment was performed based on the Klason lignin determination method obtained from NREL (Templeton and Ehrman, 1995). In this experiment, 1 g of ground lentil husk (W_1) was taken in digestion tubes and 15 mL of concentrated sulphuric acid (72%, v/v) chilled to 4°C was added to it. They were mixed using a glass rod and then incubated in a water bath (approximately 20°C) for 2 h for hydrolysis accompanied by occasional stirring every 15 min to ensure complete wetting and mixing. After hydrolysis, the hydrolysate is transferred to a 1000 mL Erlenmeyer flask and diluted to a 3% acid concentration with 560 mL of deionized water. Then, the flask is attached to a reflux condenser and heated to boil. The boiling is continued for 4 h. The hydrolysate is then filtered through filtering crucibles using excess deionized water at the end of filtration to remove excess acid present on the filtered residue. The crucibles are then dried at 105°C for 2 h until constant weight is achieved. The weight of the crucible is noted (W_2) and then ignited at 575°C in the muffle furnace for 3 h to eliminate all the carbon

present in the residue. The crucible is then cooled in the desiccator and weighed (W_3).

$$\%\ Lignin\ \left(\frac{w}{w}\right) = \frac{W_2 - W_3}{W_1} \times 100 \qquad \textbf{3.7}$$

where:

W_1= initial sample weight.

W_2= weight of crucible, lignin, and ash.

W_3= weight of crucible and ash.

3.2.10 Analytical extraction methods

3.2.10.1 Total carbohydrates for lentil husk

The total carbohydrates present in lentil husk was determined using the method described by Foyle et al. (2007). Lentil husk (2.5 g) was extracted with 200 mL of concentrated sulfuric acid for 15 min. Then, the solution was diluted with water to 80% (w/w) and then extracted for 15 min. Sulfuric acid was further diluted to 30% (w/w) with water and extracted for 15 min. Finally, the extraction was terminated by using 5M NaOH to reach a pH of 7. The extract was centrifuged at 5000 rpm for 10 min to remove any residual matter. The carbohydrates present in the extract was determined using the method described in Section 3.2.5 of this chapter.

3.2.10.2 Total phenolics

Free phenolics were extracted using the method described by Dueñas et al. (2002). In this method, 5 g of lentil husk or lentil cotyledon was mixed three

times with 80 mL methanol-HCl (1%) - water (80:20, v/v) in a water bath at room temperature (~25°C) while stirring it for 10 h. The supernatant was separated by centrifugation at 4000 rpm for 5 min. The volume of the supernatant was maintained to 240 mL with methanol-HCl (1%) - water (80:20, v/v). The resulting extract was extracted 4 times with 50 mL diethyl ether. The extract was then dried under vacuum in a rota-vapor to 50 mL volume. The phenolics of the resultant extract were determined using the method described in Section 3.2.6 of this chapter.

Alkaline hydrolysis was used to extract bound phenolics. Three grams of lentil husk residue or cotyledon residue obtained after extraction of free phenolics was digested with 30 mL of 4M NaOH in a water bath at 65°C for 4 h. The mixture was then acidified to pH of 2-3 with hydrochloric acid. The solution was centrifuged at 4000 rpm for approximately 5 min. The supernatant was extracted twice with 100 mL of petroleum ether using a separatory funnel (Bonoli et al., 2004, Madhujith and Shahidi, 2009). Then, total phenolics was measured using the method described in Section 3.2.6 of this chapter.

3.2.11 Refractive index (RI) measurement

Refractive index was determined using an automatic refractometer Mettler-Toledo RE50D (Columbus, OH, USA) with a resolution of $\pm 10^{-5}$, an uncertainty in the experimental measurements of $\pm 2 \times 10^{-5}$ and an uncertainty for the temperatures of ±0.01°C.

The refractive index vs. sugar concentration was plotted for different concentrations of glucose (Appendix A.1.a) and lactose (Appendix A.5.a)

solution. Using this calibration curve, the mass of sugar in the solution of unknown concentration was obtained. A polynomial equation was used to fit the calibration curve using a nonlinear estimation. The equation was used to calculate the sugar concentration of the saturated solutions.

3.2.12 Conductivity and pH measurements

Conductivity and pH were measured using a universal pH meter. Probes for measuring pH and conductivity were purchased separately from ColeParmer (Montreal, QC, Canada). Both the pH and conductivity probe were first calibrated with standards. For pH, five standards (pH of 2, 4, 7, 10 and 12) were used and for conductivity 55 mS/m standard was used. The probe was kept inside the extract till the reading on the screen stabilized. After each reading, the probe was thoroughly washed with deionised water.

3.3 Subcritical water (SCW) unit

The SCW system used in this study is the same used earlier by Singh and Saldaña (2011) with some modifications. This system has four main components, a high pressure vessel, one high pressure liquid pump, a heating and a cooling unit and a back pressure regulator (TESCOM, Elk River, MN, USA) (Fig. 3.1). The high pressure vessel was designed to work up to 300 bar. The system also has unions, reducers, on-off and flow control valves. Band heaters were used to heat both the high pressure vessel and the pre-heater. Temperature of both pre-heater and high pressure vessel was monitored by using K-type thermocouples connected to a digital thermometer and controlled by a rheostat (Staco Energy Products Co., Dayton, OH, USA). A Gilson 305 pump with a 10SC pressure head from Mandel

Scientific (Guelph, ON, Canada) was used to pump water into the high pressure vessel from the water reservoir.

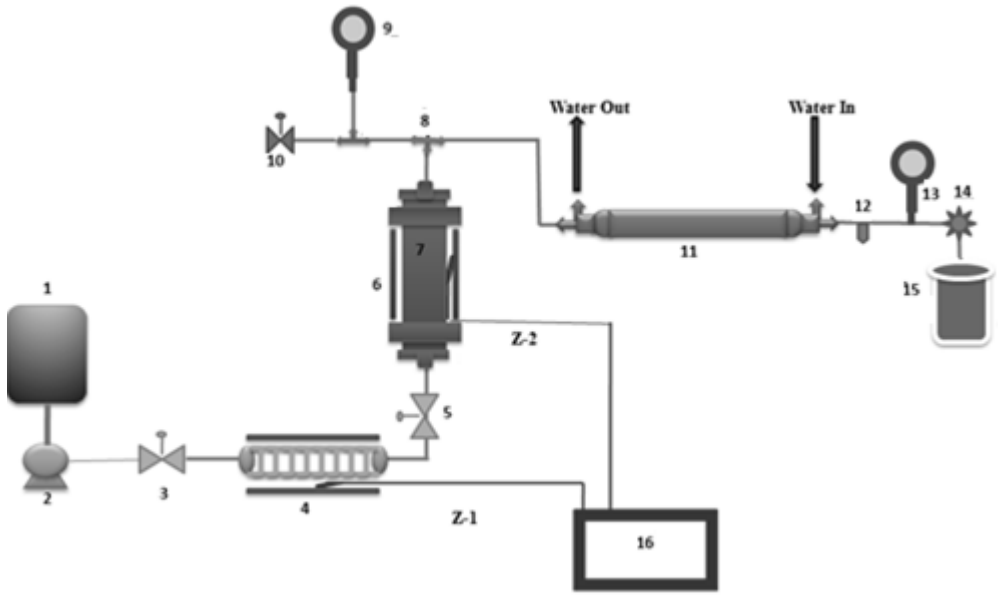


Figure 3.1 SCW extraction system.
1. Water reservoir, 2. HPLC pump, 3 and 5. Valves, 4.Pre-heater, 6. Extraction vessel heater, 7. Extraction vessel, 8. T-connection, 9 and 13. Pressure gauge, 10. Pressure relief valve, 11. Cooler, 12. Filter, 14. Back pressure regulator, 15. Sampling container, 16. Temperature controller, Z-1 and Z-2 are thermocouples.

3.3.1 SCW system for determination of sugar solubility

The SCW system used for determination of solubility had two pumps. One GILSON 307 pump (Guelph, ON, Canada) was attached to the mixing tee after the high pressure vessel as shown in Fig. 3.2. This pump was used to add dilution water to the exiting solution to prevent sugar crystallization.

In a typical solubility experiment, a known amount of sugar (~21 g) mixed with 2 mm glass beads (5:2, sugar:glass beads) was poured in the high pressure vessel with a volume of 17 mL. Distilled water was then pumped into the system at a flow rate of 1mL/min. Then, the back pressure regulator was set at a desired

pressure. As the water started to fill the system the pressure started to rise in the vessel as indicated by the pressure gauges. When the pressure reached close to its set point, the heaters were started and set to a desired temperature. After the desired temperature and pressure were achieved, the high pressure vessel was held at these conditions for 5-15 min, before starting to collect the samples. This is known as the equilibration time and it depends on the temperature of the experiment. Simultaneously, the dilution solvent pump was run between 4-6 times the flow rates of the main pump to avoid sugar crystallization inside the cooling unit. The experiments were carried out at temperatures ranging from 30 to 160°C in duplicates at 15, 80 and 120 bar. The samples were collected every 30s for 30 min. The concentration of sugar in the solution at this time was measured for solubility determination.

The SCW system was first used to determine the glucose at similar temperatures reported by Zhang et al. (2010) in order to evaluate the efficiency and reproducibility of the system. Then, the solubility of glucose, sucrose and lactose were studied at various temperatures and pressures. Lactose was selected as this compound is found in many dairy products, such as cheese, cultured milk, butter, etc. (National Dairy Council, 1993).

The sample composition was determined by refractometric analysis (Kurtz and Eliason, 1979, Aas, 1996). The refractive index of the samples was measured at 20°C and the results were calculated with the equation obtained from the standard calibration curve.

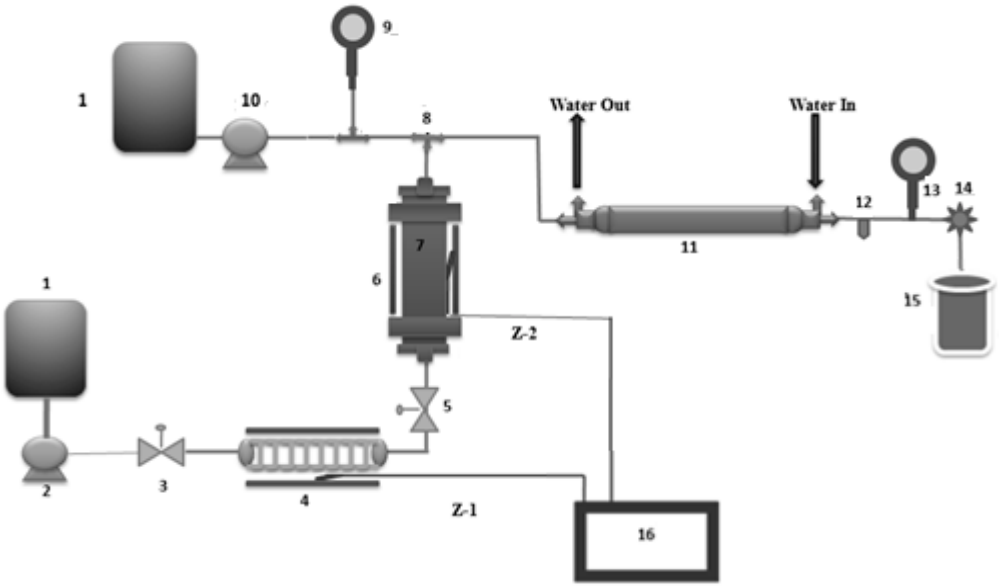


Figure 3.2 High pressure apparatus to measure solubility of sugars in SCW. 1. Water reservoir, 2 and 10. HPLC pump, 3 and 5. Valves, 4. Pre-heater, 6. Extraction vessel heater, 7. Extraction vessel, 8. T-connection, 9 and 13. Pressure gauge, 11. Cooler, 12. Filter, 14. Back pressure regulator, 15. Sampling container, 16. Temperature controller, Z-1 and Z-2 are thermocouples.

3.3.2 SCW extraction system

The system is described in Section 3.3 (Fig. 3.1). For the experiments, two types of high pressure vessels were used. The first with a capacity of 17 mL (1 cm internal diameter, i.d. and 22 cm length) and the second with a capacity of 50 mL (1.7 cm i.d. and 22 cm length) were used. To operate the flow-through SCW extraction system, lentil husk or cotyledon was loaded into the high pressure vessel (Swagelok, Edmonton, AB, Canada) along with glass beads (1:10, lentil:glass beads). The two ends of the vessel were fitted with sintered flat filters (20 μm) to prevent lentil husk from entering the system. The high pressure vessel was connected to the system using reducers and unions. Distilled water at room temperature was pumped into the system using a HPLC pump (Gilson 307,

Guelph, ON, Canada). The pressure was maintained by a back pressure regulator (Tescom, Elk River, MN, USA). After the system reached the required pressure, the pump was stopped and the heaters were turned on. Rheostats were used to maintain the temperature of the pre-heater and the pressure vessel heater. After the temperature was achieved, the system was held at that constant temperature and pressure for approximately 15 min to equilibrate the temperature inside the pressure vessel, known as equilibration time. Then, the pump was turned on to start the extraction. This time was taken as time zero. While exiting the high pressure vessel, the extract passed through a cooling unit to bring its temperature to ambient conditions, avoiding any reactions and preventing degradation of the extract. The pressure of the high pressure vessel was monitored by two pressure gauges, one near the high pressure vessel (Swagelok, Edmonton, AB, Canada) and the other one after the cooling system (GE Druck, Billerica, MA, USA).

3.4 Experimental design

For extraction, first a factorial design was used to identify the significant process parameters and then the response surface methodology (RSM) was used to optimize these parameters. A full factorial design was used for the SCW extraction of lentil husk and cotyledon using three factors with two levels. For the RSM, central composite design (CCD) with center point was used. The CCD was obtained using the Design Expert v6.06 software (Minneapolis, MN, USA).

CCD contains an imbedded factorial design with center points that is augmented with a group of star points that allow estimation of curvature. If the distance from

the center of the design space to a factorial point is ±1 unit for each factor, the distance from the center of the design space to a star point is ±α with |α| > 1. The value of α depends on the number of experimental runs in the factorial portion of the central composite design:

$$\alpha = [2^k]^{\frac{1}{4}} \qquad \textbf{3.8}$$

where, k is the number of factors and α is the distance from the center point of the design. The central point was repeated 6 times to estimate the experimental error.

The optimum conditions obtained with the factorial design were used to perform a kinetic study for the extraction of carbohydrates, pentosans and phenolics from lentil husk and lentil cotyledon.

3.4.1 Experimental design for conventional extraction

Ground lentil husk/cotyledon (~0.25 g) with particle size of 0.7 to 1.0 mm was used with 45 mL of ethanol+water mixture to perform the experiments (Table 3.1). The experiments were carried out in plastic capped tubes of 50 mL volume. Water bath was used to maintain the extraction temperature and magnetic stir bars were used to mix the solvent and lentil continuously. The experiments were conducted based on a CCD design with three factors and two levels. In this design, the three parameters, temperature (40 to 70°C), ethanol concentration (0 to 80%) and pH (4 to 10) were identified based on previous conventional extraction of lentil husk and cotyledon (Lee et al., 2007, Ronzio et al., 1996, El-Nahry and Mourad, 1980, Lee et al., 2009). The desired pH of the solution was achieved by

adding glacial acetic acid (0.1%) or sodium hydroxide (1%) solution for acidic or basic pH, respectively.

Table 3.1 Factorial design for the conventional extraction of lentil husk or cotyledon

Temperature (°C)	Ethanol (%, v/v)	pH
40	40	7
55	40	10
55	80	7
46	64	9
46	16	9
55	40	4
70	40	7
55*	40	7
55*	40	7
55	0	7
55*	40	7
64	16	9
55*	40	7
64	64	5
46	64	5
64	64	9
55*	40	7
64	16	5
55*	40	7
46	16	5

*Central points

A maximum temperature of 70°C was used for the extractions as the boiling point of ethanol is 78.4°C. The extraction time was determined after a constant refractive index value was obtained for the highest concentration of ethanol + water solutions from the factorial design (Table 3.1).

The resultant slurry was filtered under light vacuum with Whatman #4 filter paper and the solid residue was dried in a hot air oven at 40°C overnight. The filtrate was then kept refrigerated at 4°C for further analysis.

3.4.2 Experimental design and statistical analysis for extraction using SCW

Extractions were performed using two factorial designs. The literature shows pressure, temperature, flow rate, pH and percentage of ethanol as main parameters affecting the extraction with SCW. This experimental design was used to evaluate the influence of these process parameters. In the first design, the effects of temperature, pressure and flow rate were evaluated. The second design was used to find the optimal conditions for the yield of total carbohydrates and phenolics using SCW.

Factorial design 1: It had two levels of temperatures (120 and 180°C), pressures (15 and 180 bar) and flow rates (2 and 5 mL/min) (Table 3.2). The experiments were performed using a high pressure vessel with a volume of 50 mL. Approximately, 5 g of ground lentil husk/cotyledon (particle size of 0.7 to 1.0 mm) mixed with 30 g of glass beads (2 mm) in a ratio of 1:6 (lentil:glass bead) was used for the extractions. The extraction time was based on the stabilization of the refractive index reading of the extract. The samples were collected every 10 min for 90 min and stored at -18°C for analysis of total carbohydrates, total phenolics, total pentosans and lignin content.

Table 3.2 Factorial design 1 for the SCW extraction of lentil husk and cotyledon

Pressure (bar)	Temperature (°C)	Flow rate (mL/min)
15	180	2
120	120	2
15	120	5
120	180	5
120	120	5
15	180	5
15	120	2
120	180	2

Factorial design 2: Based on the results obtained from factorial design 1, three parameters were selected and CCD was used to design the experiments to determine the optimum conditions for yield of carbohydrates and phenolics. For these experiments, a high pressure vessel with an internal volume of 17 mL was used. Approximately, 1 g of ground lentil husk/cotyledon (particle size of 0.7 to 1.0 mm) mixed with 10 g of glass beads (2 mm) in a ratio of 1:10 (lentil:glass bead) was used for extraction. The extraction time was based on the stabilization of the refractive index of the extract. The samples were collected every 10 min for 90 min and stored at -18°C for further analysis.

4 Results and discussion

4.1 Solubility of sugars in SCW[1]

The solubility of two sugars (glucose and lactose) in subcritical water (SCW) was studied at temperatures from 30 to 160°C and pressures of 15, 80 and 120 bar. Water was pumped at 1 mL/min through the high pressure vessel (capacity of 17 mL) loaded with pure sugar (~21 g). Flow rates ranging from 1-4 mL/min were investigated for the highest dissolving capacity of glucose at 30°C and 15 bar with a holding time of 15 min (Fig. 4.1a, Appendix A.1b). ANOVA analysis (Appendix A.2) showed that the dissolving capacity of glucose was significantly highest at a flow rate of 1 mL/min. The flow rate of water used for dilution was determined from the dissolving capacity of glucose in water at 160°C and 15 bar using water flow rates of 3, 5 and 7 mL/min, corresponding to dilutions of 4, 6 and 8 times, respectively (Appendix A.3). Figure 4.1b shows that dissolved glucose was significantly higher with dilution of 6 times compared to 8 times and 4 times (refer to ANOVA in Appendix A.4) as it prevented crystallization of dissolved glucose when water cools from 160°C to ambient temperature (approximately 25°C). Therefore, 6 times dilution (pump flow rate of 5mL/min) was used for solubility experiments. Therefore, to determine the solubility of glucose in water, a flow rate of 1mL/min was used to pump distilled water through the high pressure vessel and 5 mL/min of distilled water was used as the dilution solvent to avoid clogging.

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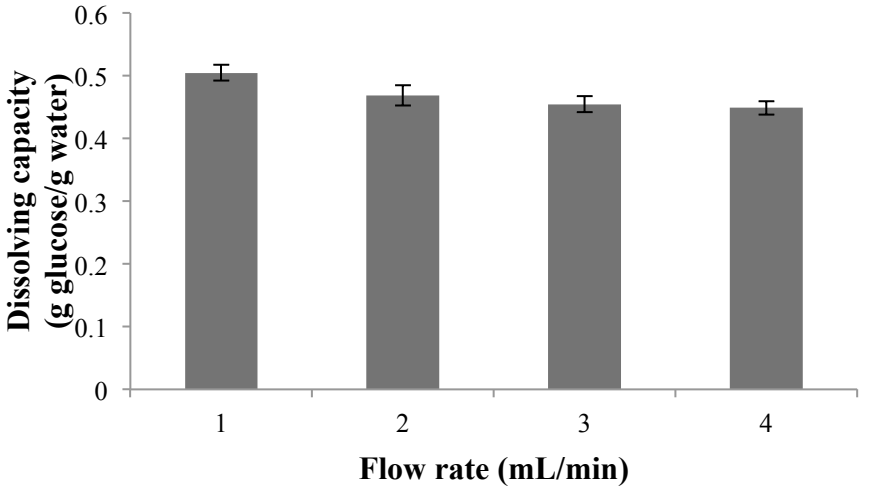
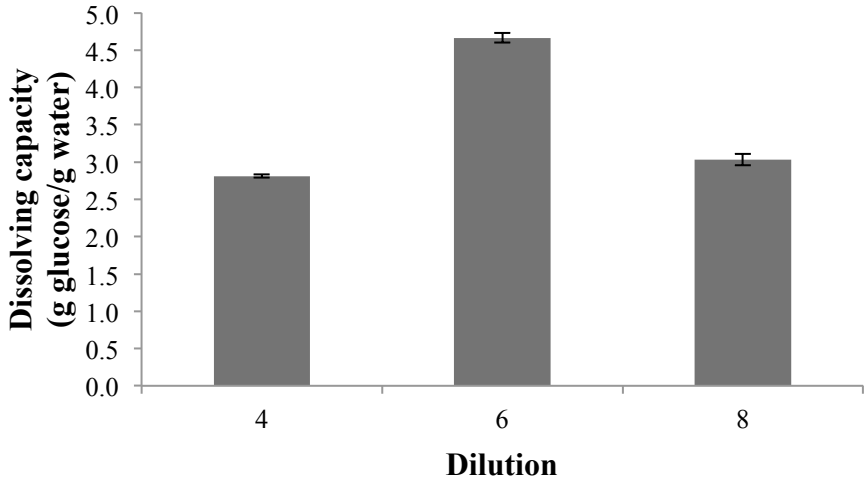
[1]A version of this chapter has been published. Saldaña 2012. J. Chem. Thermodynamics 55:115–123.



(a)



(b)

Figure 4.1 Dissolution of glucose in water at: (a) 30°C, 15 bar and different flow rates, (b) 160°C, 15 bar and 1 mL/min with different dilution factors.

A constant value for the dissolution of lactose in SCW at a constant temperature and pressure was achieved after 7 min with a dilution factor of 7 (6 mL/min, dilution pump) at a flow rate of 1 mL/min in the main pump (Fig. 4.2, Appendix A.5b). This time was required to reach the static equilibrium inside the high pressure vessel.

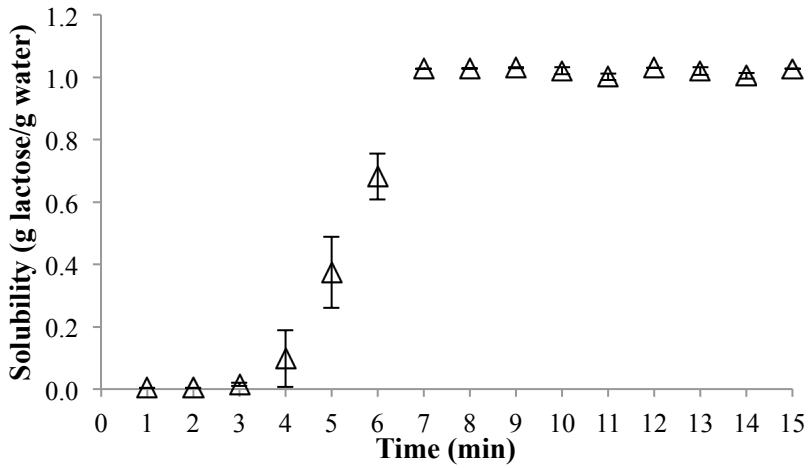


Figure 4.2 Dissolving capacity of lactose as a function of time at 80 bar and 150°C.

To validate the technique, experimental solubility data for the system glucose + SCW was first determined and compared with published data (Zhang et al., 2010). As observed in Figure 4.3, a good agreement up to 140°C between this study and the data reported by Zhang et al. (2010) was achieved. Zhang et al. (2010) did not mention the pressure used in their study, so fair comparison of the data is not possible.

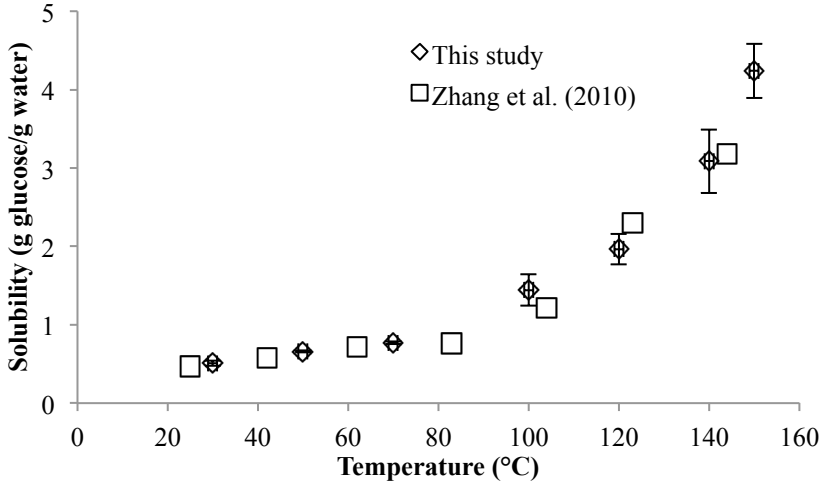


Figure 4.3 Solubility of glucose in SCW as a function of temperature at 15 bar and 15 min of holding time.

Solubility for the binary systems, glucose+water and lactose+water, was determined at 15, 80 and 120 bar as reported in Table 4.1. There is almost a 5-fold increase in solubility for both sugars in SCW at 140°C, as compared to their solubility obtained at 30°C (Table 4.1). This could be attributed to the interactions of sugar and water molecules at subcritical conditions. Water and sugar at these conditions undergo the following changes. First, the O-H (-hydroxyl) groups in sugars are attracted to the water molecules by dipole-dipole forces. The high number of hydroxyl groups on the surface of the molecules increases the force of attraction. The strength of these forces can be greater than the glucose-glucose interactions thus making them soluble. The hydrogen bonding between water and glucose molecules also makes the glucose more soluble in water (Zumdahl, 2009). At subcritical conditions, the water undergoes self-ionization more rapidly than at ambient temperature (25°C). Thus with increase in temperature of water, the dissociation of water to hydronium ions (H_3O^+) and hydroxyl ions (OH^-) increases, decreasing the ionic strength (pKw) of water from 14 (at 25°C) to 11.3 (at 180°C). This enables O-H (hydroxyl-) groups of sugar to bind covalently to excess hydronium ions (H_3O^+), which are stronger and more stable compared to hydrogen bond.

Second, the high enthalpy of solution allows breaking of the hydrogen bonding between the sugar molecules in the crystal lattice and the resulting ions are hydrated much more rapidly in SCW as compared to normal water. Moreover, enthalpy of solution increases with an increase in temperature at a constant

pressure (Alvarez and Saldaña, 2011). Effect of pressure is also observed (Table 4.1), where increase in pressure decreases the solubility of sugars in the SCW.

The relative standard deviation for solubility of glucose and lactose in SCW at various temperatures are provided in Table 4.1. For glucose solutions, standard deviations ranged from 0.010 at 50°C/120 bar to 0.405 at 140°C/15 bar, while for lactose solutions the standard deviations were less compared to those of glucose solutions and ranging from <0.001 at 30°C/120 bar to 0.088 at 140°C/120 bar which indicates the influence of the pressure in the repeatability of the data.

Table 4.1 Experimental solubility data for glucose and lactose at different pressures and temperatures (Adapted from Saldaña et al., 2012)

T (°C)	Glucose (g/g water)			Lactose (g/g water)		
	15 bar	80 bar	120 bar	15 bar	80 bar	120 bar
30	0.508±0.031	0.411±0.014	0.424±0.012	0.165±0.001	0.154±0.001	0.144±<0.001
50	0.659±0.013	0.520±0.006	0.514±0.010	0.274±0.013	0.241±0.001	0.244±0.001
70	0.768±0.017	0.632±0.034	0.635±0.016	0.440±0.043	0.36±0.001	0.341±0.001
100	1.442±0.200	1.201±0.058	1.165±0.010	0.643±0.083	0.554±0.006	0.527±0.001
120	1.964±0.193	1.613±0.043	1.554±0.035	0.781±0.028	0.715±0.007	0.711±0.022
140	3.086±0.405	2.313±0.086	2.167±0.103	0.940±0.033	0.886±0.071	0.841±0.088
160/150*	4.239±0.345	2.883±0.301	2.606±0.025	1.109±0.036	1.014±0.028	0.981±0.002

*The equilibrium temperatures of 150°C (lactose + water) and 160°C (glucose + water).

The limited solubility of lactose in water compared to glucose in water can be explained by the excess molar refractivity (R_m^E) of the lactose solution. The excess molar refractivity (R_m^E) calculated from the refractive index data for the sugar solutions is:

$$R_m^E = R_m - \sum_{i=1}^{N} x_i R_{mi} \qquad \textbf{4.1}$$

where, R_m is the molar refraction of the solution. R_{mi} and x_i are the molar refraction and mole fraction of the pure component *i*, respectively. The Lorentz–Lorenz equation is:

$$R_m = \left(\frac{n_D^2 - 1}{n_D^2 + 2}\right)\left(\frac{\sum_i^N x_i M_i}{\rho}\right) \qquad \textbf{4.2}$$

where, ρ is the density and M_i is the molar mass of the i^{th} component of the solution.

The excess molar refractivity is directly proportional to the molecular packing (Urbanczyk and Van Hook, 1996). The molecular packing structure of the SCW aqueous lactose is higher than the aqueous glucose as found from the density study of lactose solutions at different temperatures and pressures in our laboratory (Saldaña et al., 2012). This prevents the lactose from moving into the aqueous phase and binding with the hydrogen ions present in the water. Therefore, there is very low dissolution of lactose as compared to glucose in water.

Limited data was obtained for the third binary mixture of sucrose + water due to its high solubility compared to glucose and lactose (Appendix A.6). Literature data previously suggested that sucrose solubility is four times higher than that of glucose at 80°C and atmospheric pressure (Browne, 1912). Therefore, for 1 mL of water, four times more weight of sucrose is needed in the high pressure vessel. Solubility of sucrose was not achieved using the current experimental setup which meant that solubility of highly soluble sugars in water (> 4 g/g water) using this SCW system is challenging because of the volume limitations of the high pressure vessel and flow rates provided by the pump (maximum 10 mL/min).

4.2 Extraction of lentil husk

The main objective of this study was to extract carbohydrates and phenolics from lentil husk and to determine the optimum conditions for extraction. The specific objectives were: (i) to optimize the processing parameters, such as temperature, pressure, flow rate, pH and ethanol concentration, which influence the yield of carbohydrates and phenolics during extraction, (ii) to perform a kinetic study at the optimum conditions.

4.2.1 Proximate compositional analysis

Proximate compositional analysis of lentil husk included determination of moisture, ash, carbohydrates, pentosans and phenolics content. The components were calculated on dry basis (Table 4.2). Lentil husk is mainly composed of carbohydrates, which are primarily celluloses and hemicelluloses (e.g. pentosans). Cellulose content was determined by deducting the pentosans content from the total carbohydrates content (Liu and Wyman, 2003). Pentosans content (30.29±0.8 g/ 100 g lentil husk) was slightly higher, while cellulose content (45.00±1.9 g/ 100 g lentil husk) was lower than the values reported by Rani and Kawatra (1994). In their study, they measured the total dietary fiber (TDF) and cellulose content and then they subtracted the cellulose content from TDF to get the hemicellulose content (e.g. pentosans). This difference in the approach for calculating the pentosans and cellulose content caused deviation in the reported pentosans and cellulose contents.

Lignin content (2.88±0.6 g/ 100 g lentil husk) was similar to the reported values (Rani and Kawatra, 1994) because the same measurement technique was used (Section 3.2.9). The total phenolics content (3.98±0.9 g/ 100 g lentil husk) was also similar to the reported values (Dueñas et al., 2002). Dueñas et al. (2002) found that the total phenolics present in the lentil husk is 4.05 g/ 100 g lentil husk in which proanthocyanin (2.22 g/ 100 g) and catechin (1.49 g/ 100 g) are two major phenolics present. Fat and ash contents were relatively negligible in the lentil sample, while protein content was relatively low.

Table 4.2 Proximate analysis of lentil husk

Component	This study (g/100 g, db)	Literature (g/100 g, db)
Moisture	8.00 ± 0.80	n.a.
Cellulose	45.00 ± 1.90	52.37 ± 0.20[1]
Pentosan	30.29 ± 0.80	28.11 ± 0.24[1]
Lignin	2.88 ± 0.60	2.50±0.14[1]
Phenolics	3.98 ± 0.90	4.05 ± 0.18[2,3]
Ash	2.20 ± 0.005	n.a.
Fat	0.15±0.001	n.a.
Protein	7.13±0.02	9.71[4]

db – dry basis, n.a. – not available, [1]Rani and Kawatra, 1994, [2]Dueñas et al. (2002), [3]Dueñas et al. (2003), [4]Tiwari et al. (2006).

4.2.2 Conventional extraction

Extraction time was first determined by measuring the refractive index (RI) of the extracts obtained at 55°C, pH of 7 and ethanol concentration of 80% for 180 min with samples being collected every 10 min. When the RI values were plotted against time (Fig. 4.4, Appendix B.1) the curve reached a plateau stage after 150

min of extraction. This is why the remaining experiments were carried out for 180 min to ensure maximum extraction.

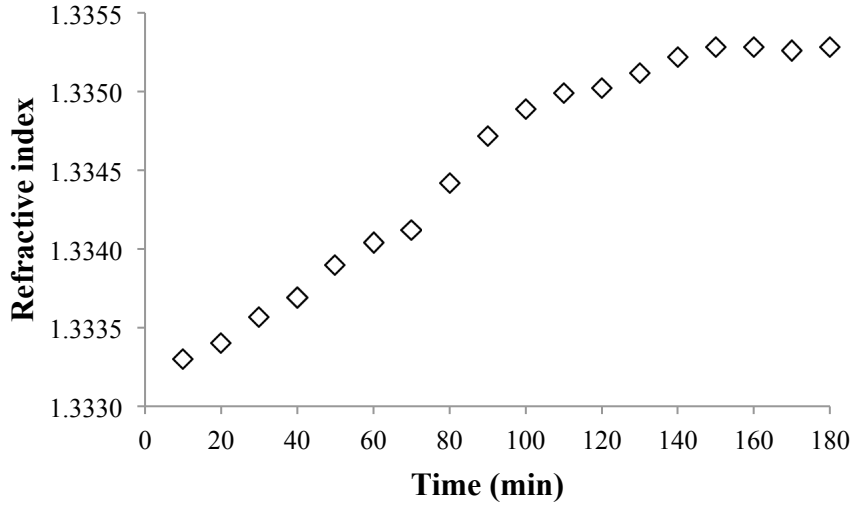


Figure 4.4 RI curve plotted as a function of time at 55°C, pH of 7 and 80% ethanol content for conventional extraction of lentil husk.

Analysis of the total carbohydrates content of all the extracts obtained using water+ethanol solutions (40-70°C, ethanol content of 0-80% and pH of 4-10) showed that temperature and pH have significant effect (ANOVA, Table 4.4). The effect of temperature was in agreement with the findings of Doner and Hicks (1997), who reported an increase in carbohydrates yield from 35 to 42% during the extraction of corn fiber from 25 to 60°C, at alkaline pH of 11.5 and atmospheric pressure. In the current study, there was 2 times increase in the yield, when the temperature of extraction increased from 40°C (5.62 g/100 g) to 70°C (11.47 g/100 g) at a constant pH of 7 and ethanol content of 40% (Table 4.3).

Apart from Doner and Hicks (1997), alkaline solvent (10% potassium hydroxide) has also been used for aqueous extraction of lupin (Monro et al., 1975) at low temperatures (0°C and 18-22°C) resulting in 35% yield of pentosans. Significant effect of alkaline solvent (pH of 10) on the yield of carbohydrates has also been observed in this study (Fig. 4.5). In Table 4.3, higher yield of carbohydrates was reported at pH of 10 (11.73 g/100 g) compared to pH of 7 (8.35±0.46 g/100 g) when lentil husk was extracted at 55°C in a water (60%) + ethanol (40%) solution.

Table 4.3 Yields of carbohydrates and phenolics of lentil husk by conventional extraction

Temperature (°C)	Ethanol (%, v/v)	pH	Yield (g/100 g lentil husk)	
			Carbohydrates	Phenolics
40	40	7	5.62	1.62
55	40	10	11.73	3.81
55	80	7	6.79	4.17
46	64	9	9.07	2.83
46	16	9	10.95	2.44
55	40	4	8.95	3.56
70	40	7	11.47	4.43
55*	40	7	8.52	3.18
55*	40	7	9.10	2.98
55	0	7	9.89	2.82
55*	40	7	8.52	3.31
64	16	9	10.94	2.81
55*	40	7	8.13	3.13
64	64	5	8.76	3.55
46	64	5	6.68	2.47
64	64	9	7.79	3.92
55*	40	7	8.05	3.35
64	16	5	10.15	2.91
55*	40	7	7.81	3.25
46	16	5	8.36	2.14

*Central point average: carbohydrates (8.35±0.46 g/100 g lentil husk) and phenolics (3.24±0.14 g/100 g lentil husk).

ANOVA (Table 4.4) for the yield of phenolics by using conventional extraction showed that temperature and ethanol content were significant parameters. The increase in temperature from 40 to 70°C increased the phenolics yield by 2.5 times (at 40% ethanol content in the solvent and pH of 7). While an increase in ethanol in the binary solvent (0-80%) increased the phenolics yield by 1.5 times (at 55°C and pH of 7) (Table 4.3). Similar behavior of temperature and ethanol concentration (Fig. 4.7) have been reported by Chew et al. (2011) when they studied extraction temperature (25°C to 65°C) and ethanol concentration (0 to 100%) for the extraction of phenolic compounds. They found approximately 1.3 times increase in the yield of phenolics as the extraction temperature increased from 25°C to 65°C and ethanol content in the water+ethanol binary solvent increased from 0 to 70%.

Table 4.4 ANOVA for yields of carbohydrates and phenolics of lentil husk by conventional extraction

Factor	Coefficient	Sum of squares (SS)	df	Mean square	*F value*	*P value*
			Carbohydrates			
A	0.91	11.31	1	11.31	13.81	0.0040
B	-0.97	12.98	1	12.98	15.85	0.0026
C	0.69	6.58	1	6.58	8.04	0.0177
AB	-0.12	0.12	1	0.12	0.15	0.7101
AC	-0.64	3.31	1	3.31	4.04	0.0721
BC	-0.24	0.48	1	0.48	0.58	0.4637
A^2	0.06	0.05	1	0.05	0.06	0.8070
B^2	-0.01	0.00	1	0.00	0.00	0.9635
C^2	0.70	6.97	1	6.97	8.51	0.0154
ERROR		1.06	5	0.21		
TOTAL SS		50.03	19			
			Phenolics			
A	0.59	4.72	1	4.72	32.22	< 0.0001
B	0.35	1.65	1	1.65	11.24	0.0040
C	0.10	0.13	1	0.13	0.91	0.3546
ERROR		0.09	5	0.02		
TOTAL SS		8.84	19			

Significant – $p<0.05$, A – temperature (°C), B – ethanol content (%) in the solvent, C – pH.

Fig. 4.5 showed that the effect of both acidic (pH of 4) and alkaline (pH of 10) solvents are prominent at high extraction temperature (70°C). Earlier efforts to understand the effect of acid/alkaline treatment on the extraction of lignocellulose had showed that both methods have significant effect on the polymeric structure of carbohydrates. Dilute acid treatment was performed by Saska and Ozer (1994) using 0.7% H_2SO_4 in water solution with a yield of 60-80% of total hemicellulose at 132°C. Alkaline hydrolysis facilitates the removal of lignin, improving the availability of the remaining polymers (Sun and Cheng, 2002). Sun and Cheng (2002) also reported that saponification of intermolecular ester bonds, cross

linking xylan hemicelluloses occur during alkaline hydrolysis, further weakening the lignocellulosic structure.

Decrease in the yield of carbohydrates occurred with an increase in the concentration of ethanol in the solvent (Fig. 4.6). This is because ethanol acts as an anti-solvent for carbohydrates, as reported by Flood and Puagsa (2000), after crystallizing glucose and fructose with ethanol (80%)+water (20%) binary mixtures at 25°C.

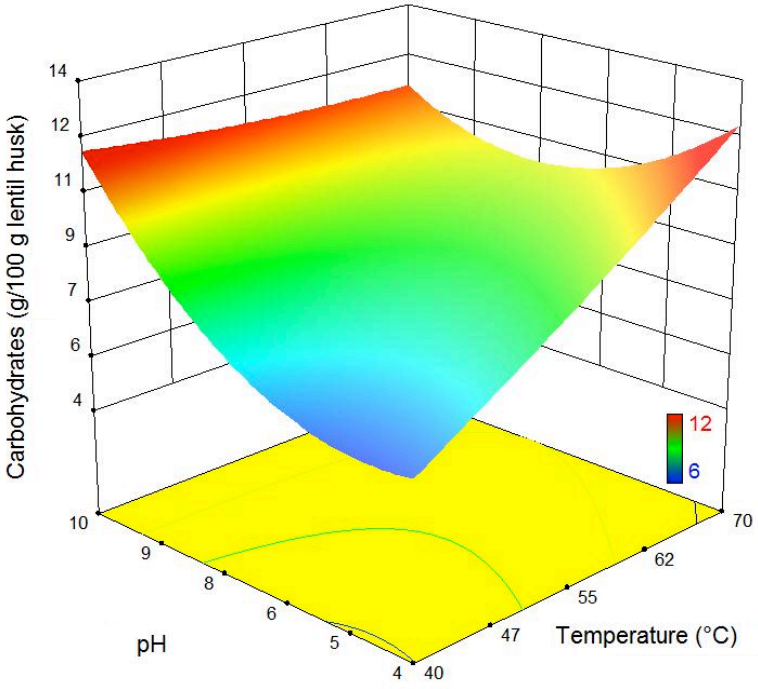


Figure 4.5 Yield of carbohydrates as a function of pH and temperature with 40% ethanol content for conventional extraction of lentil husk.

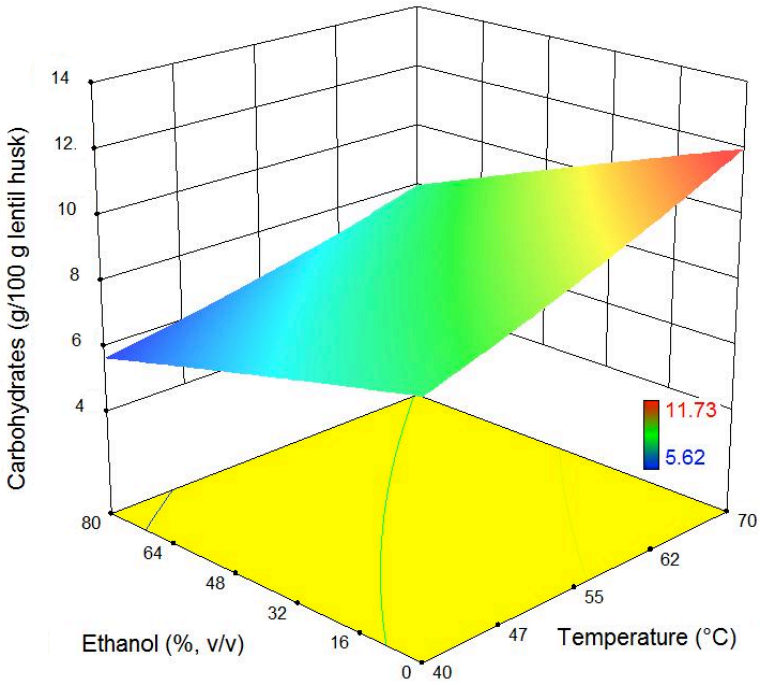


Figure 4.6 Yield of carbohydrates as a function of ethanol content and temperature at pH of 7 for conventional extraction of lentil husk.

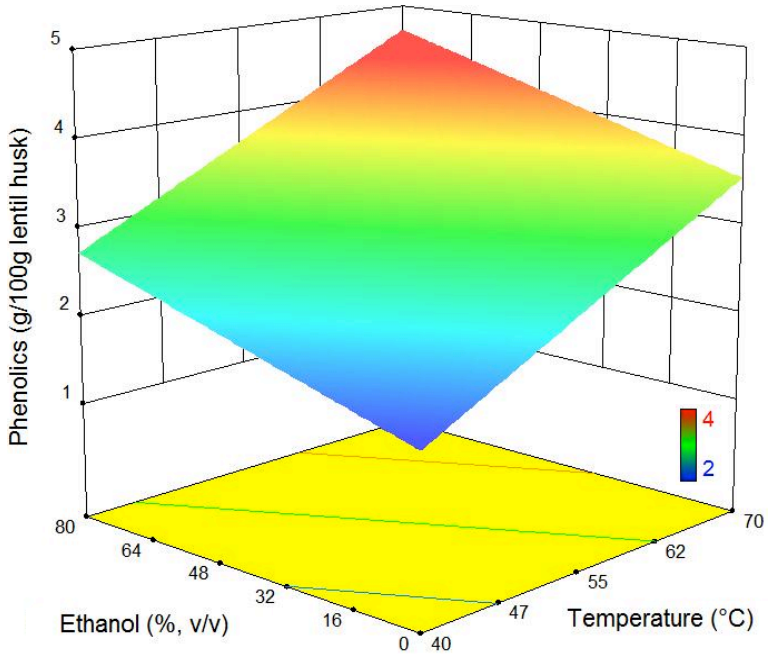


Figure 4.7 Yield of phenolics as a function of temperature and ethanol content at pH of 7 for conventional extraction of lentil husk.

By using the Design Expert ^{TM} software, the optimized conditions for maximum yields of carbohydrates and phenolics were predicted using the following equations:

Carbohydrates (Yield %) =8.36+0.91A-0.97B+0.69C-0.12AB-0.64AC-0.24BC+0.06A^2-0.01B^2+0.70C^2, $p<0.05$ **4.3**

Phenolics (Yield %) = 3.14+0.59A+0.35B+0.10C, $p<0.05$ **4.4**

where, A = temperature, B = ethanol content and C = pH.

Extractions were performed in duplicates at the optimized conditions of 69.96°C, ethanol content of 66.84% in the solvent and pH of 4 for 3 h. The experimental yield obtained at these optimized conditions was 8.60±0.12 g carbohydrates/100 g lentil husk and 4.32±0.04 g phenolics /100 g lentil husk. Both the yields of carbohydrates and phenolics were within the range of predicted yields, which

were 10.78±1.72 g carbohydrates/ 100 g lentil husk and 4.00±1.00 g phenolics/100 g lentil husk, respectively. Due to the low amounts of carbohydrate extracted (8.60±0.12 g /100 g lentil husk), SCW extraction was used for the first time for the extraction of lentil husk.

4.2.3 SCW extraction

Temperature, pressure, flow rate, pH and ethanol content of the solvent were considered as the major parameters, influencing the SCW extraction. Carbohydrates, phenolics and pentosans contents were determined to evaluate the effect of these parameters on the extraction. Here, the pentosans are the amount of pentose sugar (or, hemicelluloses) present in the extract, which is part of the total carbohydrates. Among the aforesaid factors, temperature, pressure and flow rate were first evaluated using a factorial design with water as the solvent (Table 4.5).

First, the total extraction time was decided based on the RI values of the extracts collected after every 10 min of extraction. When the RI of the extracts became constant, it was assumed that the extraction was complete. The extracts were collected for 250 min at a temperature of 180°C, pressure of 15 bar and a flow rate of 2 mL/min. These conditions were used because high temperatures (above 180°C) solubilizes hemicellulose and limited cellulose (Ho et al., 2007), a pressure of 15 bar is enough to maintain water in its liquid state at 180°C and a low flow rate provides a high residence time inside the high pressure vessel to facilitate extraction. It was observed that a flow rate of 1 mL/min caused burning of the extract due to long residence time, so a flow rate of 2 mL/min was used for

this experiment. The RI values decreased gradually indicating less total solids being extracted at each sampling interval. The RI value of the collected sample became nearly constant after 110 min of extraction (Fig. 4.8, Appendix B.3). Therefore, an extraction time of 120 min was used for all extractions.

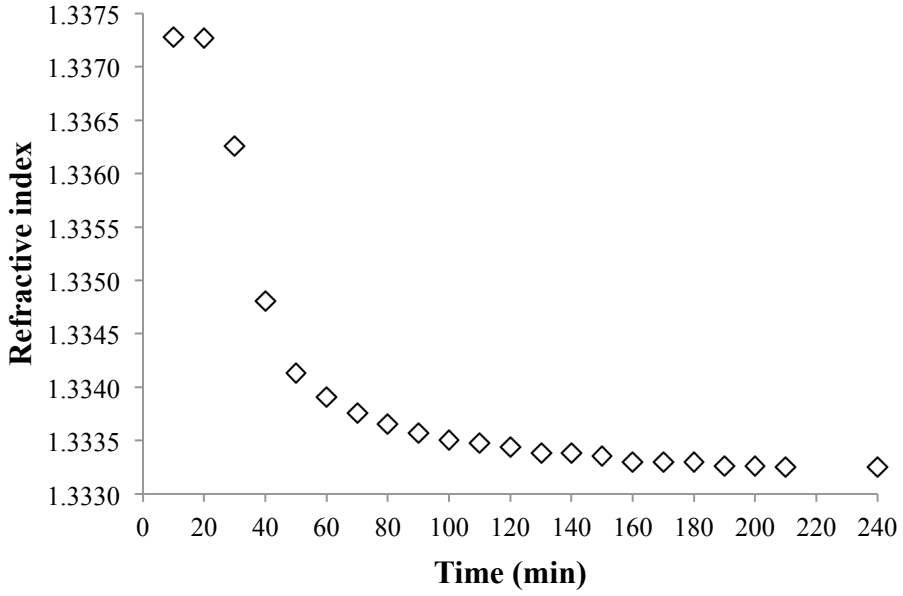


Figure 4.8 RI curve plotted as a function of time at 180°C, 15 bar and a flow rate of 2 mL/min for extraction of lentil husk.

In Table 4.5, the yields of carbohydrates, pentosans and phenolics are provided which are calculated from the calibration curve provided in Appendix B.4a. The yield of carbohydrates at 15 bar and 5 mL/min is higher at 180°C (49.40±1.41 g/ 100 g lentil husk) compared to 120°C (45.60±2.55 g/ 100 g lentil husk). ANOVA analysis (Table 4.6) confirmed that the effect of temperature was significant for the extraction of carbohydrates, pentosans and phenolics. But, the effects of pressure and flow rate were not significant. Temperature is effective because the heat capacity of water increased with increase in temperature from 120°C (4.25 kJ/kg°C) to 180°C (4.38 kJ/kg°C) under subcritical conditions, helping to

breakdown the glycosidic bond between the glucose molecules in the lignocellulosic structure.

Table 4.5 Yields of carbohydrates, pentosans and phenolics of lentil husk by SCW extraction using factorial design 1

Flow rate (mL/min)	Temperature (°C)	Pressure (bar)	Yield (g/100 g lentil husk)		
			Carbohydrates	Pentosans	Phenolics
2	120	15	39.80±2.55	17.50±0.45	0.93±0.08
5	180	120	43.57±0.24	25.28±0.38	1.57±0.17
5	120	120	45.90±1.84	25.91±0.47	1.15±0.16
5	120	15	45.60±2.55	26.06±0.48	2.89±0.17
5	180	15	49.40±1.41	29.55±0.47	2.95±0.15
2	180	15	45.00±3.68	20.55±0.49	2.42±0.07
2	180	120	41.40±1.41	16.03±0.61	0.54±0.07
2	120	120	20.19±3.69	3.69±0.38	0.05±0.01

Table 4.6 ANOVA for yields of carbohydrates, pentosans and phenolics of lentil husk by SCW extraction using factorial design 1

Factors	Value factor	Sum of squares (SS)	df	Mean square	*F value*	*P value*
			Carbohydrates			
A	1.74	181.26	1	181.26	2.96	0.0335
B	-1.80	97.16	1	97.16	1.59	0.4273
C	2.38	103.25	1	103.25	1.69	0.4179
AB	0.62	77.75	1	77.75	1.27	0.4622
AC	-1.56	39.07	1	39.07	0.64	0.5710
BC	1.11	12.20	1	12.20	0.20	0.7328
ERROR		61.27	1	61.27		
TOTAL SS		571.97	7			
			Pentosans			
A	0.16	300.49	1	300.49	13.37	0.0170
B	-0.37	41.63	1	41.63	1.85	0.4034
C	0.29	64.70	1	64.70	2.88	0.3391
AB	-0.04	19.63	1	19.63	0.87	0.5216
AC	-0.10	24.19	1	24.19	1.08	0.4884
BC	-0.02	3.34	1	3.34	0.15	0.7657
ERROR		22.48	1	22.48		
TOTAL SS		476.45	7			
			Phenolics			
A	2.92	2.67	1	2.67	11.54	0.0182
B	-1.09	0.76	1	0.76	3.27	0.3215
C	-2.71	4.32	1	4.32	18.69	0.1447
AB	0.28	0.28	1	0.28	1.22	0.4689
AC	-1.04	0.02	1	0.02	0.07	0.8353
BC	-0.05	0.05	1	0.05	0.22	0.7200
ERROR		0.23	1	0.23		
TOTAL SS		8.33	7			

Significant ($p<0.05$), A – temperature (°C), B – flow rate (mL/min), C – pressure (bar).

Two flow rates of 2 and 5 mL/min were used for extraction of carbohydrates, pentosans and phenolics in the first factorial design. Figure 4.9 showed that the rate of extraction was higher at a flow rate of 5 mL/min compared to 2 mL/min. The extraction is higher because the increase in the solvent flow rate means that

greater amount of solvent is available for extraction. Also, a higher flow rate decreases the residence time of the solvent inside the high pressure vessel. Approximately 40 g carbohydrates/100 g lentil husk was extracted after 45 min using 5 mL/min of water; while it took 150 min at the lower flow rate (Appendix B.4b) to extract the same amount of carbohydrates. But, flow rate was not significant in the yields of carbohydrates or phenolics (Table 4.6). Liu and Wyman (2003) reported no change in the final recovery of xylan from corn stover for water flow rates of 1-10 mL/min at 180-220°C and 21-24 bar after 60 min of extraction.

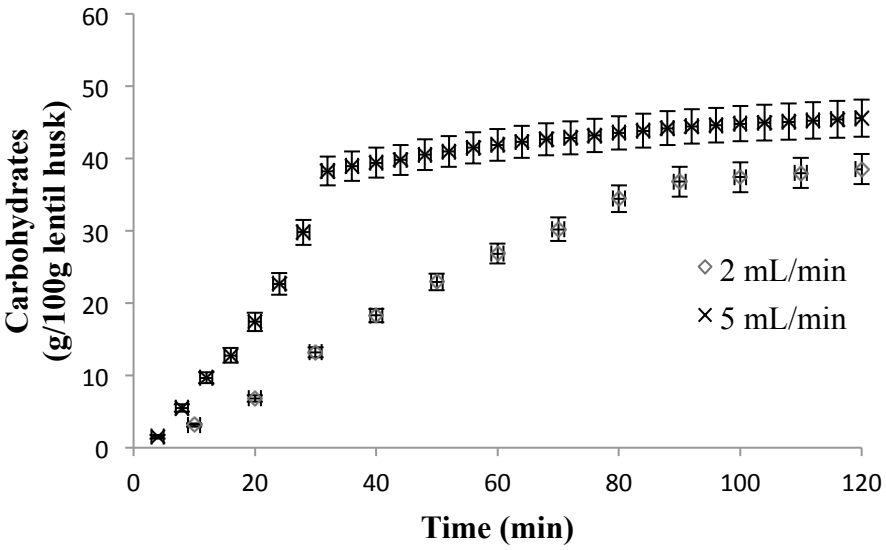


Figure 4.9 Total carbohydrates extracted of lentil husk at 120°C, 15 bar and flow rates of 2 and 5 mL/min.

On basis of the results obtained using the factorial design 1 (Table 4.6), a new factorial design 2 was developed with temperature, ethanol concentration of the solvent and pH as the three variables (Table 4.8). A high pressure vessel of 17 mL volume was used for these experiments. Since the vessel volume decreased by

60% (from 50 mL to 17 mL), the flow rate of the solvent also decreased to 2 mL/min against 5 mL/min used in the earlier experiments in order to maintain a constant residence time for the solvent inside the vessel. Temperature range of 120 to 200°C, with the pH of the solvent varying from highly acidic (pH=4) to highly basic (pH=10) was used for this design. The extractions were carried out at different concentrations of ethanol (0 to 80%) to determine its effect on the yieldsof total carbohydrates and phenolics.

The extraction time was decided based on the refractive index of the extract measured every 10 min for 90 min (Fig. 4.10). The RI increased rapidly in the first 20 min of extraction indicating maximum extraction of bio-compounds during that time (Appendix B.5). The RI dropped gradually after that, indicating gradual decrease in the available bio-compounds.

After each experiment, the pH and conductivity of the extracts were measured. The extracts were found to be alkaline (pH>7). Contrary to this observation, pH change was less prominent in extracts from conventional extraction (Appendix B.2). The alkalinity of the extracts could be attributed to the high amount of salts extracted during the extraction as per their conductivity values (Appendix B.6). The high conductivity of the extracts is caused due to dissolution of the mineral salts during extraction (Urbano et al., 2007). Since lentil husk is rich in minerals such as, calcium, phosphorus, iron, sodium and potassium (Muehlbauer et al., 1995), higher extraction temperatures results in greater dissolution of these salts. The anionic salts, such as carbonate, acetate or phostphates served as the conjugate base of a weak acid. These are slightly basic and increase the pH of

water when they are dissolved. Similarly, 0.1 mol of phosphate ions/L of water raises its pH from 7.0 to 7.2-7.6 at 25°C (Riché et al., 2006).

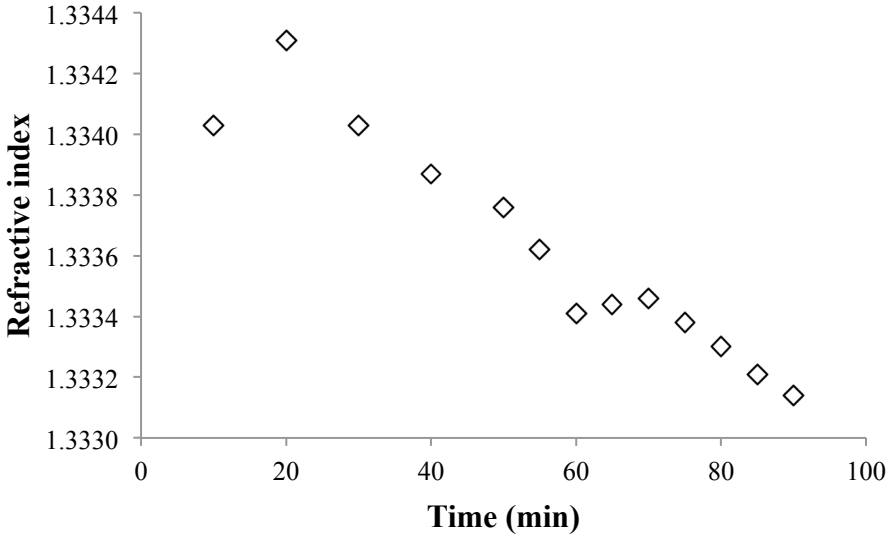


Figure 4.10 RI curve plotted as a function of time at 184°C, pH of 8.8, ethanol content of 63.78% and 65 bar for extraction of lentil husk.

To maintain liquid state inside the vessel, ethanol+water solutions were used at minimum 65 bar (over the saturation pressure of the mixture). This pressure was calculated in our laboratory using the Peng-Robinson equation of state (Table 4.7) for different ethanol mixtures.

Table 4.7 Vapor pressure for the mixture water+ethanol

Mole fraction		Vapor pressure (bar)			
Water	**Ethanol**	**70°C**	**80°C**	**180°C**	**200°C**
0.00	1.00	0.74	1.08	19.67	29.59
0.05	0.95	1.04	1.56	24.91	35.32
0.20	0.8	1.04	1.56	29.87	45.98
0.40	0.6	1.05	1.56	28.93	43.48
0.60	0.4	1.05	1.55	27.28	39.81
0.80	0.2	1.03	1.52	23.69	33.41
1.00	0.00	0.31	0.47	10.01	15.53

The results from the factorial design 2 (Table 4.8) were used to determine the optimum conditions for the maximum yieldsof carbohydrates and phenolics. Effect of each of the significant parameters and the statistical analysis to determine the optimum conditions are discussed in subsequent sections.

4.2.3.1 Effect of temperature

In this study, the maximum temperature of extraction was 200°C because above that temperature the carbohydrates monomers convert to degradation products such as, furan, furfural, hydroxymethyl furfural and levulinic acid. Figure 4.11 shows typical breakdown of glucose during auto-hydrolysis in SCW. The mechanism is known as autohydrolysis because of the absence of any exogenous acid to perform hydrolysis. During this process, the hemicellulose and cellulose break down to oligomers (xylo-oligomers, oligosaccharides, cellobiose) and monosaccharides (glucose, xylose, arabinose). The oligomers breakdown to corresponding monosaccharides as the extraction proceeds. With increase in

temperature above 200°C, these monomers degrade to furans, furfurals, hydroxymethyl furfurals and levulinic acid. There is formation of acetic acid and formic acid during this process which increases the hydrolyzing capacity of the SCW (Ramos, 2003). Therefore, in our study, the highest temperature of extraction was 200°C where maximum solubilisation of hemicellulose could be obtained, minimizing degradation.

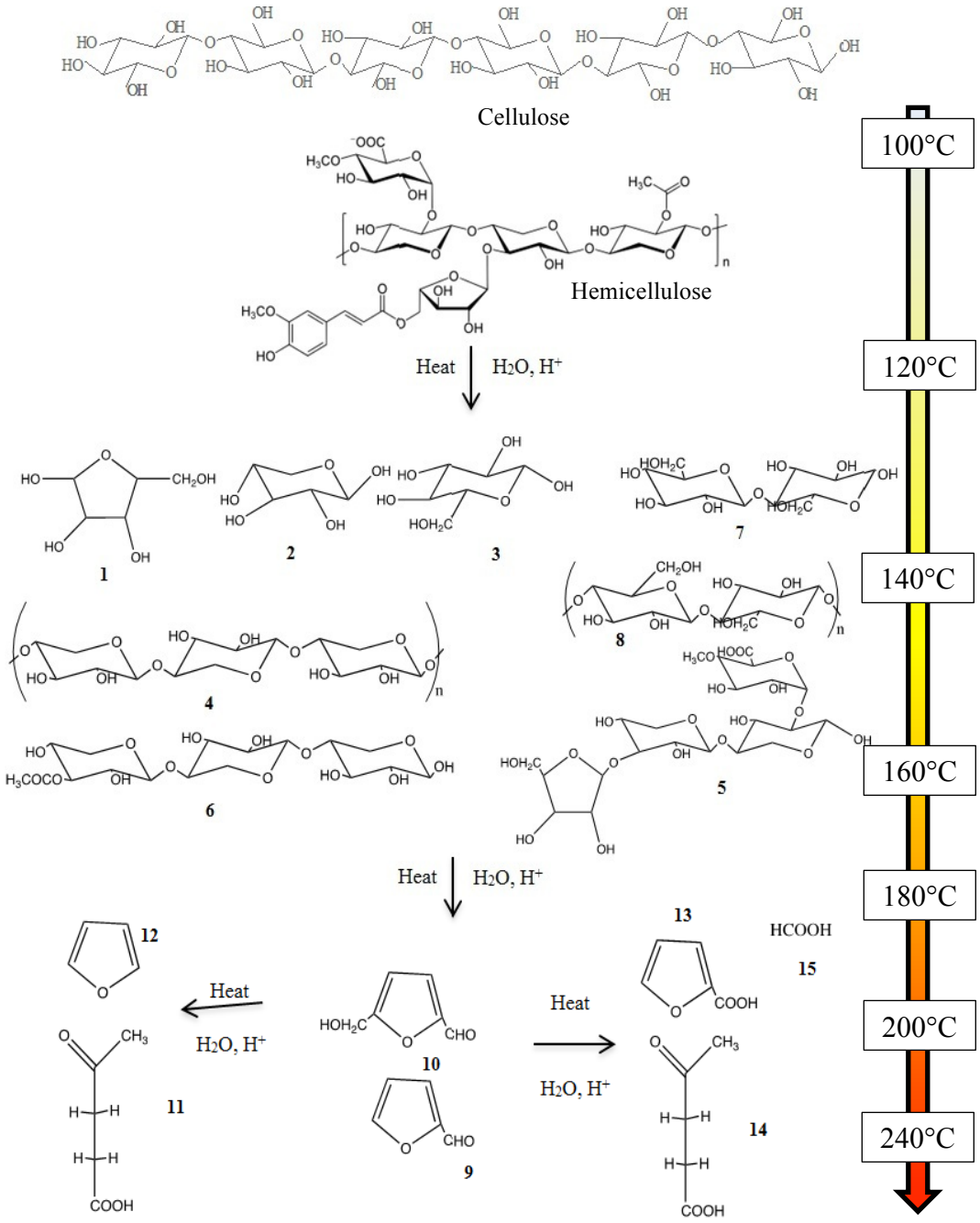


Figure 4.11 Hydrolysis of hemicellulose and cellulose as a result of steam explosion of hardwood from 120 to 240°C
(1) Arabinose, (2) xylose, (3) glucose, (4) xylo-oligomers of higher molecular mass, (5) acidic branched oligosaccharides, (6) acetylated xylo-oligomers (DP of 3), (7) cellobiose, (8) cello-oligomers, (9) furfural, (10) hydroxymethylfurfural, (11) levulinic acid, (12) furan, and (13) 2-furoic acid (pyromucic acid), (14) acetic acid and (15) formic acid (adapted from Ramos, 2003) (DP – Degree of polymerization).

Figure 4.12 shows that the extraction of carbohydrates increased significantly with an increase in temperature. This behavior was also observed in extracts obtained from the factorial design (Table 4.5). Maximum yield of carbohydrates was obtained at 160°C, neutral pH of 7 and in absence of ethanol (57.80 g/100 g lentil husk) (Table 4.8). This yield was considerably higher than the one obtained by conventional extraction method (8.60±0.12 g/100 g lentil husk). The conventional extraction method can dissolve only a part of the hemicelluloses and soluble carbohydrates but is unable to dissolve cellulose as it is insoluble in water at temperatures below 180°C and less influenced by moderate acidic (pH-4)/basic conditions (pH-10). During the SCW extraction process, cellulose starts solubilizing above 180°C. Garrote et al. (1999) reported recovery of 15-29% of solubilized cellulose during extraction of agricultural residues, such as corn stover, vine shoots and wheat straw between 180-200°C, with water flow rates of 1-40 mL/min and residence time of 0.1 – 10s. They obtained 52% of xylose at 230°C and a pressure of 34 bar with a flow rate of 10 mL/min from corn stalks.

Table 4.8 Yields of carbohydrates, pentosans and phenolics of lentil husk by SCW extraction using factorial design 2

Temperature (°C)	pH	Ethanol (%, v/v)	Yield (g/100 g lentil husk)		
			Carbohydrates	Pentosans	Phenolics
160*	7	40	35.60	17.64	2.83
160*	7	40	35.50	17.85	2.85
183	5.2	63.8	33.74	16.39	4.22
200	7	40	52.02	24.15	4.81
136	5.2	16.2	25.90	12.87	1.84
136	8.8	63.8	9.20	4.98	1.99
136	8.8	16.2	15.79	7.15	1.67
136	5.2	63.8	25.10	12.66	2.27
160	7	0	57.80	28.85	2.56
160*	7	40	35.30	17.64	2.79
120	7	40	12.70	6.64	1.75
183	8.8	16.2	46.20	23.66	3.09
160	7	80	13.10	6.64	3.81
160	4	40	23.47	9.58	3.28
160*	7	40	34.90	17.44	2.83
160	10	40	46.12	22.62	2.57
183	5.2	16.2	53.20	26.57	3.38
183	8.8	63.8	26.60	13.28	3.67
160*	7	40	35.80	17.85	2.77
160*	7	40	35.30	17.44	2.93

*Central point average: carbohydrates = 35.40±0.31 g/100 g lentil husk, pentosans = 17.64±0.1831g/100 g lentil husk and phenolics = 2.83±0.06 g/100 g lentil husk.

Table 4.9 ANOVA for yields of carbohydrates, pentosans and phenolics of lentil husk by SCW extraction using factorial design 2

Factors	Coefficient	Sum of squares (SS)	df	Mean square	*F value*	*P value*
		Carbohydrates				
A	11.00	1653.32	1	1653.32	28.76	< 0.0001
B	-0.09	0.11	1	0.11	0.00	0.9662
C	-8.87	1073.24	1	1073.24	18.67	0.0005
ERROR		0.36	5	0.07		
TOTAL SS		3646.52	19			
		Phenolics				
A	0.13	0.24	1	0.24	295.51	< 0.0001
B	-0.03	0.01	1	0.01	12.93	0.0024
C	0.05	0.03	1	0.03	35.99	< 0.0001
ERROR		0.00036	5	0.000072		
TOTAL SS		0.29	19			

Significant – $p<0.05$, A – temperature (°C), B – pH, C - ethanol content (%) in the solvent.

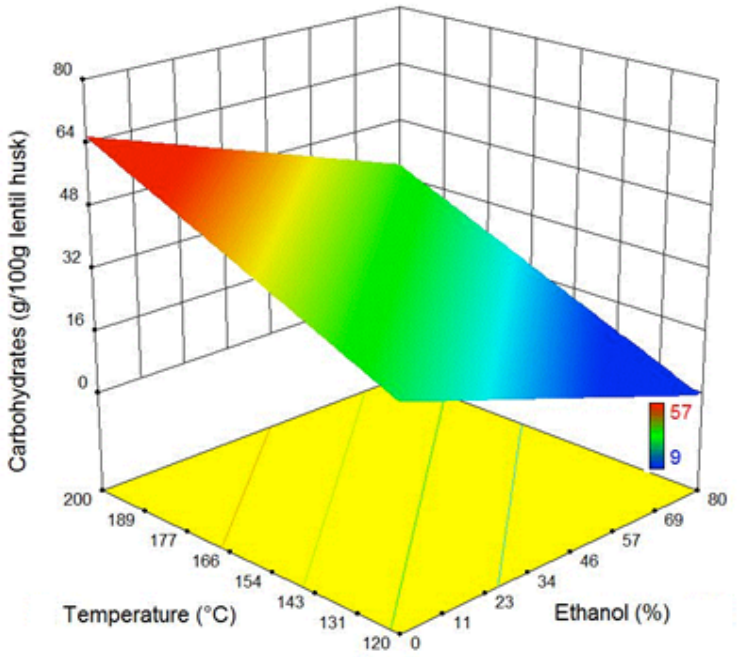


Figure 4.12 Yield of carbohydrates as a function of temperature and ethanol content at constant pH of 7, pressure of 65 bar and a flow rate of 2 mL/min for the extraction of lentil husk using SCW.

Even though temperature had a positive effect on carbohydrates extraction, a maximum of 200°C was used in this study. Degradation of monosaccharides above 180°C has been well documented by Haghighat Khajavi et al. (2005). They studied degradation kinetics of glucose in SCW using a tubular high pressure vessel in the temperature range of 180-260°C and a constant pressure of 100 bar. The degradation of glucose increased with increase in temperature from 180 to 260°C, leading to the formation of secondary degradation compounds of which hydroxyl methyl furfural (HMF) was identified as the main product as a result of dehydration of the hexoses. Qi and Xiuyang (2008) also reported that the HMF decomposes further at subcritical conditions (180-260°C) to generate levuinic acid (LA), which is fairly stable under subcritical conditions (180-260°C) with a maximum decomposition of 7.08% at 280°C for 32 h.

A change in color of the extract was observed with the color darkening with increase in temperature from 160 to 200°C. Pritchard and Adam (1994) hypothesized it as a result of Maillard reaction. Maillard reaction is reported to occur at temperatures of 55°C for model systems (Laroque et al., 2008) between a reducing sugar (e.g. glucose) and an amino acid (e.g. lysine). But the color of the product is intensified after 120°C and increases seven fold as it reaches 150°C, producing a dark colored solution when glucose and lysine are heated together (Fogliano et al., 1999). During SCW extraction, these compounds percolate into the extracting water, contributing to the dark color of the extract (Fig. 4.13).

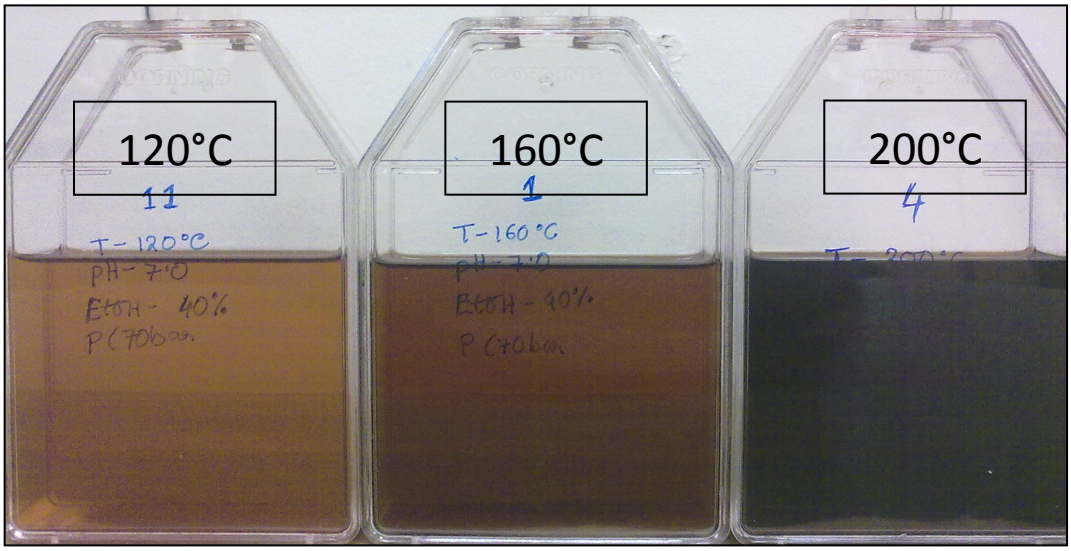


Figure 4.13 Effect of temperature on the color of the extracts of lentil husk at 65 bar with pH of 7, ethanol content of 40% and flow rate of 2 mL/min for three different temperatures (120, 160 and 200°C).

Effect of temperature on the yield of phenolics had been reported by Rangsriwong et al. (2007) (for *Terminalia chebula)*, Shalmashi (2008) (for black tea leaf) and Singh and Saldaña (2011) (for potato peel). Rangsriwong et al. (2007) found that the yield of gallic acid increased from 1 mg/g (d.w.) to 5 mg/g (d.w.) when the temperature increased from 120 to 200°C at 6 bar and 5 mL/min. Shalmashi (2008) reported that increase in temperature from 100 to 175°C at 20 bar and 4 mL/min increased the recovery of phenolics from 40 to 80%. Recently, Singh and Saldaña (2011) reported the highest yield of phenolics from potato peel at 180°C using SCW. In the current study, a similar observation was confirmed. Extraction of phenolics was the highest at 200°C (4.81 g/100 g) compared to 120°C (1.75 g/100 g) (Table 4.8). ANOVA analysis (Table 4.9) of the extraction of phenolics showed that temperature and ethanol content positively affected the yield.

The use of different concentrations of ethanol + water binary solvents improved the recovery of phenolics (Fig. 4.14). The use of ethanol + water (40%, v/v) increased the total yield of phenolics by approximately 10% at 160°C (2.83±0.06 g/100 g) compared to pure water (2.56 g/100 g) (Table 4.8). The effect of ethanol + water on the total yield is discussed in the next section.

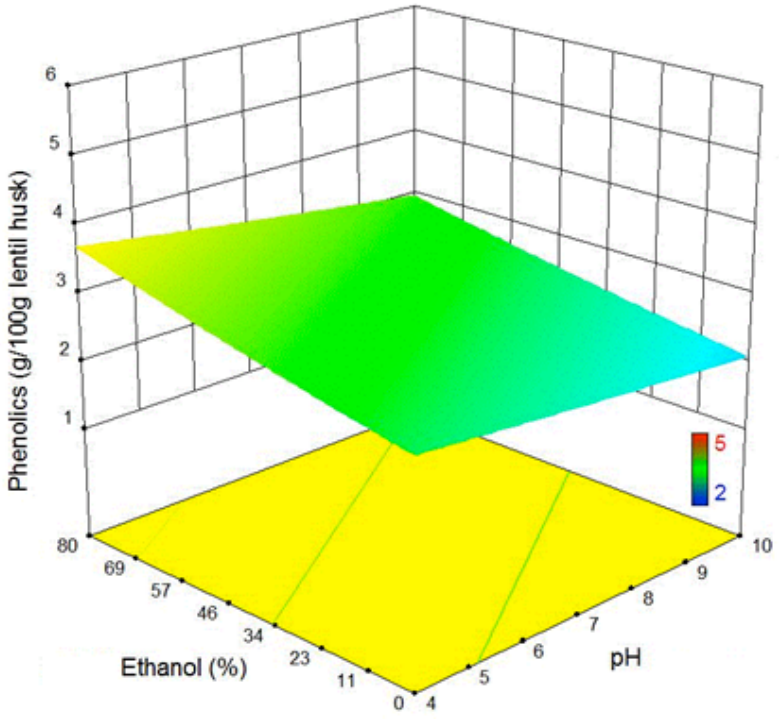


Figure 4.14 Yield of phenolics as a function of pH and ethanol content at constant temperature of 160°C, pressure of 65 bar and a flow rate of 2 mL/min for the extraction of lentil husk using SCW.

4.2.3.2 Effect of pressurized ethanol + water solvents

Ethanol is sometimes a better solvent for phenolics compared to pure water as reported by Lee et al. (2009). They extracted phenolics from olive leaf with different solvents, such as water, ethanol, butanol, hexane and chloroform at temperatures of 20-60°C. Ethanol (80%) and butanol (78%) had better yield of phenolics compared to hexane (65%) and chloroform (62%). In another study

with olive leaf (Mylonaki et al., 2008), optimization of phenolics extraction with ethanol + water mixtures was done. The highest recovery of phenolics was obtained at a concentration of 60% (v/v) ethanol at room temperature (22 ± 2°C). A similar study using the binary mixture solvent was conducted by Cacace and Mazza (2003) and Libran et al. (2010). They used ethanol with concentrations of 40-80% (v/v) to extract phenolics from black currant and grapes at temperatures of 20-60°C. They found the highest yield of phenolics at the highest concentration of ethanol (80%, v/v).

In this study, the yield of phenolics increased with increase in concentration of ethanol from 0% (2.56 g/100 g) to 80% (3.81 g/100 g) at 160°C, pH of 7 and a flow rate of 2 mL/min (Table 4.8 and Fig. 4.14). ANOVA showed that the change in ethanol concentration had a significant effect ($p<0.05$) on the yield of phenolics (Table 4.9) along with temperature and pH.

Ethanol also acts as an antisolvent for monosaccharides like glucose. Flood and Puagsa (2000) proposed a method to crystallize glucose and fructose with ethanol + water binary mixtures at 25°C and ethanol concentrations of 40-80% (v/v). This explains the decrease in the recovery of carbohydrates with the increase in ethanol concentration (Fig. 4.12) at 160°C from 0% ethanol (57.80 g/100 g) to 80% ethanol (13.10 g/100 g) content. Moreover, precipitation of soluble carbohydrates was visually observed in the extracted samples in agreement with the experiments reported by Alves et al. (2007). They measured solubility of sugars in ethanol + water binary mixtures with ethanol concentrations of 50-80% at 60°C on basis of their refractive index. Solubility of glucose was reduced by 50% when ethanol

concentration increased from 50 to 80%, resulting in crystallization of the remaining glucose.

4.2.4 Statistical analysis

The optimum conditions and maximum recovery of carbohydrates and phenolics with the factorial design 2 was evaluated using the statistical software Design Expert^{TM}. Data obtained for the yield of carbohydrates and phenolics was fitted into a polynomial equation. The best fit was obtained with a first order linear equation. This provided two linear polynomial equations that fitted the response surface for the corresponding yields.

$$\text{Carbohydrates (Yield \%)} = 32.56 + 11.00A - 0.088B - 8.87C, \; p<0.05 \quad \textbf{4.5}$$

$$\text{Phenolics (Yield \%)} = 0.45 + 0.13A - 0.028B + 0.046C, \; p<0.05 \quad \textbf{4.6}$$

where, A = temperature, B = pH and C = ethanol content.

The optimization was performed to achieve maximum yield of carbohydrates and phenolics. The optimized conditions were: 200°C, 22.83% (v/v) ethanol content and pH of 4.

Table 4.10 compares the yields carbohydrates, pentosans and phenolics obtained from the optimized conditions using conventional and SCW extraction. The yield of carbohydrates was higher for SCW extraction when compared to conventional extraction. This is due to higher degree of hydrolysis of polymeric carbohydrates at elevated temperatures. The yield of pentosans was much lower than the predicted value, which can occur as a result of reduction to hydroxyl methyl furfural. This hypothesis could be supported by the high absorbance values of the

extracts at 420 nm for the first 30 min of extraction (Appendix B.7). Fogliano et al. (1999) had used absorbance at 420 nm to express the extent of Maillard reaction occurring in a carbohydrate-protein system using wheat pasta as their reference material at different temperatures (80-150°C) and extraction times (15-60 min). In this study, high rate of Maillard reaction occurred for the first 30 min of extraction due to the high extraction temperature (200°C) and abundance of carbohydrate in the initial stages of extraction.

Both the conventional and SCW extraction methods were successful in extracting most of the phenolics present in the extracts.

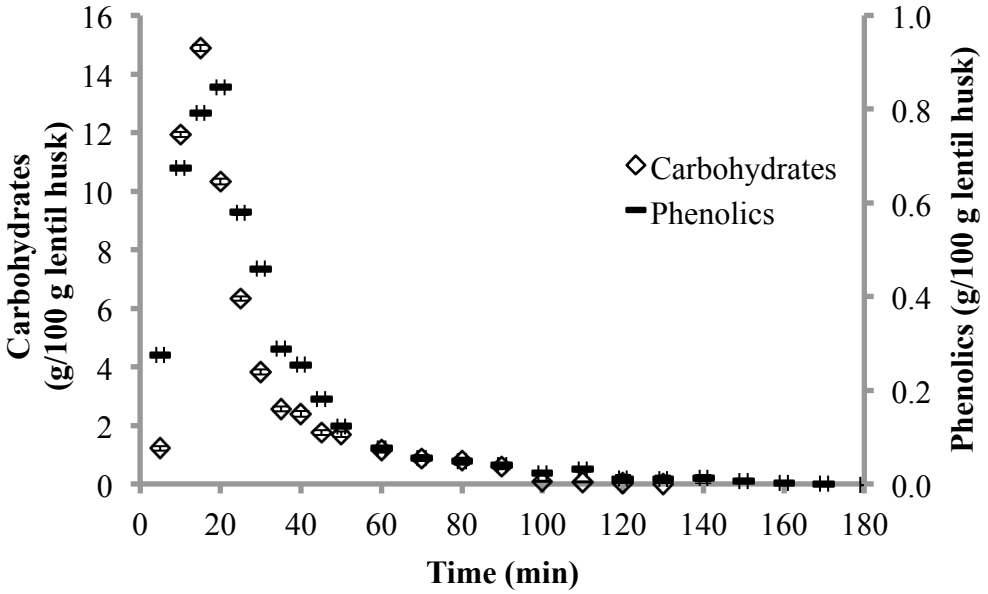
Table 4.10 Yields of carbohydrates, pentosans and phenolics of lentil husk by different extraction methods

Component	Standard	Conventional*		SCW extraction*	
		Experiment	Predicted	Experiment	Predicted
Carbohydrates (g/100 g lentil husk)	75.29±1.90	8.60±0.12	10.78±1.72	60.54±1.32	57.62±5.66
Pentosans (g/100 g lentil husk)	30.29±0.80	n.d.	n.d.	18.26±1.47	27.8±4.06
Phenolics (g/100 g lentil husk)	3.98±0.90	4.32±0.04	4.00±1.00	4.78±0.15	4.80±0.26

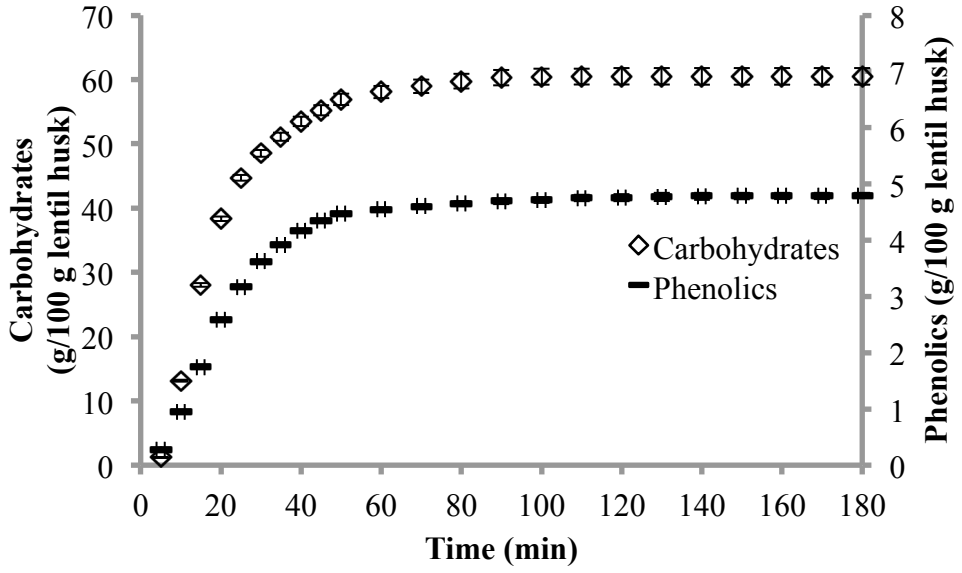
*Optimal conditions: Conventional – 69.96°C, 66.84% ethanol content and pH of 4.0 for 360 min, SCW – 200.00°C, 22.83% ethanol content, pH of 4.0 and 65 bar for 180 min and flow rate of 2 mL/min, n.d. - not determined.

Figure 4.15 shows kinetic curves of lentil husk at optimal conditions. During the kinetics experiment, pH and conductivity were measured every 10 min of extraction. The maximum extraction of both carbohydrates and phenolics was obtained during 15-20 min of extraction (Fig. 4.15a). As the extraction proceeded, the carbohydrates and phenolics content in the lentil husk decreased, resulting in the lower availability of extractable components till the extraction reached a

plateau stage after which further extraction was not possible (Fig 4.15b) (Appendix B.7).



(a)



(b)

Figure 4.15 (a) Extraction rate and (b) cumulative extraction of carbohydrates and phenolics from lentil husk at 200°C, pH of 4 and 22.83% ethanol content.

The pH of the extract was higher than the solvent, but remained almost constant during the whole experiment. The increase in the pH of the extract is due to dissolved protein and anionic salts (Riché et al., 2006) extracted from the lentil husk (Appendix B.7). The behavior of the conductivity of the extracts collected after every 10 min of extraction was similarly to the extraction rate of carbohydrates and phenolics. It increased rapidly during the first 20 min of extraction, after which it slowly decreased. Since lentil husk is rich in minerals (Muehlbauer et al., 1995), the hot water and pressure forced the minerals present in the lignocellulose matrix to leach out rapidly in the first 20 min of extraction after which it slowly decreased.

4.3 Extraction of lentil cotyledon

The main objective of this work was to extract carbohydrates and phenolics from lentil cotyledon. The specific objectives include: (i) determination and optimization of process conditions (e.g. temperature, pressure, flow rate and pH) that affect the yields of carbohydrates and phenolics, and (ii) perform a kinetic study at the optimum conditions.

4.3.1 Proximate compositional analysis

Lentil cotyledon was analyzed for its proximate contents, such as moisture, fat, carbohydrates, protein, starch and phenolics. The cotyledon was rich in starch (52.31±1.20%) and protein (23.42±0.40%). The quantity of fat (1.83±0.12%) and ash (2.64±0.13%) was comparatively low. The amount of soluble carbohydrates (glucose, sucrose, mannose, rafinose, stachyose and ciceritol) was obtained by the difference between the total carbohydrates to the amount of starch present in the cotyledon. Table 4.11 also shows that the proximate composition of the lentil cotyledon from the 'Redberry' variety is similar to other lentil varieties reported in the literature.

Table 4.11 Proximate compositional analysis of lentil cotyledon

Components	This study (g/100 g, db)	Literature (g/100 g, db)
Total carbohydrates	61.81±1.40	59.7-62.5%[1-3,6]
Starch	52.31±1.20	34.7-52.8%[2,3,6]
Soluble carbohydrates	9.52±0.40	4.6-9.5%[1,4,5,7]
Moisture	11.24±0.60	8.05 – 9.06%[2,3]
Protein	23.42±0.40	25.3-26.9%[2,3]
Fat	1.83±0.12	1.1-2.3%[3]
Ash	2.64±0.13	2.3-2.8%[3]
Total phenolics	2.75±0.10	n.a.

d.b. – dry basis, [1]Berrios et al. (2010), [2]Ofuya and Akhude (2005),[3]García-Alonso (1998),[4] Sánchez-Mata et al. (1998), [5]Quemener and Brillouet (1983) (type of lentil not specified), [6]El-Nahry and Mourad (1980) and [7]Han and Baik (2006) worked on both red and green lentils.

4.3.2 Conventional extraction

Conventional extraction of lentil cotyledon was performed for 180 min at 40-80°C, with pH from 4-10 and ethanol concentration of 0-80%. The experiment at 55°C, pH of 7 and ethanol concentration of 80% was used to determine the extraction time by measuring the refractive index (RI) of the extract at intervals of 10 min for 180 min (Appendix C.1). Figure 4.16 shows that the RI became constant after 170 min of extraction.

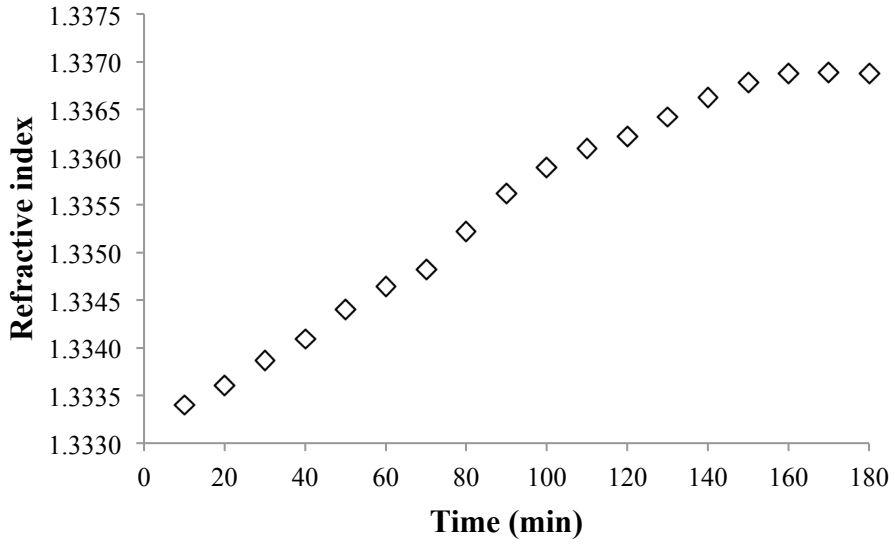


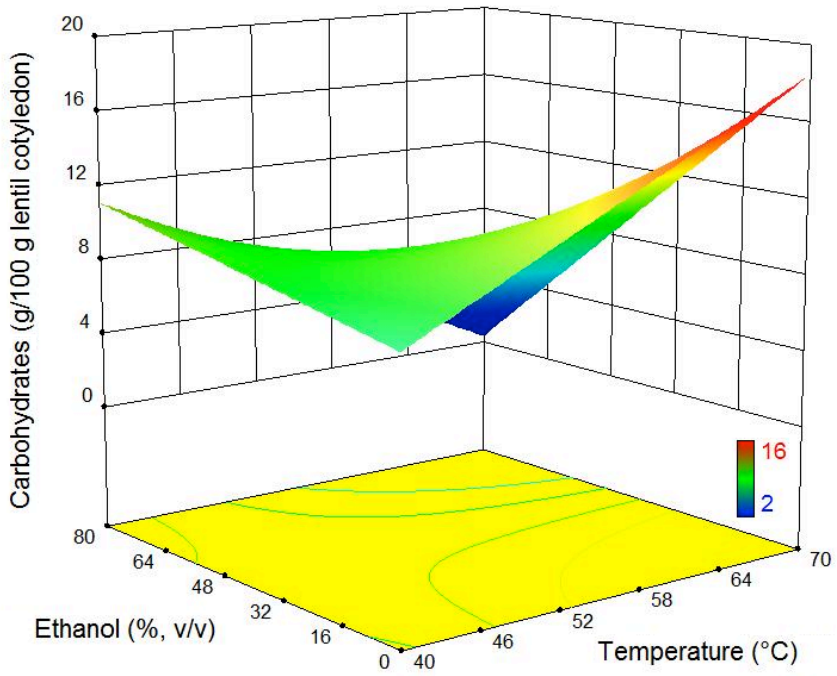
Figure 4.16 RI curve plotted as a function of time at 55°C, pH of 7 and ethanol concentration of 80% for conventional extraction of lentil cotyledon.

As discussed earlier, carbohydrate in lentil cotyledon is mostly composed of starch with a high concentration of soluble sugars, which are more accessible to extraction at lower temperatures than lignocellulose of husk. Thus, conventional extraction of lentil cotyledon yielded higher carbohydrates than lentil husk. Maximum amount of carbohydrates (15.97 g/100 g lentil cotyledon) was recovered during extraction at 63°C in presence of 16.2 % ethanol and pH of 5.2. Both temperature of extraction and ethanol content of the solvent were found to affect the extraction process. As reported in Table 4.12, an increase in extraction temperature from 40°C to 70°C improved the yield of carbohydrates by 1.7 times, while the increase in ethanol concentration from 0 to 80% had resulted in decrease of the yield of carbohydrates by 1.5 times (Fig. 4.17). This negative effect of ethanol concentration in the solvent affecting the solubility of

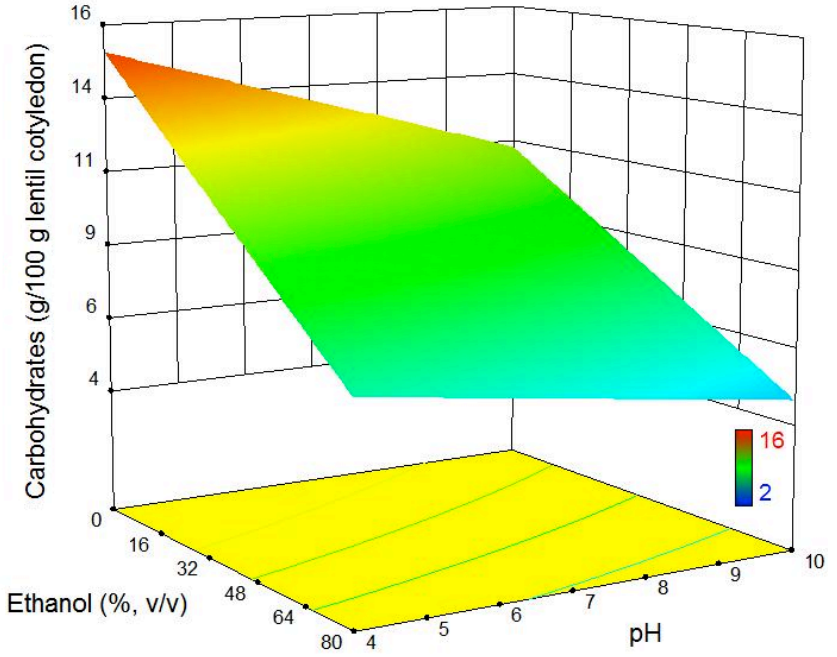
carbohydrates monomers have also been observed by Bockstanz et al. (1989) for α-anhydrous glucose at 35°C and Peres and Macedo (1997) for D-glucose at 40 and 60°C with concentration of ethanol ranging from 0 to 100% (v/v).

In this study, a change of pH from basic to acidic improved the yield of carbohydrates significantly (Table 4.13). Similar observation had been reported by Daiuto et al. (2005) when they used oxalic acid (10%, v/v) for starch extraction from Yam tubers at room temperature. Oxalic acid yielded 18% more starch compared to 0.3M NaOH solutions.

ANOVA (Table 4.13) of the obtained data and Figure 4.17 show that pH and ethanol content adversely affect the yield of carbohydrates, thus proving to be the most influential factors affecting the extraction.



(a)



(b)

Figure 4.17 Yield of carbohydrates for conventional extraction of lentil cotyledon as a function of: (a) temperature and ethanol content at pH of 7 and (b) ethanol content and pH at 55°C.

Maximum recovery of phenolics was achieved at 70°C, pH=7 and ethanol concentration of 40%. ANOVA showed that the yield of phenolics was influenced by temperature of extraction and ethanol concentration of the solvent (Table 4.13). The increase in temperature from 40 to 70°C increased the yield of phenolics by almost 25% and increase in ethanol concentration from 0 to 80% improved the yield of phenolics by 5% (Table 4.12 and Fig. 4.18).

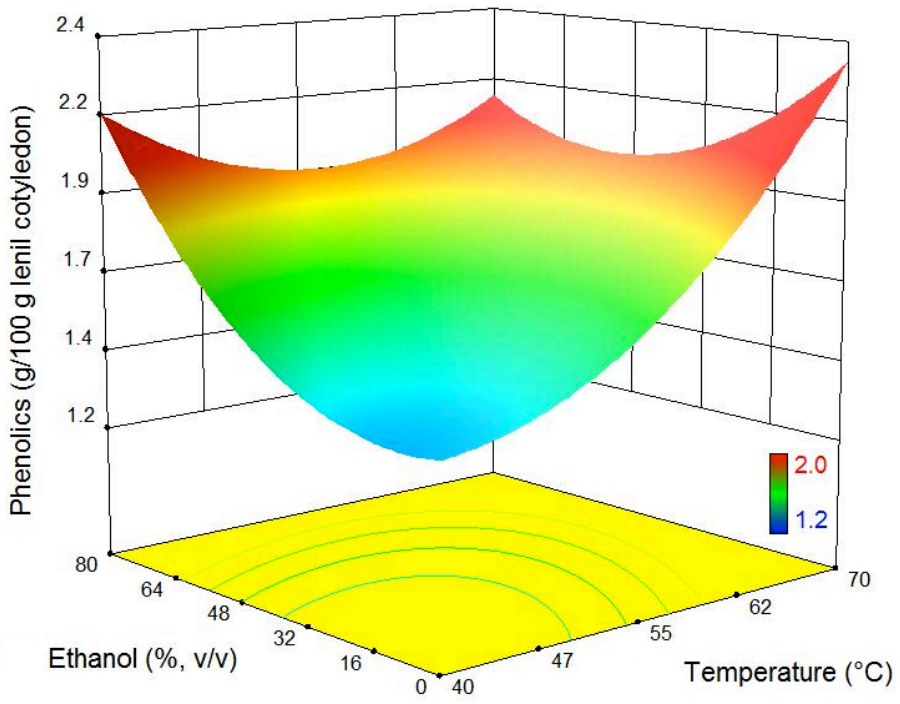


Figure 4.18 Yield of phenolics as a function of temperature and ethanol content at pH of 7 for conventional extraction of lentil cotyledon.

Table 4.12 Yields of carbohydrates and phenolics of lentil cotyledon by conventional extraction

Temperature (°C)	Ethanol (%, v/v)	pH	Yield (g/100 g lentil cotyledon)	
			Carbohydrates	Phenolics
40	40	7	6.57	1.64
55	40	10	8.63	1.58
55	80	7	7.71	1.91
46	64	9	10.90	1.74
46	16	9	11.86	1.24
55	40	4	11.65	1.57
70	40	7	11.14	2.04
55*	40	7	8.91	1.55
55*	40	7	8.75	1.53
55	0	7	14.20	1.82
55*	40	7	9.50	1.58
64	16	9	9.66	1.78
55*	40	7	7.81	1.51
64	64	5	6.60	1.86
46	64	5	9.07	1.67
64	64	9	1.94	1.76
55*	40	7	8.32	1.54
64	16	5	15.97	1.72
55*	40	7	9.38	1.57
46	16	5	9.89	1.34

*Central point average: carbohydrates = 8.78±0.64 g/100 g lentil cotyledon, and phenolics = 1.55±0.02 g/100 g lentil cotyledon.

Table 4.13 ANOVA for yields of carbohydrates and phenolics of lentil cotyledon by conventional extraction

Factor	Coefficient	Sum of squares (SS)	Df	Mean square	*F value*	*P value*
		Carbohydrates				
A	0.01	0.00	1	0.00	0.00	0.9800
B	-2.18	64.94	1	64.94	28.59	0.0001
C	-0.90	10.99	1	10.99	4.84	0.0465
AB	-1.91	29.32	1	29.32	12.91	0.0033
AC	-1.85	27.27	1	27.27	12.01	0.0042
BC	0.19	0.29	1	0.29	0.13	0.7283
ERROR		2.04	5	0.41		
TOTAL SS		162.34	19			
		Phenolics				
A	0.13	0.24	1	0.24	23.00	0.0007
B	0.08	0.09	1	0.09	8.68	0.0146
C	0.00	0.00	1	0.00	0.01	0.9131
AB	-0.09	0.06	1	0.06	6.16	0.0324
AC	0.00	0.00	1	0.00	0.00	0.9775
BC	0.00	0.00	1	0.00	0.00	0.9730
A^2	0.07	0.08	1	0.08	7.78	0.0191
B^2	0.08	0.10	1	0.10	9.89	0.0104
C^2	-0.02	0.01	1	0.01	0.49	0.5012
ERROR		0.003	5	0.001		
TOTAL SS		0.68	19			

Significant – $p<0.05$, A – Temperature (°C), B – Ethanol content (%), C – pH.

By using Design expert ^{TM} software, the optimized condition for maximum yields of carbohydrates and phenolics was predicted using the following equations:

Carbohydrates (Yield %) = 9.42+0.01A-2.18B-0.90C-1.91AB-1.85Ac+0.19BC, ($p<0.05$) **4.7**

Phenolics (Yield %) = 1.55+0.13A+0.08B +0.001C-0.09AB+0.001AC+0.001BC+0.07A^2+0.08B^2-0.02C^2, ($p<0.05$) **4.8**

where, A = temperature, B = ethanol content and C = pH

Extraction was done at the optimized condition of 69.60°C, ethanol content of 0.67% and pH of 5.91. The yields obtained at these optimized conditions were 17.01±0.17 g carbohydrates /100 g lentil cotyledon and 2.02±0.02 g phenolics /100 g lentil cotyledon which were slightly lower than the predicted values of carbohydrates (20.83±0.93 g /100 g lentil cotyledon) and phenolics (2.30±0.10 g /100 g lentil cotyledon) (Table 4.18).

A kinetic study of extraction at the optimal conditions (Fig. 4.19) shows that both the carbohydrates and phenolics follow similar trend for extraction. The rate of extraction is high for the first 120 min, and then it slows down (Appendix C.3). The extraction reached completion at the end of 180 min as curve reaches a plateau stage after 180 min of extraction.

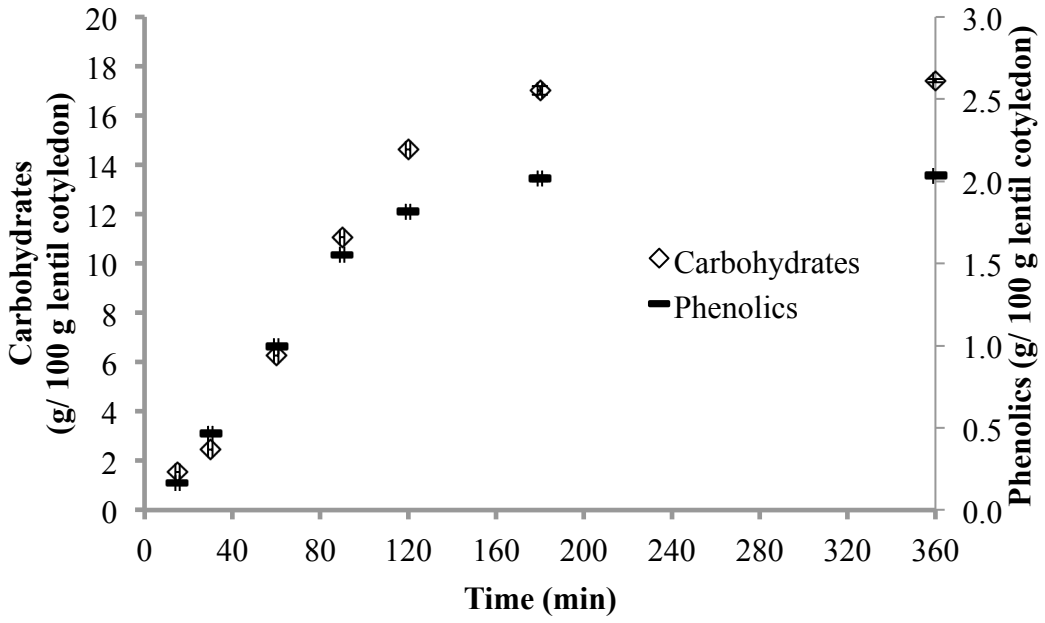


Figure 4.19 Kinetic curve for extraction of carbohydrates and phenolics from lentil cotyledon using conventional extraction at 69.60°C, ethanol content of 0.67% and pH of 5.91.

4.3.3 SCW extraction

The SCW extraction process of lentil cotyledon was optimized on the basis of yield of total carbohydrates and total phenolics. The process conditions that influenced the yield were temperature, pressure, flow rate and pH. Factorial design 1 was used to determine the effect of the first three parameters. The total yield of carbohydrates included both the starch and the soluble sugars dissolved in the SCW. In the first factorial design, the total time of extraction was determined on basis of the RI of the extract measured after intervals of 10 min, where the experimental conditions were 180°C, 15 bar and a flow rate of 2 mL/min. A decrease in RI value was observed after 50 min of extraction, reaching the minimum after 210 min of extraction (Fig. 4.20, Appendix C.4). Thus, the total extraction time was 210 min for all experiments conducted.

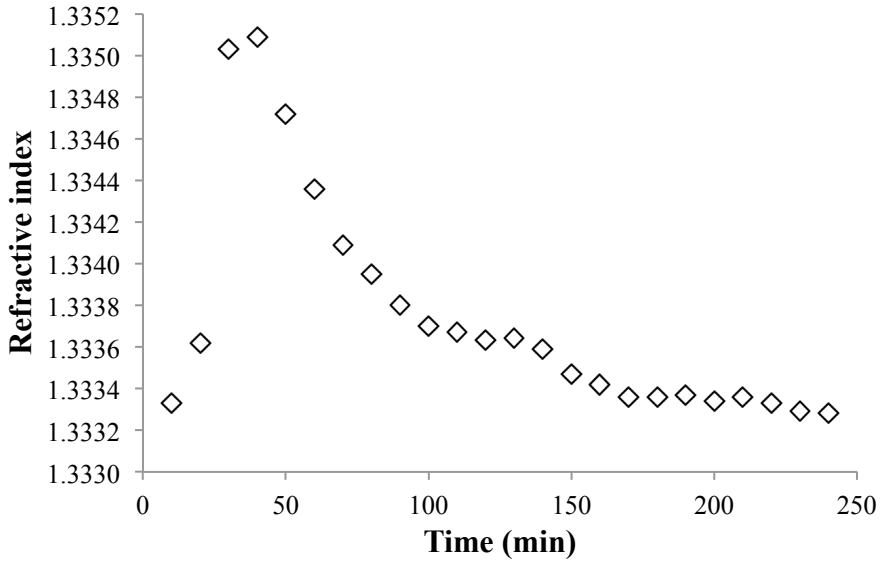


Figure 4.20 RI curve plotted as a function of time at 180°C, 15 bar, and a flow rate of 2 mL/min for extraction of lentil cotyledon.

The yield of carbohydrates (Table 4.14) increased from 11.18±1.03 g/100 g at 120°C to 37.36±5.92 g/100 g at 180°C and the same pressure (120 bar) and a flow rate of 2 mL/min. This is due to high solubility of starch at temperatures above 180°C. Miyazawa et al. (2008) found that starch (source not mentioned) solubilised in less than 6 min of residence time inside a semi-batch high pressure vessel at 180°C (pressure not mentioned). The yield of phenolics increased with increase in temperature as reported by Singh and Saldaña (2011). The yield of phenolics (Table 4.14) increased from 1.05±0.25 g/100 g at 120°C to 1.38±0.10 g/100 g at 180°C, and same conditions of pressure (120 bar) and flow rate (2 mL/min). Even though, pressure significantly influenced the yield of carbohydrates, it did not have a significant effect on phenolics. The yield of carbohydrates increased from 15 bar (23.71±0.05 g/100 g) to 120 bar (37.36±5.92 g/100 g) at constant temperature (180°C) and flow rate (2mL/min) due to breakdown of the starch matrix (Zheng et al., 1995).

The ANOVA analysis (Table 4.15) of the carbohydrates results showed that the effect of flow rate, temperature and pressure were significant ($p<0.5$). For phenolics, both temperature and pressure did not have significant effect on the extraction, but an increase in flow rate improved the extraction.

Table 4.14 Yields of carbohydrates and phenolics of lentil cotyledon by SCW extraction using factorial design 1

Temperature (°C)	Pressure (bar)	Flow rate (mL/min)	Yield (g/100 g lentil cotyledon)	
			Carbohydrates	Phenolics
180	15	2	23.71±0.049	0.94±0.023
120	120	2	11.18±1.027	1.05±0.251
120	15	5	25.08±6.165	2.14±0.435
180	120	2	37.36±5.921	1.38±0.100
180	120	5	42.75±3.182	2.55±0.448
180	15	5	34.10±2.970	2.30±0.034
120	15	2	4.84±0.925	1.01±0.033
120	120	5	33.67±0.882	2.36±0.210

Table 4.15 ANOVA for yields of carbohydrates and phenolics of lentil cotyledon by SCW extraction using factorial design 1

Factors	Value Factor	Sum of squares (SS)	Df	Mean square	*F value*	*P value*
Carbohydrates						
A	3.82	931.81	1	931.81	90.62	< 0.0001
B	2.46	386.93	1	386.93	37.63	0.0002
C	3.53	795.72	1	795.72	77.39	0. 0100
AB	0.59	22.43	1	22.43	2.18	0.1738
AC	-1.82	210.96	1	210.96	20.52	0.0014
BC	-0.04	0.11	1	0.11	0.01	0.9214
ERROR		85.88	8	10.73		
TOTAL SS		2440.48	15			
Phenolics						
A	0.04	0.10	1	0.10	1.65	0.2316
B	0.06	0.23	1	0.23	3.83	0.082
C	0.31	6.14	1	6.14	102.71	< 0.0001
AB	0.03	0.05	1	0.05	0.78	0.3996
AC	0.01	0.00	1	0.00	0.04	0.8542
BC	0.00	0.00	1	0.00	0.00	0.9643
ERROR		0.50	8	0.06		
TOTAL SS		7.06	15			

Significant - $p<0.05$, A- temperature (°C), B – pressure (bar), C- flow rate (mL/min).

Flow rate had a significant effect on the yield of total carbohydrates using SCW extraction of lentil cotyledons. Figure 4.25 shows that the increase in flow rate from 2 to 5 mL/min increased the yield of carbohydrates from 37.36±5.92 g/100 g to 42.75±3.18 g/100 g at 180°C and 120bar in factorial design 1. The same trend was observed for the yield of total phenolics, where the yield increased from 1.38±0.10 g/100 g to 2.55±0.45 g/100 g with increase in flow rate from 2 to 5mL/min at 180 °C and 120 bar, respectively.

A high flow rate (5 mL/min), reached the maximum yield of carbohydrates faster than a low flow rate (2 mL/min) (Fig. 4.21). At 90 min of extraction, a higher water flow rate of 5 mL/min extracted 37.48 g carbohydrates /100 g lentil cotyledon which is higher compared to a lower flow rate of 2 mL/min (24.99 g/ 100 g lentil cotyledon) (Appendix C.5). This increase in the yield of carbohydrates was because of the increased solvent availability in the high pressure vessel at a given time. Since 5 mL/min flow rate had higher volume of solvent passing through the high pressure vessel compared to 2 mL/min at any given time interval, 5 mL/min had a higher solvent to solid ratio which increased the rate of extraction.

The high pressure vessel (17 mL) was almost one-third the volume of the vessel used for factorial design 1 (50 mL). The residence time of the solvent inside the high pressure vessel was maintained by decreasing the flow rate pumped through the vessel. For factorial design 2, a flow rate of 2 mL/min was used instead of 5 mL/min to maintain the residence time of the solvent inside the vessel.

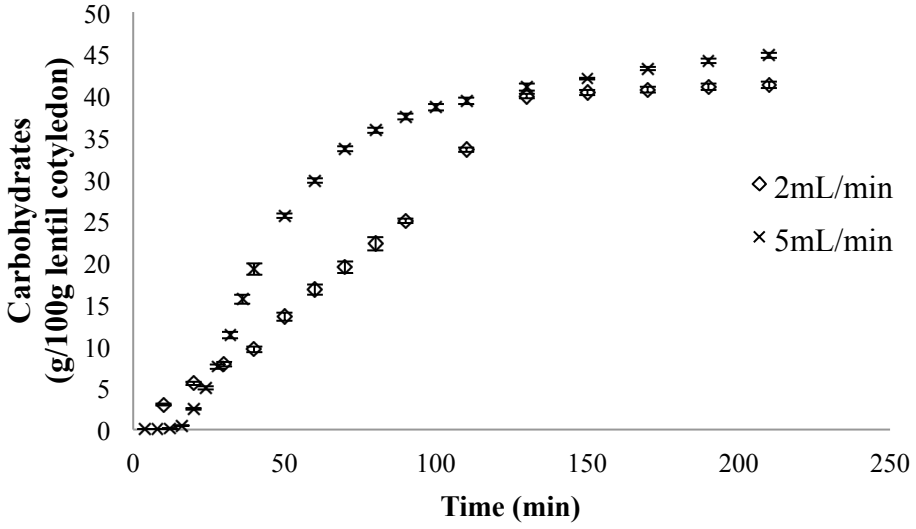


Figure 4.21 Total carbohydrates extracted of lentil cotyledon at 180°C, 120 bar and flow rates of 2 and 5 mL/min.

The effect of pH was studied in the factorial design 2 because Lee et al. (2007) reported that alkaline pH (9.5) increased the yield of carbohydrates from 85% (pH=7) to 95% at 40 °C. Therefore, factorial design 2 (Table 4.16) consisted of three parameters: temperature, pressure and solvent pH. The solvent pH ranged from acidic (pH=4) to basic (pH=10) conditions. The pH was modified using glacial acetic acid (1%) or sodium hydroxide (1%) solutions. The extraction time for this experiment was decided after monitoring the RI at 200°C, pH of 7 and pressure of 65 bar. Since the minimum RI was obtained at 90 min of extraction (Fig. 4.22, Appendix C.6), all the experiments in this factorial design were performed for 90 min.

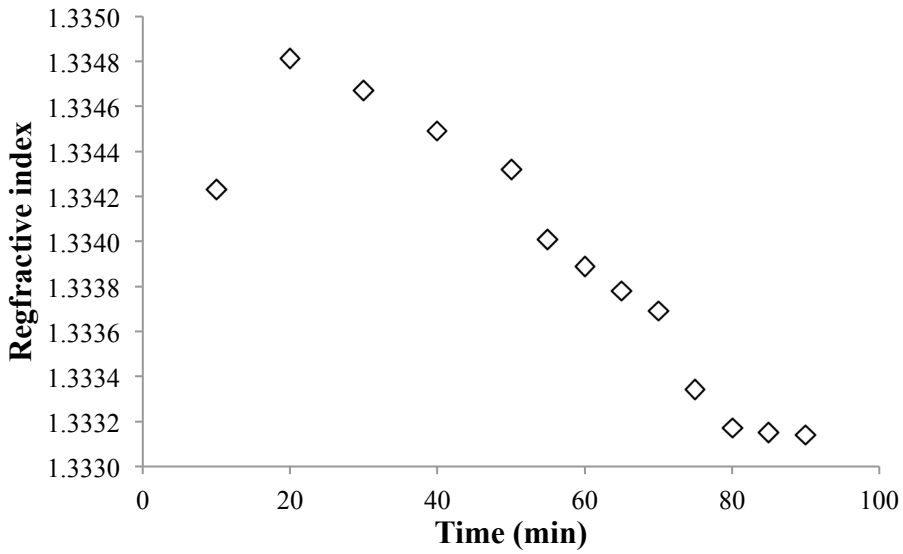


Figure 4.22 RI curve plotted as a function of time at 200°C, pH of 7 and 65 bar for extraction of lentil cotyledon.

The results from the factorial design 2 (Table 4.16) were used to determine the optimum conditions for the maximum yield of carbohydrates and phenolics. The conductivity of the extracts was measured at each experimental condition. Change in temperature and pH was found to affect the conductivity of the extracts. The conductivity of the extract increased with increase in temperature with a maximum of 868.5 mS/m at 200°C. The conductivity values of lentil cotyledon extract were higher compared to the values for lentil husk extract (Appendix B.2 and Appendix C.7). This is due to the high protein concentration in lentil cotyledon, because wet protein is a good conductor of electricity (Rosenberg, 1962). But an opposite trend was observed during conventional extraction, where the conductivity of lentil husk extracts was high compared to lentil cotyledon (Appendix B.2 and Appendix C.2). This is because the protein remained bound to the surface of starch during conventional extraction. Also, husk is rich in mineral

content compared to cotyledon. That is why the conductivity of cotyledon extracts are lower compared to husk extracts in conventional extraction.

Effect of each process parameter and the statistical analysis to determine the optimum conditions are discussed in subsequent sections.

4.3.3.1 Effect of temperature

Experiments carried out using the factorial design 1 for lentil cotyledon as a function of temperature, pressure and flow rate yielded low carbohydrates content (maximum of 42.75±3.18 g/100 g). While a high yield of carbohydrates (66.21 g/100 g) was obtained at a temperature of 160°C, pH of 7.0 and pressure of 65 bar as reported in Table 4.16. Statistical analysis of the results showed that the yield of total carbohydrates was significantly low ($p>0.05$) at 120°C (27.90 g/100 g) as compared to 160°C (65.18±0.87 g/100 g) at a pH of 7 and a pressure of 65 bar (Table 4.16). ANOVA analysis (Table 4.17) for the SCW extraction of carbohydrates and phenolics shows that temperature has a positive effect on the yields of carbohydrates and phenolics. This is because the β-1,6-glycosidic linkages in starch is broken due to high temperature. This phenomenon was studied on corn starch (Rogalinski et al., 2008a) and sweet potato (Toor et al., 2011) at temperatures of 180 – 310°C with unspecified pressures. During hydrolysis of corn starch, the complete solubilisation was achieved at 180°C within 6 min of extraction. Whereas, Toor et al. (2011) reported breakdown of carbohydrates in sweet potato to oligosaccharides at 180°C after 10 min of extraction in batch processing conditions and then converted to glucose with

higher extraction time and temperature (200°C and 30 min, 220°C and 10 min). The results of Toor et al. (2011) confirms the findings of Nagamori and Funazukuri (2004), who found the highest yield of glucose at 200°C after 30 min of extraction.

Table 4.16 Yields of carbohydrates and phenolics of lentil cotyledon by SCW extraction using factorial design 2

Temperature(°C)	pH	Pressure(bar)	Yield (g/100 g lentil cotyledon)	
			Carbohydrates	Phenolics
200	7.0	65	20.46	2.21
184	8.8	98	57.40	1.65
160	4.0	65	52.55	2.18
160*	7.0	65	65.80	1.93
160*	7.0	65	64.00	1.89
136	8.8	32	16.30	1.15
184	5.2	32	37.00	1.86
136	8.8	98	32.70	1.21
120	7.0	65	27.90	1.24
160*	7.0	65	64.80	1.94
160*	7.0	65	65.10	1.96
160	7.0	120	64.68	1.78
184	5.2	98	56.80	2.06
160	10.0	65	29.60	1.33
160*	7.0	65	64.90	1.87
136	5.2	98	17.74	1.62
160*	7.0	65	66.50	1.94
136	5.2	32	23.38	1.53
184	8.8	32	40.46	1.75
160	7.0	10	20.10	1.99

*Central point average: carbohydrates = 65.18±0.87 g/100 g cotyledon and phenolics = 1.92±0.04 g/100 g cotyledon.

Table 4.17 ANOVA for yields of carbohydrates and phenolics of lentil cotyledon by SCW extraction using factorial design 2

Factors	Coefficient	Sum of squares (SS)	df	Mean square	*F value*	*P value*
		Carbohydrates				
A	6.52	580.46	1	580.46	4.73	0.0548
B	-1.95	52.11	1	52.11	0.42	0.5294
C	8.97	1098.36	1	1098.36	8.95	0.0136
AB	-0.48	1.83	1	1.83	0.01	0.9053
AC	3.25	84.42	1	84.42	0.69	0.4263
BC	2.40	46.03	1	46.03	0.37	0.5540
A^2	-14.26	2930.67	1	2930.67	23.87	0.0006
B^2	-8.29	989.70	1	989.70	8.06	0.0176
C^2	-7.82	881.98	1	881.98	7.18	0.0231
ERROR		3.75	5	0.75		
TOTAL SS		7200.14	19			
		Phenolics				
A	0.25	0.14	1	0.14	94.26	< 0.0001
B	-0.20	0.08	1	0.08	57.53	< 0.0001
C	-0.01	0.00	1	0.00	0.00	0.9901
AB	0.03	0.01	1	0.01	6.27	0.0313
AC	-0.01	0.00	1	0.00	0.33	0.5788
BC	-0.04	0.00	1	0.00	0.52	0.4890
A^2	-0.11	0.04	1	0.04	24.42	0.0006
B^2	-0.10	0.02	1	0.02	16.84	0.0021
C^2	-0.05	0.00	1	0.00	2.37	0.1548
ERROR		0.0005	5	0.0001		
TOTAL SS		0.30	19			

Significant - $p<0.05$, A – temperature (°C), B – pH, C- pressure (bar).

The maximum yield of phenolics (2.21 g/100 g) was obtained at 200°C, 65 bar and pH of 7 and the minimum yield was obtained at 136°C, 32 bar and pH of 8.8. At the same pressure (65 bar) and pH (7), the yield was high at 200 °C (2.21 g/100 g) which decreased to 1.92±0.04 g/100 g at 160 °C and further to 1.24 g/100 g at 120 °C. ANOVA analysis (Table 4.17) confirmed that the increase in

temperature positively affected the yield of phenolics which were in agreement with results reported by Singh and Saldaña (2011) discussed earlier.

4.3.3.2 Effect of pressure

Increase in pressure for the SCW extraction of lentil cotyledon helps to break the starch matrix (Zheng et al., 1995). The hydrothermal extraction of ginger and turmeric bagasse using binary mixtures of water+CO_2 (Moreschi et al., 2004, 2006) proved this hypothesis, where the authors found higher degree of hydrolysis at pressures of 200 bar (70.8%) compared to 100 bar (66.0%) at all temperatures studied (150-200°C). Moreover, scanning electron microscopy (SEM) analysis of both ginger and turmeric starches after treatment with $SCCO_2$ and 150 bar at 80°C showed ruptures of cellulosic cell walls of the starch granules due to high pressure treatment 100 – 200 bar.

Similar results were obtained in this study (Fig. 4.23). Table 4.16 shows the yield of carbohydrates increased with pressure from 10 bar (20.10 g/100 g lentil cotyledon) to 65 bar (65.18±0.87 g/100 g lentil cotyledon) ($p<0.05$) at 160°C and pH of 7. ANOVA analysis (Table 4.17) also supports this observation where temperature and pressure are significant factors.

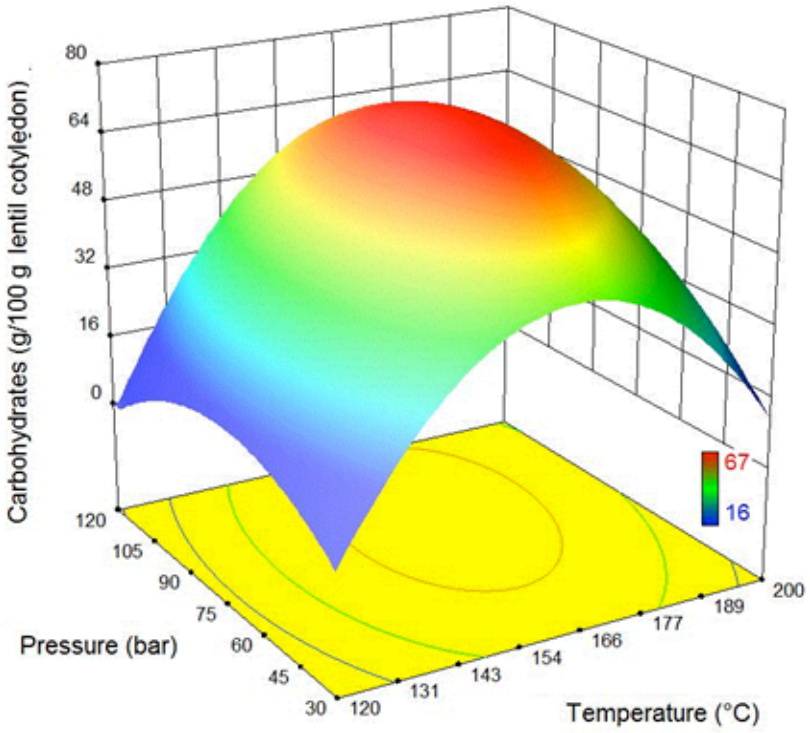
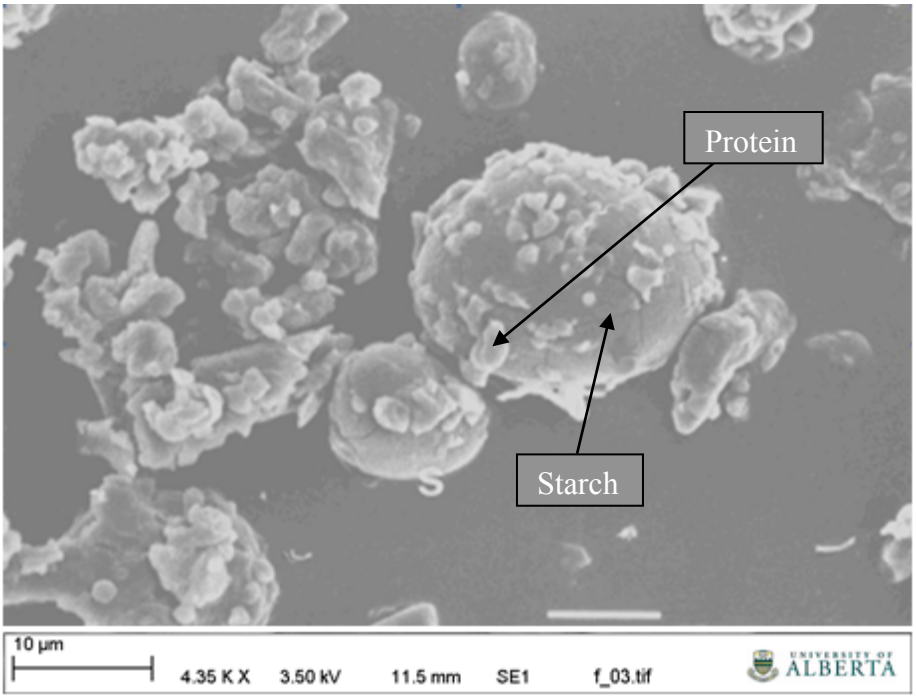
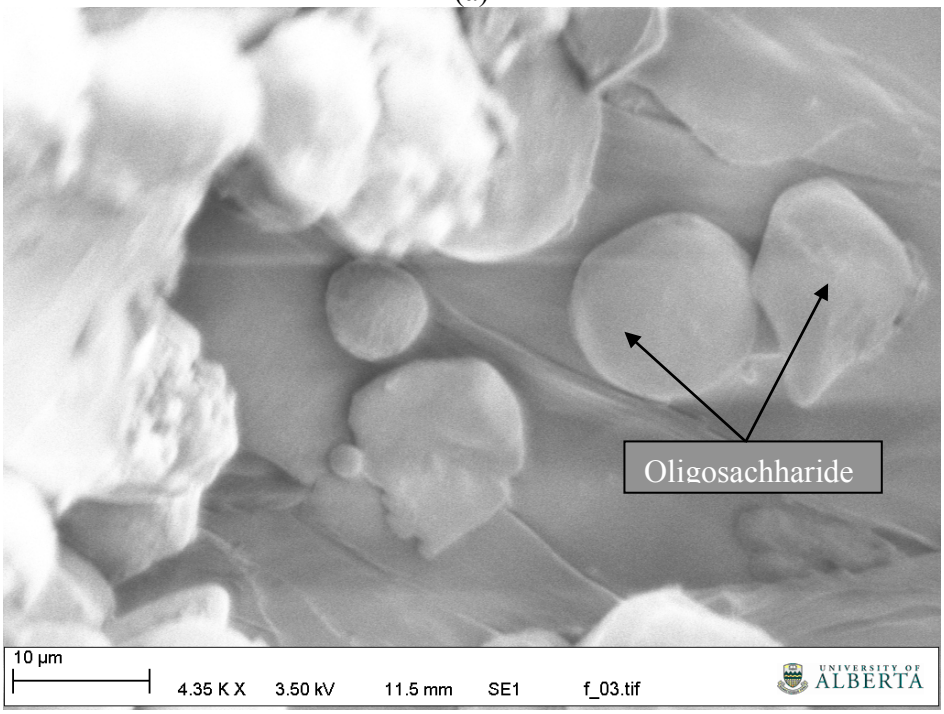


Figure 4.23 Yield of carbohydrates as a function of temperature and pressure at pH of 7 and a flow rate of 2 mL/min for extraction of lentil cotyledon using SCW.

The starch molecule observed in Figure 4.24a by SEM showed that the particle size ranged from 3-30µm in diameter, differing in shape from ovoid to spherical. The starch molecule had a coating on top which could be protein or cellulosic cell wall as described earlier by Moreschi et al. (2006), which could prevent its hydrolysis during conventional extraction. These cell structures had the majority of starch molecules inside their cavity and thus unavailable for extraction. An increase in pressure helped to break the cellulosic or protein structure to release the carbohydrates from the lentil cotyledon. In Figure 4.24b obtained from the extract, the starch molecule was devoid of cellulosic or protein structure.



(a)



(b)

Figure 4.24 Scanning electron microscopy (SEM) of: (a) untreated lentil flour, and (b) extract from the SCW treatment at 182°C, 56 bar and pH of 6.9 of lentil cotyledon.

4.3.3.3 Effect of pH

The effect of pH on increased yield of carbohydrates has been discussed by Tumaalii and Wootton (1988). They hypothesized that the starch granules swell rapidly in alkaline solvents (2-5%, v/v), and their swelling results in subsequent cell rupture and leaching of enclosed carbohydrates with increase in temperature. Similar results were obtained by Maher (1983a, 1983b) for other starchy grains like barley, buckwheat, corn, rice, wheat and rye. He observed an increase in starch viscosity with increasing concentration of sodium hydroxide from 0.94 to 8.47 milliequivalents per g flour at 25°C with a solid-solvent ratio of 1:10.

In this study, the pH was not significant to improve the yield of carbohydrates, but did significantly improve the yield of phenolics (Table 4.17). Phenolics yield increased with decrease in the pH of the solvent, thus making acidic solvent favorable for phenolics extraction. Figure 4.25 confirms this observation, showing that temperature and pH influence the extraction of phenolics from lentil cotyledon.

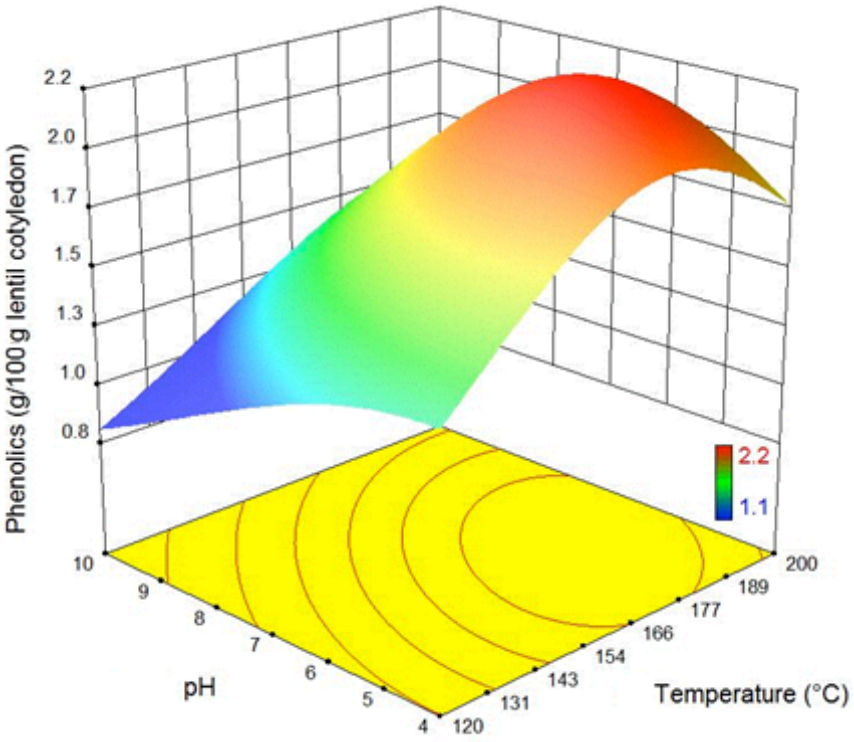


Figure 4.25 Yield of phenolics as a function of temperature and pH at a pressure of 65 bar for extraction of lentil cotyledon using SCW.

4.3.4 Statistical analysis

The recovery of carbohydrates and phenolics using factorial design 2 was evaluated with the statistical software Design Expert^{TM}. The behavior of the data obtained for the yields of carbohydrates and phenolics were fitted into a polynomial equation. The two linear polynomial equations to obtain the maximum yields of carbohydrates and phenolics were:

Carbohydrates (Yield %) = 32.56+11.00A-0.088B-8.87C, ($p<0.05$) **4.9**

Phenolics (Yield %) = 0.45+0.13A-0.028B -0.046C, ($p<0.05$) **4.10**

where, A = temperature, B = pH and C= pressure

The optimization was performed with the goal of achieving maximum yields of carbohydrates and phenolics. The optimized conditions were: 172.87°C, 80.21 bar and pH of 6.17.

Table 4.18 compares the yields of carbohydrates and phenolics from lentil cotyledon. SCW extraction of lentil cotyledon at optimized conditions yielded higher total carbohydrates (61.66±0.72 g/ 100 g lentil cotyledon) compared to conventional method (17.01±0.17 g/ 100 g lentil cotyledon). Moreover the yield of carbohydrates was within the range of the predicted yield at the optimal conditions which validates the accuracy of the experiments.

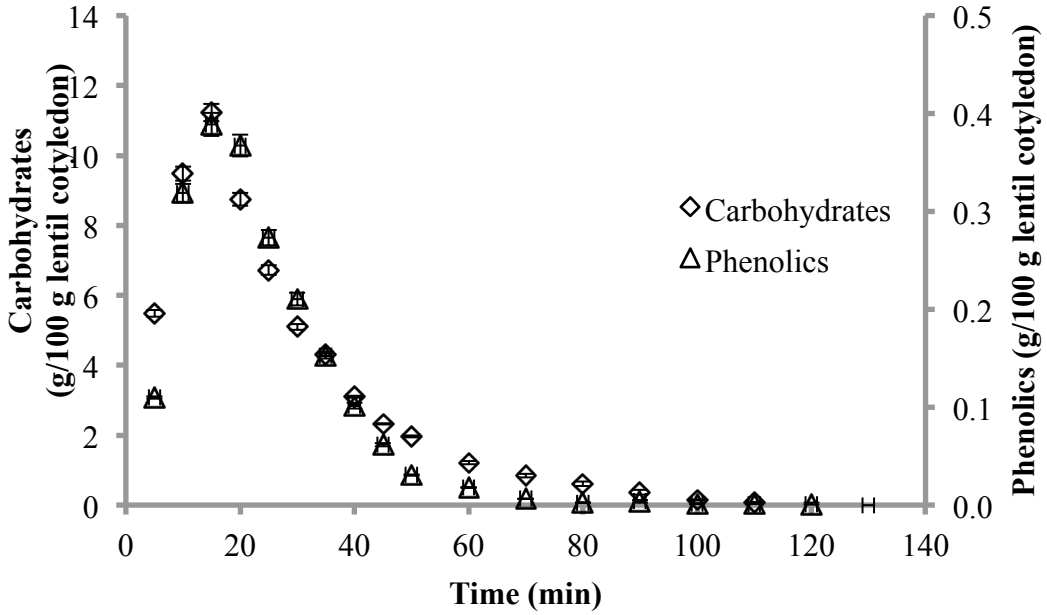
Similar to the lentil husk, the recovery of phenolics using SCW (2.05±0.06 g/100 g) and conventional method (2.02±0.02 g/100 g) were similar. Therefore SCW conditions did not show any advantage over the conventional method.

Table 4.18 Yields of carbohydrates and phenolics of lentil cotyledon by different extraction methods

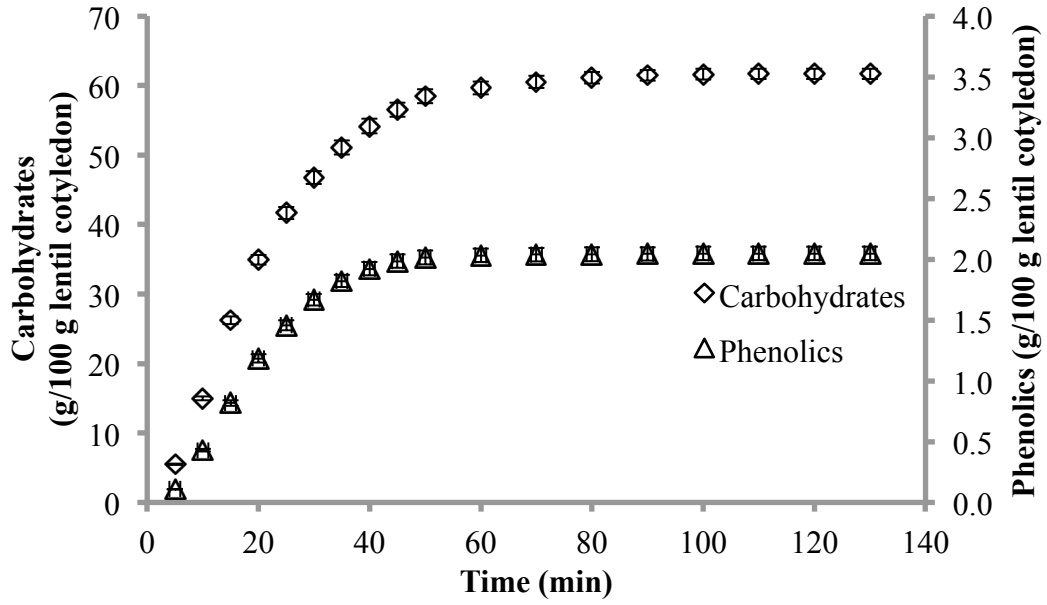
Component	Standard	Conventional*		SCW extraction*	
		Experiment	Predicted	Experiment	Predicted
Carbohydrates (g/100 g lentil cotyledon)	61.81±1.40	17.01±0.17	20.83±0.93	61.66±0.72	66.51±10.35
Phenolics (g/100 g lentil cotyledon)	2.6±0.01	2.02±0.02	2.30±0.10	2.05±0.06	2.09±0.16

*Optimized conditions: Conventional – 69.60°C, 0.67% ethanol content and pH of 5.91 for 360 min, SCW - 172.87°C, 80.21 bar and pH of 6.17 for 130 min and a flow rate of 2 mL/min

The results from the kinetics experiment showed that the yields of carbohydrates and phenolics increased rapidly in the first 15 min of the experiment but then decreased slowly towards the end of the experiment (Fig. 4.26a, Appendix C.8). After 80 min of extraction, the yields of carbohydrates and phenolics reached a plateau stage, indicating that the maximum extraction capacity had been achieved (Fig. 4.26b).



(a)



(b)

Figure 4.26 (a) Extraction rate and (b) cumulative extraction of carbohydrates and phenolics of lentil cotyledon at 172.87°C, 80.21 bar and pH of 6.17.

Since there was no ethanol in the solvent, the water did not react to release hydroxyl ions, keeping the pH of the extracts similar to the pH of the solvent, throughout the experiment. The behavior of conductivity with respect to

extraction time shows that most of the protein and minerals were extracted during the initial 30 min of extraction (Appendix C.8). However, protein and minerals should be analyzed in the extracts to confirm this hypothesis.

5 Conclusions and Recommendations

5.1 Conclusions

The objectives of this research have been met through a series of experiments by using factorial design and response surface methodology. The subcritical water (SCW) system was used effectively to extract carbohydrates and phenolics from the lentil biomass.

5.1.1 Solubility of sugars

- Solubility of glucose increased with temperature but decreased with an increase in pressure.
- Increase in glucose-glucose cohesive interaction with an increase in pressure decreased their solubility in water.
- Lactose solubility increased with increase in temperature, and pressure had less influence than in glucose solubility.

5.1.2 SCW extraction

- Conventional extraction was less effective compared to SCW extraction of carbohydrates from lentil husk and cotyledon.
- For lentil husk, carbohydrates yield increases with increase in temperature till 200°C. For lentil cotyledon, carbohydrates yield maximise at 180°C, after which degradation of carbohydrates occur, resulting in lower yield.
- Yield of phenolics using SCW was not significantly higher than conventional extraction.

5.2 Recommendations

- Additional solubility data for disaccharides need to be obtained to understand their behavior in subcritical water.
- Study of residence time is needed to avoid degradation of the extract.
- Separation of protein after extraction will be a significant post-processing step. This can be achieved by optimization of its iso-electric pH.
- Stability and functionality of the extract after protein separation should be determined by microbial inoculation to establish the shelf stability of the extract.

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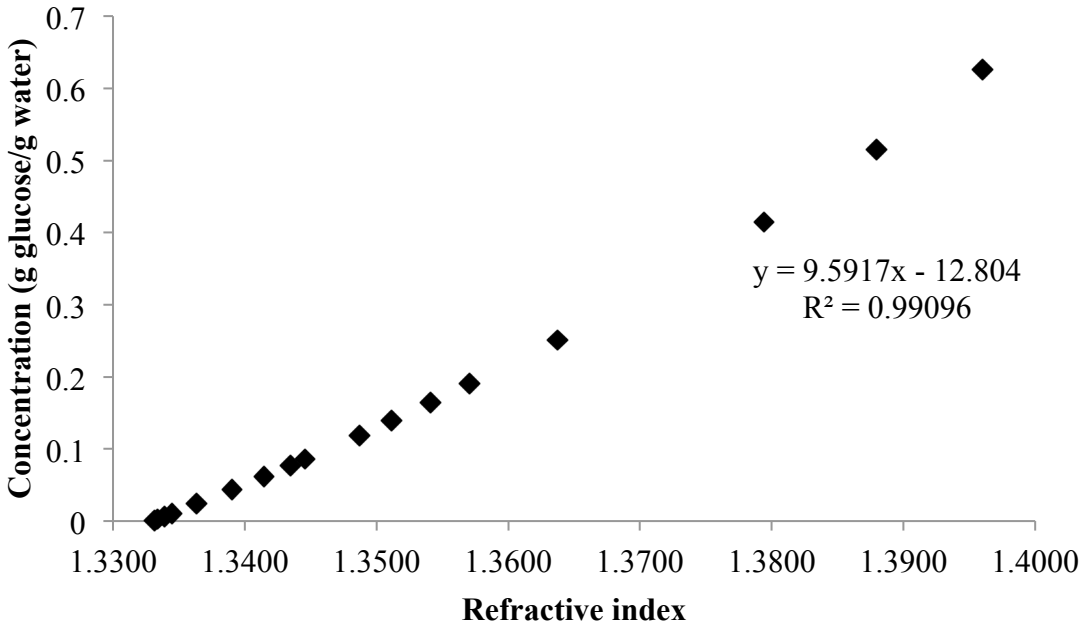
APPENDIX A: SOLUBILITY OF SUGARS IN SCW

A.1.a Calibration curve of glucose



Amount of glucose (g/g water) calculated from the above equation where x is obtained by the method mentioned in Section 3.2.11.

A.1.b Dissolving capacity of glucose in water at 30°C, 15 bar and flow rates of 1-4 mL/min

Flow rate (mL/min)	Temperature (°C)	Pressure (bar)	Refractive index (RI)	Glucose (g/g water)	Average glucose (g/g water)	Standard Deviation
1	30.1	15.4	1.39067	0.512	0.505	0.012
1	30.2	15.6	1.39133	0.518		
1	30.4	15.5	1.38913	0.498		
1	30.3	15.2	1.38836	0.491		
2	29.7	15.7	1.38803	0.488	0.469	0.016
2	29.8	15.9	1.38637	0.473		
2	30.0	15.5	1.38384	0.450		
2	30.1	15.6	1.38527	0.463		
3	29.9	15.2	1.38560	0.466	0.455	0.012
3	29.8	15.6	1.38296	0.442		
3	30.1	16.0	1.38340	0.446		
3	30.3	15.2	1.38549	0.465		
4	29.4	15.5	1.38516	0.462	0.449	0.011
4	29.9	15.3	1.38384	0.450		
4	30.2	15.8	1.38241	0.437		
4	30.1	15.1	1.38351	0.447		

A.2 ANOVA for dissolving capacity of glucose in water at 30°C, 15 bar and flow rates of 1-4 mL/min

Factor	Sum of squares	df	Mean square	*F-value*	*P-value*
Between flow rates	0.007	3	0.003	15.92	0.0001
Within flow rates	0.002	12	0.0002		
Total	0.010	15			

A.3 Dissolving capacity of glucose in water at 160°C, 15 bar and dilutions of 4, 6 and 8 times

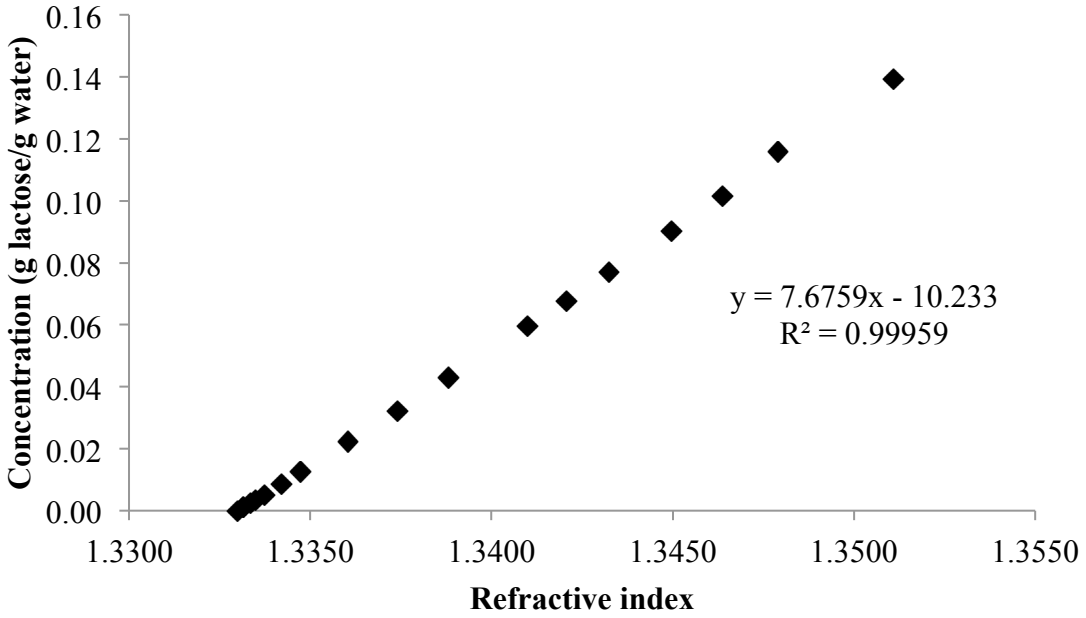
Flow rate (mL/min)		Dilution	Temperature (°C)	Pressure (bar)	Refractive index		Glucose (g/g water)			Standard Deviation
Pump 1	Pump 2				E1	E2	E1	E2	Average	
1	3	4	159.3	15.7	1.41221	1.41111	2.83	2.79	2.81	0.02
1	5	6	159.8	16.3	1.41855	1.42148	4.59	4.75	4.67	0.11
1	7	8	159.4	16.5	1.37668	1.37544	3.08	2.99	3.03	0.06

E1, E2 – experiments in duplicate

A.4 ANOVA for dissolving capacity of glucose in water at 160°C, 15 bar and dilutions of 4, 6 and 8 times

Factor	Sum of squares	df	Mean square	*F-value*	*P-value*
Between dilutions	4.11	2	2.05	355.55	0.0003
Within dilutions	0.02	3	0.01		
Total					

A.5.a Calibration curve of lactose



Amount of lactose (g/g water) calculated from the above equation where x is obtained by the method mentioned in Section 3.2.11.

A.5.b Dissolving capacity of lactose (150°C, 80 bar, a flow rate of 1 mL/min and 7 times dilution)

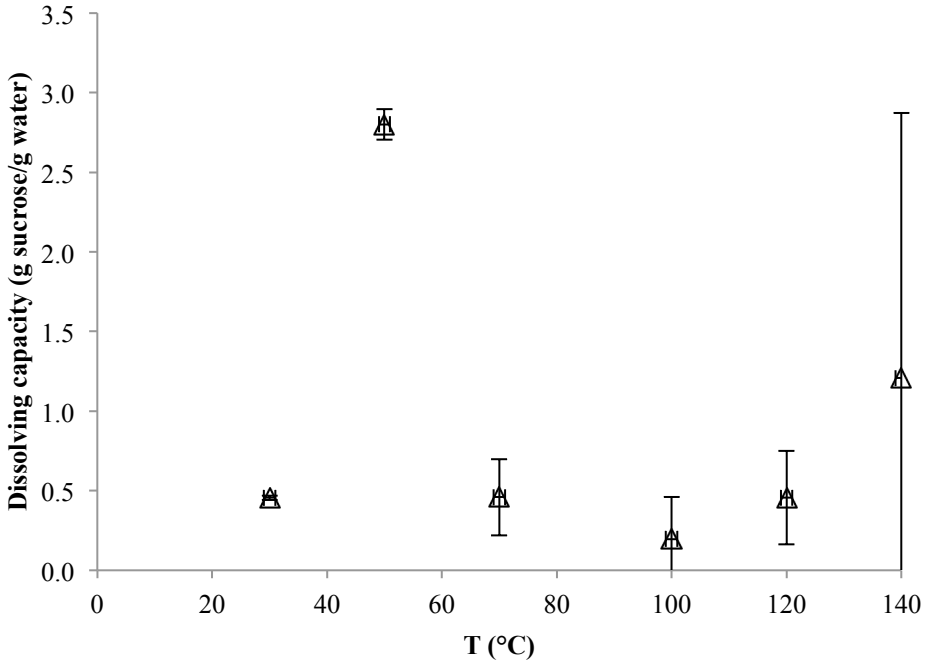
Time (min)	Temperature (°C)	Pressure (bar)	Refractive index (RI)		Lactose (g/g water)			
			E1	E2	E1	E2	Average	Standard Deviation
1	150	80	1.33313	1.33311	0.0039	0.0029	0.0034	0.001
2	150	80	1.33314	1.33310	0.0045	0.0024	0.0035	0.001
3	150	80	1.33340	1.33327	0.0182	0.0113	0.0148	0.005
4	150	80	1.33612	1.33369	0.1614	0.0335	0.0975	0.090
5	150	80	1.33863	1.34168	0.2937	0.4543	0.3740	0.114
6	150	80	1.34501	1.34698	0.6297	0.7335	0.6816	0.073
7	150	80	1.35258	1.35255	1.0285	1.0269	1.0277	0.001
8	150	80	1.35259	1.35258	1.0290	1.0285	1.0288	0.000
9	150	80	1.35260	1.35264	1.0295	1.0317	1.0306	0.002
10	150	80	1.35260	1.35228	1.0295	1.0127	1.0211	0.012
11	150	80	1.35193	1.35222	0.9943	1.0095	1.0019	0.011
12	150	80	1.35260	1.35261	1.0295	1.0301	1.0298	0.000
13	150	80	1.35259	1.35224	1.0290	1.0106	1.0198	0.013
14	150	80	1.35201	1.35226	0.9985	1.0116	1.0051	0.009
15	150	80	1.35255	1.35256	1.0269	1.0274	1.0272	0.000

E1, E2 – experiments in duplicate

A.6 Dissolving capacity of sucrose at different temperatures and 15 bar with a flow rate of 1 mL/min and 8 times dilution

Temperature (°C)	Sucrose (g/g water)			
	E1	E2	Average	Standard Deviation
30	0.47	0.45	0.455	0.013
50	2.908	2.772	2.801	0.096
70	0.598	0.181	0.459	0.241
100	0.382	0.012	0.197	0.262
120	0.664	0.248	0.456	0.294
140	2.385	0.031	1.208	1.665

E1, E2 – experiments in duplicates



APPENDIX B: EXTRACTION OF LENTIL HUSK

CONVENTIONAL EXPERIMENT

B.1 Refractive index (RI) as a function of time at 55°C, pH of 7 and 80% ethanol content for conventional extraction of lentil husk

Time (min)	RI
10	1.3333
20	1.3334
30	1.33357
40	1.33369
50	1.3339
60	1.33404
70	1.33412
80	1.33442
90	1.33472
100	1.33489
110	1.33499
120	1.33502
130	1.33512
140	1.33522
150	1.33528
160	1.33528
170	1.33526
180	1.33528

CONVENTIONAL EXPERIMENT

B.2 Refractive index (RI), pH and conductivity of extracts obtained by conventional extraction of lentil husk

Experimental conditions			Extract		
Temperature (°C)	pH	Ethanol (%, v/v)	RI	pH	Conductivity (mS/m)
40	40	7.0	1.33421	7.68	80.2
55	40	10.0	1.34096	10.27	100.6
55	80	7.0	1.33531	7.72	76.4
46	64	8.8	1.33901	9.14	92.5
46	16	8.8	1.33989	9.20	99.8
55	40	4.0	1.33862	4.83	90.0
70	40	7.0	1.34133	7.33	102.4
55*	40	7.0	1.33809	7.41	92.4
55*	40	7.0	1.33894	7.32	93.2
55	0	7.0	1.33926	7.56	98.3
55*	40	7.0	1.33806	7.43	91.9
64	16	8.8	1.33986	9.16	98.2
55*	40	7.0	1.33814	7.36	94.3
64	64	5.2	1.33899	5.83	94.4
46	64	5.2	1.33536	5.46	77.4
64	64	8.8	1.33882	8.96	96.2
55*	40	7.0	1.33759	7.61	89.3
64	16	5.2	1.33901	5.89	99.2
55*	40	7.0	1.33803	7.43	92.1
46	16	5.2	1.33802	5.46	94.3

*Central point average: RI – 1.33814, pH – 7.43, Conductivity – 92.2 mS/m

SCW EXPERIMENTAL DESIGN 1

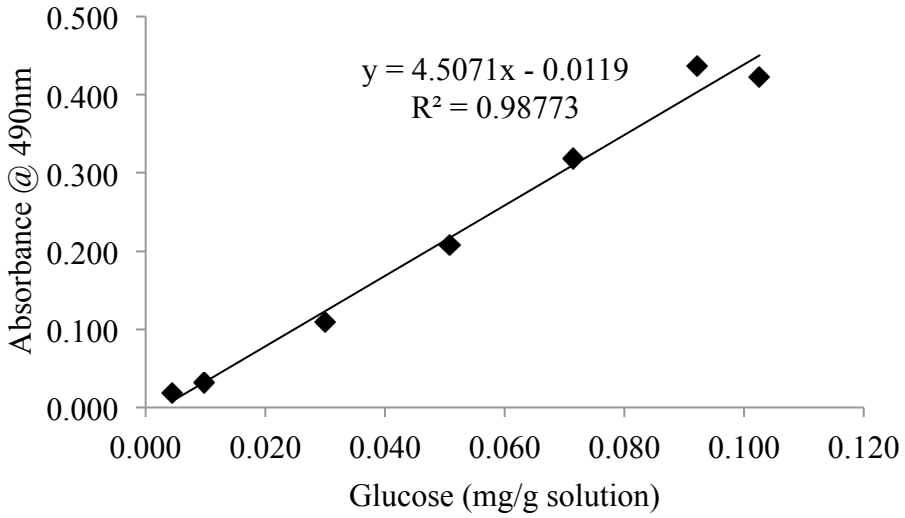
B.3 Refractive index (RI) as a function of time at 180°C, 15 bar and a flow rate of 2 mL/min for extraction of lentil husk

Time (min)	RI
10	1.33728
20	1.33727
30	1.33626
40	1.33481
50	1.33413
60	1.33391
70	1.33376
80	1.33365
90	1.33357
100	1.33351
110	1.33348
120	1.33344
130	1.33338
140	1.33338
150	1.33335
160	1.33330
170	1.33330
180	1.33330
190	1.33326
200	1.33326
210	1.33325
240	1.33325

SCW EXPERIMENTAL DESIGN 1

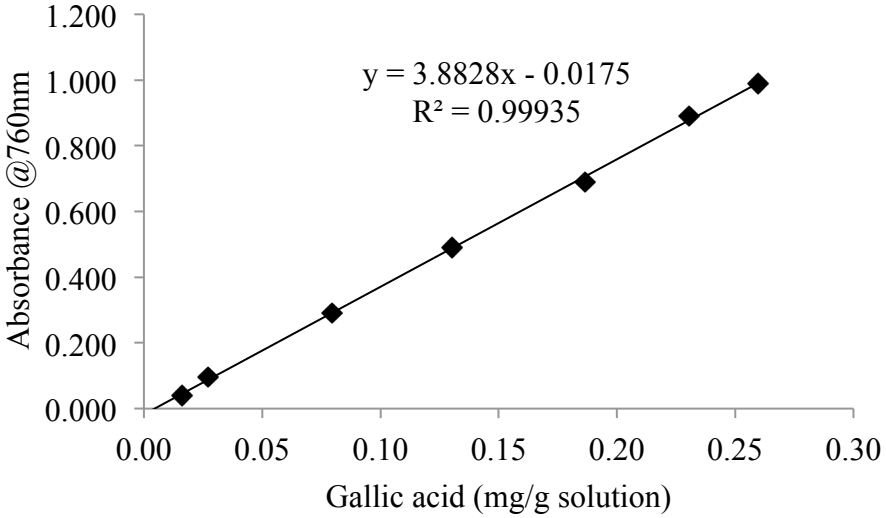
B.4.a Calibration curve for spectrophotometric determination of carbohydrate, phenolics and pentosans

Glucose standard curve



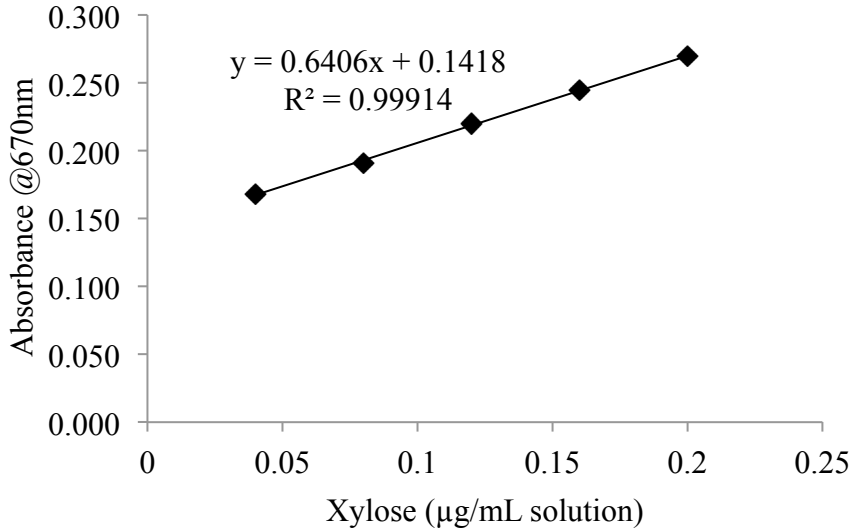
Total carbohydrate (x) calculated in terms of glucose equivalent from the above equation where y is obtained by the procedure detailed in Section 3.2.5.

Gallic acid standard curve



Total phenolics (x) calculated in terms of gallic acid equivalent from the above equation where y is obtained by the procedure detailed in Section 3.2.6.

Xylose standard curve



Total pentosans is calculated in terms of xylose equivalents by using the slope of the calibration curve in the equation mentioned in Section 3.2.7 and the absorbance obtained by the procedure detailed in the same section.

B.4.b Total carbohydrates extracted of lentil husk at 120°C, 15 bar and flow rates of 2 and 5 mL/min

Flow rate – 2 mL/min

Time	Carbohydrates (g/100 g)			
(min)	E1	E2	Average	SD
10	3.3	2.98	3.14	0.23
20	7.16	6.51	6.83	0.46
30	13.69	12.72	13.2	0.69
40	18.93	17.64	18.29	0.92
50	23.75	22.13	22.94	1.15
60	27.81	25.86	26.84	1.38
70	31.36	29.08	30.22	1.61
80	35.75	33.14	34.45	1.84
90	38.27	35.34	36.81	2.07
100	38.9	35.96	37.43	2.08
110	39.49	36.53	38.01	2.09
120	40.04	37.06	38.55	2.1
130	40.59	37.6	39.1	2.11
140	41.05	38.05	39.55	2.12
150	41.57	38.56	40.07	2.13

Flow rate – 5 mL/min

Time	Carbohydrates (g/100 g)			
(min)	E1	E2	Average	SD
4	1.34	1.69	1.52	0.25
8	5.13	5.85	5.49	0.5
12	9.13	10.2	9.67	0.75
16	12.05	13.48	12.76	1.01
20	16.51	18.29	17.4	1.26
24	21.61	23.75	22.68	1.51
28	28.55	31.04	29.79	1.76
32	36.85	39.7	38.27	2.01
36	37.51	40.4	38.96	2.04
40	37.97	40.89	39.43	2.06
44	38.35	41.3	39.83	2.09
48	39.03	42.02	40.53	2.12
52	39.45	42.47	40.96	2.14
56	39.96	43.02	41.49	2.17
60	40.34	43.44	41.89	2.19
64	40.73	43.87	42.3	2.22
68	41.08	44.25	42.67	2.24
72	41.25	44.45	42.85	2.26
76	41.56	44.8	43.18	2.29
80	41.92	45.2	43.56	2.31
84	42.2	45.51	43.86	2.34
88	42.54	45.89	44.22	2.36
92	42.77	46.15	44.46	2.39
96	42.89	46.31	44.6	2.42
100	43.08	46.53	44.81	2.44
104	43.22	46.71	44.97	2.47
108	43.34	46.86	45.1	2.49
112	43.49	47.05	45.27	2.52
116	43.62	47.22	45.42	2.54
120	43.75	47.38	45.56	2.57

E1, E2 – experiments in duplicate.
SD – standard deviation

SCW EXPERIMENTAL DESIGN 2

B.5 Refractive index (RI) as a function of time at 184°C, pH of 8.8, ethanol content of 63.78% and 65 bar for extraction of lentil husk

Time (min)	RI
10	1.33403
20	1.33431
30	1.33403
40	1.33387
50	1.33376
55	1.33362
60	1.33341
65	1.33344
70	1.33346
75	1.33338
80	1.33330
85	1.33321
90	1.33314

SCW EXPERIMENTAL DESIGN 2

B.6 Refractive index (RI), pH and conductivity of extracts obtained by SCW extraction of lentil husk

Experimental conditions			Extract		
Temperature (°C)	Ethanol (%, v/v)	pH	RI	pH	Conductivity (mS/m)
160*	40	7.0	1.35104	9.83	128.9
160*	40	7.0	1.35106	11.4	132.8
183	64	5.2	1.36329	10.94	94.4
200	40	7.0	1.35267	9.44	187.0
136	16	5.2	1.34560	6.25	95.6
136	64	8.8	1.36345	11.03	68.2
136	16	8.8	1.34415	11.02	94.5
136	64	5.2	1.36260	7.88	47.9
160	0	7.0	1.33366	10.61	411.0
160*	40	7.0	1.35107	9.83	132.1
120	40	7.0	1.34960	9.35	86.5
183	16	8.8	1.34486	8.16	303.5
160	80	7.0	1.34436	7.48	64.5
160	40	4.0	1.35544	5.72	152.7
160*	40	7.0	1.35102	9.92	129.8
160	40	10.0	1.35422	9.72	156.9
183	16	5.2	1.34407	11.3	285.6
183	64	8.8	1.36043	9.08	122.6
160*	40	7.0	1.35105	9.85	133.2
160*	40	7.0	1.35104	9.92	129.6

*Central point average: RI – 1.33497, pH – 7.59, Conductivity – 72.6 mS/m

B.7 Kinetic study at optimum experimental conditions for lentil husk

(200°C, 22.83% ethanol content, pH of 4 and 65 bar for 180 min and a flow rate of 2 mL/min)

Time (min)	Temperature (°C)	Pressure (bar)	Yield (g/100 g)							
			Carbohydrates				Phenolics			
			E1	E2	Average	Standard Deviation	E1	E2	Average	Standard Deviation
5	200.2	65.87	1.30	1.16	1.21	0.08	0.28	0.28	0.27	0.004
10	199.9	65.60	12.43	11.86	11.93	0.09	0.70	0.68	0.67	0.003
15	200.3	65.98	15.24	14.82	14.89	0.11	0.81	0.79	0.79	0.000
20	201.0	64.29	10.19	10.26	10.33	0.10	0.83	0.85	0.85	0.001
25	200.6	65.19	6.44	6.28	6.33	0.07	0.58	0.58	0.58	0.005
30	200.4	65.22	3.74	3.76	3.83	0.09	0.44	0.46	0.46	0.002
35	200.5	65.12	2.50	2.50	2.56	0.08	0.27	0.29	0.29	0.003
40	200.1	65.43	2.34	2.32	2.39	0.10	0.24	0.26	0.25	0.001
45	200.4	65.12	1.75	1.70	1.75	0.08	0.17	0.18	0.18	0.004
50	200.1	65.43	1.80	1.63	1.69	0.09	0.12	0.12	0.12	0.002
60	200.6	65.10	1.17	1.09	1.16	0.09	0.07	0.08	0.08	0.005
70	200.6	65.37	0.91	0.82	0.88	0.08	0.05	0.06	0.06	0.006
80	199.9	65.04	0.81	0.73	0.78	0.08	0.04	0.05	0.05	0.008
90	200.6	65.08	0.62	0.52	0.58	0.09	0.03	0.04	0.04	0.006
100	201.6	65.02	0.10	0.08	0.09	0.02	0.02	0.02	0.02	0.001
110	199.8	65.26	0.08	0.06	0.07	0.02	0.03	0.03	0.03	0.005
120	200.6	65.34	0.03	0.01	0.02	0.02	0.01	0.02	0.01	0.008
130	200.7	65.38	0.02	0.00	0.01	0.02	0.01	0.02	0.01	0.007
140	200.6	65.51	0.01	0.00	0.00	0.02	0.02	0.01	0.01	0.007
150	200.6	65.43	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.006
160	200.6	65.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.001
170	200.2	65.87		0.00	0.00			0.00	0.00	
180	199.9	65.60		0.00	0.00			0.00	0.00	
Total			**61.47**	**59.60**	**60.54**	**1.32**	**4.73**	**4.83**	**4.78**	**0.15**

E1, E2 – experiments in duplicates

B.7 Kinetic study at optimum experimental conditions for lentil husk (continued)

(200°C, 22.83% ethanol content, pH of 4 and 65 bar for 180 min and a flow rate of 2 mL/min)

Refractive index (RI), pH and conductivity

Experiment 1				
Time (min)	**RI**	**Abs at 420nm**	**pH**	**Conductivity (mS/m)**
5	1.34107	0.475	6.15	230.3
10	1.34866	1.966	5.36	359.7
15	1.35111	2.364	5.27	238.2
20	1.34621	1.505	5.38	183.9
25	1.34504	1.062	5.45	139.6
30	1.34491	0.686	5.47	113.6
35	1.34487	0.593	5.42	107.7
40	1.34480	0.582	5.49	95.5
45	1.34480	0.401	5.47	94.8
50	1.34479	0.363	5.45	93.2
60	1.34472	0.297	5.47	99.4
70	1.34477	0.205	5.38	71.3
80	1.34477	0.141	5.52	65.7
90	1.34466	0.114	5.46	33.2
100	1.34459	0.084	5.29	36.3
110	1.34449	0.082	5.36	25.4
120	1.34462	0.072	5.47	23.2
130	1.34455	0.089	5.45	21
140	1.34449	0.081	5.43	20.2
150	1.34447	0.069	5.42	18.7
155	1.34442	0.06	5.44	20.3

*Longer extraction time than experiment 1

Experiment 2				
Time (min)	**RI**	**Abs at 420nm**	**pH**	**Conductivity (mS/m)**
5	1.34287	0.491	5.78	189.9
10	1.34950	2.041	5.24	350.6
15	1.35150	2.458	5.22	299.4
20	1.34633	1.662	5.29	172.6
25	1.34535	1.13	5.18	136.8
30	1.34531	0.798	5.29	111.7
35	1.34530	0.631	5.28	105.2
40	1.34533	0.602	5.32	99.5
45	1.34529	0.519	5.26	112.2
50	1.34529	0.407	5.21	96.4
60	1.34515	0.287	5.22	106.6
70	1.34520	0.212	5.26	70.3
80	1.34505	0.156	5.28	68
90	1.34500	0.139	5.21	39.9
100	1.34501	0.118	5.24	38.3
110	1.34503	0.106	5.22	32
120	1.34504	0.091	5.26	35.4
130	1.34491	0.092	5.22	33.4
140	1.34465	0.084	5.28	32.9
150	1.34442	0.071	5.26	32
*160	1.34443	0.062	5.2	30.24
*170	1.34438	0.054	5.22	35.6
*180	1.34439	0.055	5.26	28.9

APPENDIX C: EXTRACTION OF LENTIL COTYLEDON

CONVENTIONAL EXPERIMENT

C.1 Refractive index (RI) as a function of time at 55°C, pH of 7 and ethanol concentration of 80% for conventional extraction of lentil cotyledon

Time (min)	RI
10	1.33340
20	1.33360
30	1.33387
40	1.33409
50	1.33440
60	1.33464
70	1.33482
80	1.33522
90	1.33562
100	1.33589
110	1.33609
120	1.33622
130	1.33642
140	1.33662
150	1.33678
160	1.33688
170	1.33689
180	1.33688

CONVENTIONAL EXPERIMENT

C.2 Refractive index (RI), pH and conductivity of extracts obtained by conventional extraction of lentil cotyledon

Experimental conditions			Extract		
Temperature (°C)	**pH**	**Ethanol (%, v/v)**	**RI**	**pH**	**Conductivity (mS/m)**
40	40	7.0	1.33329	7.46	40.3
55	40	10.0	1.33392	10.01	60.2
55	80	7.0	1.33346	6.94	45.3
46	64	8.8	1.33339	8.48	41.3
46	16	8.8	1.33410	9.10	40.4
55	40	4.0	1.33400	4.03	90.3
70	40	7.0	1.33395	7.21	89.1
55*	40	7.0	1.33395	7.32	65.1
55*	40	7.0	1.33390	7.14	64.3
55	0	7.0	1.34120	7.11	100.2
55*	40	7.0	1.33398	7.31	85.2
64	16	8.8	1.33399	8.94	86.3
55*	40	7.0	1.33347	6.84	50.2
64	64	5.2	1.33328	5.28	42.4
46	64	5.2	1.33348	5.42	60.2
64	64	8.8	1.33340	8.63	58.3
55*	40	7.0	1.33342	7.14	60.1
64	16	5.2	1.34421	5.33	120.1
55*	40	7.0	1.33395	7.21	84.3
46	16	5.2	1.33399	8.32	90.2

*Central point average: RI – 1.33378, pH – 7.16, Conductivity – 68.2 mS/m

C.3 Kinetic study at optimum experimental conditions - conventional extraction of lentil cotyledon

(69.60°C, pH of 5.91 and ethanol content of 0.67% v/v)

Time (min)	Yield (g/ 100 g)							
	Carbohydrates				Phenolics			
	E1	E2	Average	Standard Deviation	E1	E2	Average	Standard Deviation
15	1.55	np	1.55	0.00	0.16	np	0.16	0.00
30	2.46	np	2.46	0.00	0.46	np	0.46	0.00
60	6.28	np	6.28	0.00	0.99	np	0.99	0.00
90	11.07	np	11.07	0.00	1.55	np	1.55	0.00
120	14.63	np	14.63	0.00	1.81	np	1.81	0.00
180	17.13	16.89	17.01	0.17	2.03	2.01	2.02	0.01
360	17.45	17.36	17.405	0.06	2.04	2.02	2.03	0.01

E1, E2 – experiments in duplicates
np – experiment was not performed.

SCW EXPERIMENTAL DESIGN 1

C.4 Refractive index (RI) as a function of time at 180°C, 15 bar, and a flow rate of 2 mL/min for extraction of lentil cotyledon

Time (min)	RI
10	1.33333
20	1.33362
30	1.33503
40	1.33509
50	1.33472
60	1.33436
70	1.33409
80	1.33395
90	1.33380
100	1.33370
110	1.33367
120	1.33363
130	1.33364
140	1.33359
150	1.33347
160	1.33342
170	1.33336
180	1.33336
190	1.33337
200	1.33334
210	1.33336
220	1.33333
230	1.33329
240	1.33328

SCW EXPERIMENTAL DESIGN 1

C.5 Total carbohydrates extracted of lentil cotyledon at 180°C and 120 bar at flow rates of 2 and 5 mL/min

Flow rate – 2 mL/min				
Time	Carbohydrates (g/100 g)			
(min)	E1	E2	Average	SD
10	3.04	2.89	2.97	0.11
20	5.68	5.39	5.54	0.20
30	8.05	7.65	7.85	0.28
40	9.87	9.37	9.62	0.35
50	13.87	13.17	13.52	0.49
60	17.19	16.33	16.76	0.61
70	19.94	18.95	19.44	0.71
80	22.83	21.69	22.26	0.81
90	25.17	24.81	24.99	0.26
100	33.71	33.33	33.52	0.26
110	40.14	39.72	39.93	0.29
120	40.58	40.13	40.36	0.32
130	40.92	40.44	40.68	0.34
140	41.29	40.78	41.04	0.37
150	41.55	40.99	41.27	0.40

E1, E2 – experiment in duplicates

SD – standard deviation

Flow rate – 5 mL/min				
Time	Carbohydrates (g/100 g)			
(min)	E1	E2	Average	SD
4	0.03	0.03	0.03	0.00
8	0.06	0.06	0.06	0.00
12	0.11	0.11	0.11	0.00
16	0.47	0.45	0.46	0.02
20	2.53	2.41	2.47	0.09
24	5.11	4.86	4.99	0.18
28	7.74	7.35	7.54	0.27
32	11.62	11.04	11.33	0.41
36	16.02	15.22	15.62	0.57
40	19.73	18.74	19.24	0.70
50	25.78	25.42	25.60	0.26
60	30.01	29.63	29.82	0.26
70	33.84	33.42	33.63	0.29
80	36.07	35.62	35.84	0.32
90	37.72	37.24	37.48	0.34
100	38.84	38.32	38.58	0.37
110	39.65	39.09	39.37	0.40
130	41.28	40.69	40.98	0.42
150	42.08	41.95	42.02	0.09
170	43.35	43.09	43.22	0.18
190	44.34	43.94	44.14	0.28
210	45.00	44.59	44.80	0.29

SCW EXPERIMENTAL DESIGN 2

C.6 Refractive index (RI) as a function of time at 200°C, pH of 7 and 65 bar for extraction of lentil cotyledon

Time (min)	RI
10	1.33423
20	1.33481
30	1.33467
40	1.33449
50	1.33432
55	1.33401
60	1.33389
65	1.33378
70	1.33369
75	1.33334
80	1.33317
85	1.33315
90	1.33314

SCW EXPERIMENTAL DESIGN 2

C.7 Refractive index (RI), pH and conductivity of extracts obtained by SCW extraction of lentil cotyledon

Experimental conditions			Extract		
Temperature (°C)	**pH**	**Pressure (bar)**	**RI**	**pH**	**Conductivity (mS/m)**
200	7.0	65	1.33328	8.88	868.5
184	8.8	98	1.33383	6.37	218.7
160	4.0	65	1.33399	7.32	337.5
160*	7.0	65	1.33346	7.30	222.2
160*	7.0	65	1.33347	7.75	204.7
136	8.8	32	1.33327	9.69	215.4
184	5.2	32	1.33354	5.33	625.6
136	8.8	98	1.33345	5.46	437.6
120	7.0	65	1.33315	6.37	172.2
160*	7.0	65	1.33347	7.40	221.4
160*	7.0	65	1.33346	7.36	218.6
160	7.0	120	1.33350	7.89	299.3
184	5.2	98	1.33321	5.85	574.7
160	10.0	65	1.33340	11.04	205.7
160*	7.0	65	1.33346	7.28	214.6
136	5.2	98	1.33336	5.02	319.2
160*	7.0	65	1.33347	7.46	208.9
136	5.2	32	1.33350	5.68	178.6
184	8.8	32	1.33315	6.26	451.7
160	7.0	10	1.33347	7.68	208.6

*Central point average: RI – 1.33347, pH – 7.43, Conductivity – 215.1 mS/m

C.8 Kinetic study at optimum experimental conditions for lentil cotyledon

(172.87°C, 80.21 bar and pH of 6.17 for 130 min and a flow rate of 2 mL/min)

Time (min)	Temperature (°C)	Pressure (bar)	Yield (g/100 g)							
			Carbohydrates				Phenolics			
			E1	E2	Average	Standard Deviation	E1	E2	Average	Standard Deviation
5	172.7	80.58	5.42	5.56	5.49	0.099	0.11	0.11	0.11	0.001
10	173.5	80.22	9.34	9.62	9.48	0.200	0.31	0.33	0.32	0.010
15	173.2	79.87	11.05	11.39	11.22	0.242	0.38	0.40	0.39	0.012
20	173.6	80.42	8.61	8.87	8.74	0.183	0.36	0.37	0.37	0.011
25	173.9	80.21	6.62	6.81	6.72	0.133	0.27	0.28	0.27	0.008
30	173.7	80.28	5.03	5.16	5.10	0.089	0.21	0.21	0.21	0.006
35	173.6	80.39	4.27	4.37	4.32	0.065	0.15	0.15	0.15	0.003
40	173.8	81.2	3.08	3.13	3.10	0.032	0.10	0.10	0.10	0.003
45	173.6	81.37	2.34	2.31	2.32	0.019	0.06	0.06	0.06	0.002
50	173.6	79.56	1.98	1.94	1.96	0.030	0.03	0.03	0.03	0.001
60	173.6	80.11	1.24	1.17	1.21	0.046	0.02	0.02	0.02	0.000
70	173.1	80.58	0.89	0.81	0.85	0.055	0.01	0.01	0.01	0.000
80	173.8	80.75	0.65	0.56	0.61	0.061	0.00	0.00	0.00	0.000
90	172.3	80.94	0.41	0.32	0.36	0.065	0.00	0.00	0.00	0.001
100	172.5	80.36	0.15	0.13	0.14	0.011	0.00	0.00	0.00	0.000
110	172.6	80.92	0.07	0.06	0.07	0.013	0.00	0.00	0.00	0.000
120	172.1	80.36	0.00	0.00	0.00	0.003	0.00	0.00	0.00	0.001
130	172.7	80.58		0.00	0.00			0.00	0.00	
TOTAL			**61.15**	**62.17**	**61.66**	**0.72**	**2.01**	**2.09**	**2.05**	**0.06**

E1, E2 – experiments in duplicates

C.8 Kinetic study at optimum experimental conditions for lentil cotyledon (continued)

(172.87°C, 80.21 bar and pH of 6.17 for 130 min and a flow rate of 2 mL/min)

Refractive index (RI), pH and conductivity

Experiment 1					Experiment 2				
Time (min)	RI	Abs at 420nm	pH	Conductivity (mS/m)	Time (min)	RI	Abs at 420nm	pH	Conductivity (mS/m)
5	1.33277	0.475	6.71	201.6	5	1.33282	0.409	6.68	221.5
10	1.3342	1.695	6.21	810.9	10	1.33442	1.762	6.22	808.6
15	1.33497	2.282	6.18	821.3	15	1.33506	2.393	6.21	830.3
20	1.33385	1.131	6.27	808.3	20	1.3341	1.458	6.24	801.3
25	1.33308	0.897	6.3	680.2	25	1.33308	0.982	6.32	670.6
30	1.33277	0.68	6.21	490.2	30	1.33289	0.76	6.29	430.2
35	1.33276	0.464	6.2	200.6	35	1.33277	0.481	6.21	201.5
40	1.33278	0.356	6.16	180.3	40	1.33267	0.368	6.26	175.6
45	1.33274	0.247	6.15	165.6	45	1.33272	0.262	6.18	155.3
50	1.33272	0.146	6.29	130.3	50	1.33273	0.159	6.22	130.3
60	1.33274	0.098	6.29	100.4	60	1.33271	0.099	6.29	102.4
70	1.33266	0.089	6.23	85.2	70	1.33272	0.098	6.31	86.3
80	1.33262	0.062	6.39	55.4	80	1.33263	0.069	6.26	60.3
90	1.33256	0.033	6.35	20.6	90	1.33261	0.058	6.35	40.3
100	1.33254	0.023	6.29	21.3	100	1.33259	0.039	6.29	25.3
110	1.33252	0.021	6.32	20.3	110	1.33257	0.029	6.33	20.3
120	1.33253	0.022	6.33	20.2	120	1.33257	0.025	6.32	22.3
* Longer extraction time than experiment 1.					*130	1.33256	0.022	6.24	20.3