University of Alberta

GREEN BIOMASS: CHARACTERIZATION AND FRACTIONATION OF IMMATURE CEREAL CROPS

by

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Abstract

In general, the development of a biorefining process acknowledges the feedstock characterization as it can often dictate the process technology. The maturity stages of agricultural feedstocks also have the potential for altering the process conditions. Thus, the variability of growth stages at 4-6 leaf, flowering, milk and soft dough were additionally assessed for use of immature cereal crops in a green biomass biorefinery. Hence, the primary objective of this project was to evaluate the composition of green biomass at different harvest stages. Four varieties of triticale (Pronghorn, AC Ultima, Bobcat and Fridge), two wheat varieties (AC Pathfinder and CDC Falcon) and two rye varieties (Gazelle and AC Rifle) were analyzed for starch, protein, phenols, fatty acids and sterols. To further characterize the feedstocks, an alternate extraction strategy was explored based on solvent polarity. Interestingly, the Gazelle (rye) showed an increase in campesterol, stigmasterol and stitosterol content of 18%, 10% and 18% which is significantly different than all other varieties. Furthermore, it was found that for triticale varieties, the milk to soft dough harvest stages showed a decrease in proteins and phenols content and an increase in starch content. This could potentially provide benefit to organic acid production through fermentation.

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Abbreviations

°C	decree in Celsius
ADP	Adenosine Diphosphate
DNA	Deoxyribonucleic acid
g	gram
GC-FID	Gas Chromatography - Flame Ionization Detector
GC-MS	Gas Chromatography - Mass Spectroscopy Detector
IMS	Infrared Microspectroscopy
kD	kiloDalton
Kpa	kilopascal
mg	milligram
min	minute
ml	millilitre
NIRS	Near Infrared Spectroscopy
nm	nanometre
NREL	Natural Resource Ecology Laboratory
REED	Reverse Electro-Enhanced Dialysis
SE	Standard Error
ul	microlitre
v/v	volume per volume
w/v	weigh per volume
x g	multilplied by force of gravity

1. Introduction

1.1. Background

Biorefineries represent sustainable processes that convert renewable biomass into fuels, power, materials and chemicals as defined by the International Energy Agency and BIOPOL (http://www.biorefinery.nl/biopol, accessed July 2010). The renewed interest in these processes result from a necessity to manage greenhouse gas emissions, the threat of depleting fossil fuels and the current unsustainable supply of fuels or chemicals to the industrialized nations. Though promising technologies, successful change of global goods and services production from fossil-based products to plant-based products will be gradual (Kamm and Kamm, 2004b). This is largely due to difficulties associated with developing a bioindustrial process, including logistics and quantity of feedstock, market accessibility, sound technical processes and commercial viability.

Current estimates are that 22.3 million hectares of cropland, idle cropland and cropland pasture will be used for biomass production by 2030 in the United States of America (Sanderson and Adler, 2008). This proposed additional demand on agriculture and forestry to serve as a fossil petroleum substitute is thought to create an imbalance in land use, which will ultimately create competition with the food and feed sectors (Narodoslawsky et al., 2008). In an effort to manage crop failure and reduce financial risk to producers, emerging research is focused on the use of immature material, such as forages and grasses, which is referred to as green biomass biorefining. This chapter will introduce and review the concept and current application of green biomass biorefining,

including the general structure and life cycle of a seed, potential green biomass feedstocks, processing methodologies, and considerations for industrial application.

1.2. Grain Function and Composition

Since immature cereals, such as rye, barley and triticale, have demonstrated to be candidates for feedstocks in the biorefineries, an understanding of the structure and composition of grain changes throughout the growth cycle is necessary when evaluating what metabolites or biochemistry are present at each stage. Canadian small grains are members of the *Poaceae* family in which the starchy endosperm is the most predominant part of the mature seed (Shewry and Stobart, 1993).

In the early stages of plant growth, growth rates can be exponential. Cereals will grow 5 to 6 leaves before the jointing stage, at which point the internodal tissue in the grass leaf begins to elongate, forming a stem (Seibold, 1990). This early stage represents the peak of the cereal plant's production of vegetative development factors involved in photosynthesis and plant metabolism. Subsequently, chlorophyll, protein, and vitamin content decline in proportion as the level of cellulose increases, which provides structural stability for the growing stem (Kohler, 1943). A specific sequence of growth events take place as the cereal plant develops (discussed later in this chapter), though it should be noted that because of tillering and the sequence of grain maturity, a plant stand may contain more than one stage of maturity.

Mature monocotyledon seeds all share certain distinctive characteristics in the main components of the embryo, scutellum, endosperm, pericarp and aleurone (Evers and Millar, 2002). The most distinctive feature of a monocot grain is the single cotyledon embryo within the angiosperm. The embryo of a monocot seed is a highly developed seedling with a root and shoot apex consisting of numerous leaf structures surrounding the apical meristem of the shoot called the plumule (Evers et al., 1999).

The predominant energy storage reserve is the starchy endosperm adjacent to, but not connected to, the embryo (Shewry and Stobart, 1993). The endosperm is a mass of cells in the centre of the grain comprised of mostly starch and proteins. The aleurone layer surrounds the endosperm with non-starch polysaccharides, such as arabinoxylans and beta-glucan (Rudi et al., 2006). The aleurone layers assist hydrolytic enzymes, which are secreted by the scutellum, in the breakdown of the endosperm's starch reserves when needed. To protect the embryo, scutellum, endosperm and the aleurone layer from the oxidative environment, but allowing respiration, an outer layer of cells impregnated with wax, fats and phenols called the pericarp or seed coat, surrounds the seed (Bewley and Black, 1985).

1.2.1. Seed formation stages

Seed formation involves physical and biochemical changes that are governed by species, growth regulation activity and environmental conditions including light, soil type, temperature, rainfall, and humidity. It is not the intention of this section to provide

a comprehensive review of seed formation and development, but to instead to provide a synopsis of the topic in a slightly different approach as an exploration of the characteristic changes that could be qualified as maturity markers. Agrologists use seed development stages, including anther or fertilization, milk stage, dough stage and fully ripened (Bolsen and Berger, 1976) to identify the harvest readiness of the immature crop. Seed development indicators are references to the physical and chemical development of structural components within the grain. Consideration is given to chemical and biosynthetic changes of four stages as these correspond to enzyme production or metabolic pathways.

1.2.1.1. Stage 1: Anthesis

The formation of the structural components of the seed coat and endosperm are of interest during anthesis. The subsequent stages are evaluated as "days after the anthesis" or "days after the fertilization". Benchmarking of the development changes proceed 12hrs after the fertilization of the egg cell. At fertilization, the plant proceeds through a process of cell division, cell expansion and differentiation during growth and development called embryogenesis. During this stage, a single-celled zygote develops into a multicellular sporophyte with recognizable morphological features of the mature plant, including the body axis of the plant (Milligan et al., 2004; Itoh et al., 2005). At the same time, in the angiosperm, the embryo sac is transformed into the endosperm (Kohler and Makarevich, 2006). The angiosperm contains the embryo and endosperm, surrounded by a seed coat derived from the components of the egg cells called the ovule

(Itoh et al., 2005). The embryo develops from the cytoplasm, while the larger basal cell forms the conduit for nutrients from the surrounding plant tissues. The embryo relies on the developing endosperm tissue to facilitate the transfer of glucose being generated by the sporophyte from the surrounding tissue (Martin and Smith, 1995; Milligan et al., 2004; Kohler and Makarevich, 2006). Carbohydrates are stored in the stem and leaves of the plant prior to anthesis, which are used initially to develop the endosperm contributing to 5-10% of the final kernel weight (Bewley and Black, 1978). When examining the seed coat development, the ovule portion develops after fertilization when outer cell layers of the nucleus differentiate into specialized cell types. The outermost layer of the cereal grain is the pericarp, and at the beginning of the seed development, the pericarp is permeable, and allows oxygen into the endosperm (Moise et al., 2005). When the oxygen level is high, the mitochondrial respiration is high during embryogenesis facilitating glycolysis (Bailly, 2004). Some inner cell layers in the seed coat accumulate large quantities of substances; such as mucilage or pigment contributing to the overall seed morphology, while other inner cell layers will not undergo any significant differentiation and will be crushed at maturity (Milligan et al., 2004, Mechin et al., 2007).

1.2.1.2. Stage 2: Milk, early development (8-10 days after anthesis)

After the seed structure is formed, grain seed develops and fills with the starch and protein of the endosperm tissues and aleurone layer. To identify this stage the kernel can be squeezed between your thumb and forefinger and a white, milky fluid appears (Collar and Aksland, 2001). The endosperm of the developing seed begins turning milky

white eight days after fertilization. The milk stage lasts about ten days at which time the embryo completes development (Milligan et al., 2004).

The endosperm development is typified by a series of nuclear cell divisions around a large vacuole and the process repeats until the endosperm is completely cellularized (Milligan et al., 2004). The growth of these cells is accompanied by the accumulation of starch, as well as protein moieties used for cell division, signal transduction, transcription/translation, protein synthesis/assembly, and components of the cytoskeleton (Vensel et al., 2005). The proteins involved with cell division are cytoskeleton proteins, which are assembled in microfilaments and microtubules (Mechin et al., 2007). The endosperm cells differentiate into starch-filled endosperm cells and transfer cells that facilitate nutrient uptake from the plant tissue and outer epidermal layers called aleurone endosperm cells (Milligan et al., 2004; Vicente-Carbojosa and Carbobero, 2005). These layers are developed as coordinated activities in the gene encoding for metabolic pathways, regulating enzymes and proteins (Rudi et al., 2006).

The endosperm cells contain starch granules embedded in a matrix of storage proteins. The cells are densely packed with starch granules surrounded by protein storage bodies with beta-glucan and arabinoxylan depositing in the cell walls for cohesion and structure. The granule composition, size and shape are determined by the genetics of the cereal species. The starchy endosperm biosynthesis is characterized by the ADP-glucose pyrophosphorylase pathway, starch synthase, starch branching enzymes, and debranching enzymes in addition to the hexose phosphate metabolism (Rudi et al., 2006). Vensel et al., (2005) showed that at early stages of development, this carbohydrate metabolism is a principle process by identifying enzymes related to function in glycolysis, the citric acid

cycle, sucrose synthesis and starch synthesis. Starch accumulates as glucose polymers in three-dimensional granules of alternating semi-crystalline and amorphous layers and is deposited in rings with varying amylose and amylopectin content (Martin and Smith, 1995). Svihus et al., (2005) suggests that one growth ring is added daily during the growth stage provided both light and sucrose are available.

The granular starch is surrounded by a matrix protein. Cereal beta-glucan is a linear mixed linkage glucan, consisting of cellotriose and cellotetraose. According to Rudi et al., (2006) the beta glucan biosynthetic pathway and the accumulation in the endosperm cell wall is not well understood. It is proposed that the mechanism follows a similar mechanism of cellulose synthase polymerization of the glucose molecules.

The aleurone endosperm layers are the cells closest to the surface of the seed containing lipids, protein, phytic acid, minerals and vitamins (Milligan et al., 2004). Paradiso et al., (2006) also reported high levels of frutans in the endosperm layer of wheat at the milky stage.

The seed coat is vital for directing the nutrient supplies to the embryo during seed development by the syntheses of invertase, which facilitates the transfer of nutrients by increasing the sucrose gradient in the seed coat. The high hexose-to-sucrose ratios promote embryo growth by enhancing cell division (Borisjuk et al., 1998). As the embryo grows within the seed coat, the inner seed coat layers are crushed, and due to the loss of cell wall, invertase activity decreases (Moise et al., 2005; Weber et al., 2005). Cell wall crushing increases the amount of reactive oxygen species, causing the production of proteins developed for cell rescue, defence, cell death, and aging (Mechin et al., 2007). Developing cereals have an outer chlorophyll-containing layer of pericarp

tissue and during most of the grain filling period the pericarp tissue is green and capable of producing oxygen through photosynthesis (Foxon et al., 1990). Foxon et al. (1990), suggest that because the outer pericarp tissue is impermeable to oxygen and carbon dioxide, the major function of the green layer is to provide oxygen to maintain starch synthesis in the inner endosperm. The amount and duration of available light then becomes a critical factor to the incorporation of sugars into the starch material.

1.2.1.3. Stage 3: Soft Dough, late development (14 days after anthesis)

At the third stage, called the soft dough stage, the grain is well formed and filled with starch. The identification of this stage is that when a kernel is squeezed, there is no milky fluid, and only rubbery dough like substance is liberated (Collar and Aksland, 2001). This stage begins 14 days after fertilization and lasts about a week to ten days. Research into the seed formation at this stage suggests a significant change in the biochemistry of the seed from starch biosynthesis as in stage 2, toward preparation of metabolic survival caused by environmental stresses through lipid and protein synthesis.

Environmental stresses cause an overall shift of the seed development from biosynthesis and metabolism to maintenance and storage. At this later developmental stage, the enzymes involved with glycolysis, the citric acid cycle, and oxidative pentose phosphate pathway decrease coinciding with the depletion of oxygen levels. The exception is the increase of glyceraldehyde-3-phosphate dehydrogenase, which facilitates the breakdown of glucose for energy (Vensel et al., 2005). Mechin et al., (2007) further

indicated that proteins presented a peak expression and glycolysis enzymes presented a maximum level of accumulation at 14 days after fertilization.

Research of the development of over 400 proteins by Vensel et al., (2005) showed that in late stage development, the endosperm was rich in defence and stress related proteins. Many were identified to protect against pathogens, insects and fungi by acting as digestion inhibitors, including α-amylase inhibitor, bifunctional α-amylase/trypsin, and subtilisin inhibitors, and thaumatin-like protein. Other proteins were identified for protection from biological stresses like wound healing, disease resistance and elevated temperature resistance. Other sets of enzymes found in the endosperm protected against oxidative stresses. Peroxidase enzymes are used in the defence mechanisms of cereals and their activity was located in the endosperm, the aleurone and the embryo; but little activity was detected in the husk or green layer of the pericarp (Cochrane et al., 2000). Peroxidase functions in the development of an anti-microbial barrier when the tissue is ruptured, and increases in the presence of a fungus, as well as facilitating control of plant growth and seed elongation (Bewley and Black, 1978; Cochrane et al., 2000). Da Silva et al., (2005) investigated plant lipid transfer proteins, which were small extracellular proteins containing eight cysteine residues that were able to bind and transfer lipids. Their role in the plant involves cutin biosynthesis, surface wax formation, pathogen defence, adaptation to environmental conditions and systematic signalling (Da Silva et al., 2005).

1.2.1.4. Stage 4: Hard Dough to Fully Ripe, maturation and desiccation (21-30 days after anthesis)

The seed has completed formation and is entering into the maturation phase where metabolic activity slows down, storage reserves accumulate, the protective seed coat changes, and dehydration occurs through desiccation. The maturation stage begins within 21-30 days after fertilization and lasts until the ovule has matured and fully ripened into a seed (Gutierrez et al., 2007). The maturation stage was discussed by Vicent-Carbojosa and Carbobero, (2005) suggesting the process can be divided into overlapping programs of gene expression to accomplish different tasks, like synthesis of storage compounds, acquisition of desiccation tolerance or the establishment of dormancy. The cellular and biochemical changes associated with the acquisition of desiccation tolerance in seeds include cellular destruction, synthesis of dehydrins or heat shock proteins, activation of antioxidant defence systems, accumulation of raffinose, and maintenance of DNA integrity (Lehner et al., 2006).

Maturation within the seed is a multipart process that affects both the embryo and the endosperm, leading into quiescence. Quiescent seeds are resting organs, generally with a low hydration level and lowered metabolic activity (Bewley and Black, 1985). During seed maturation, the subsequent low oxygen levels can lead to energy depletion by decreasing starch and lipid synthesis forcing the seed to counteract this effect by adapting mechanisms to control metabolic flux and prevent fermentation. There are regulators that signal hormone and metabolic changes such as abscisic acid, sugar

balance and nitrogen, all working as a network for signalling dormancy and subsequent germination in the future (Gutierrez et al., 2007).

After ripening is an important step in cereal crops to prevent premature seed germination. Research in the mechanism of seed dormancy suggests a strong involvement of the plant hormone, abscisic acid. Levels of abscisic acid are controlled by both biosynthesis, as an oxidative cleavage reaction, and catabolism into phaseic acid (Chono et al., 2006). Soluble protein synthesis can be redirected from synthesis characteristic of development to synthesis associated with germination, which causes premature desiccation of the seed. The imposed changes decrease the synthesis of protein precursors and subunit polypeptides to the synthesis of insoluble proteins (Kermode et al., 1984). The abscisic acid content increases to a maximum amount, one week before maximum dry weight occurs, before a sharp decrease in water content (Radley, 1976), indicating an inhibition response of gibberellin secretions. Gibberellin, which regulates the synthesis of α-amylase in germination, was found by Radley, (1976) to be equally distributed between the endosperm and surrounding layers such as the aleurone and pericarp. When testing immature cereal, Cornford et al., (1986) found that the aleurone tissues did not to respond to gibberellins, but a response could be induced after a drying period or temperature treatment, demonstrating that the seed must undergo a dehydration or heating period before germination. The metabolite concentration gradients of sugar and embryo secretions trigger the transitions for the onset of the seed maturation phase (Gutierrez et al., 2007). The sucrose series oligosaccharides, particularly sucrose and raffinose, and the dehydrin group proteins, within the embryos

could be used as a measure of their biochemical maturity (Black et al., 1999) but would be influenced by selective breeding.

The metabolic switch is involved at late maturation whereby the accumulation of any oil and storage proteins increase(Shewry and Stobart, 1993). These proteins involved in the free amino acid biosynthesis and pyruvate orthophosphate dikinase peak at 21 days after fertilization (Mechin et al., 2007). Maturation is also controlled by nitrogen availability because storage protein synthesis is limited by the capacity of the seed for nitrogen uptake. During this stage, as the water is lost and dehydrin participation in desiccation tolerance occurs, the only detectable oligosaccharide is raffinose (Black et al., 1999). During the last developmental stage, sucrose accumulation is a prerequisite for the acquisition of desiccation tolerance (Gutierrez et al., 2007). Vensel et al., (2005) discovered that superoxide radicals increase with response to dehydration in the final stage of maturation.

Maturation ends with a desiccation phase after the embryo enters into a quiescent state, providing maintenance and survival under a range of environmental conditions. Strong impermeable seed coats protect the embryo during dormancy and maintain an environment around the embryo (Moise et al., 2005). According to Moise et al., (2005) the seed coats separate one generation of plants from the next and ensure the survival of the offspring.

1.2.2. Methodologies: Determination of Seed Maturity

Determination of the seed formation stage becomes important when an essential desired physical attribute and chemical by-product is identified. There are multiple ways to detect the seed maturity either destructive or non-destructive.

Destructive but simple visual observation technique whereby squeezing the grain between fingers, used by agrologists, and growers (Collar and Aksland, 2001), is a good indicator of the approximate maturity level; however, the methodology is not precise. Moisture content of the grain can also be used to determine maturity level. A recent study by Ronganini et al., (2007) concluded that the maturity level could be estimated by the moisture content of a sunflower seed at different developmental stages, which correlated well with documented data of maturity. The surface of the grain can also be used to identify the growth stage by coating the aleurone layer with a hardening wax and monitoring the adsorption with Tapping Mode Atomic Force Microscopy, which will give a nanometer detail view of the surface of the grain (Tomoaia-Cotisel et al., 2006). Near Infrared Spectroscopy (NIRS) was identified as a quick method of determining chemical, nutritive and agronomic characteristic of wheat once the unit is calibrated (Garnsworthy et al., 2000). Ogawa et al., (2003) developed a simple way to study whole sections of rice where the grain was dehydrated through a graded ethanol series, transformed to xylene and embedded in paraffin wax. The grain was then sliced thinly and pasted to adhesive tape where the cross section could be observed under a Scanning Electron Microscope for determination of the maturity of the seed tissues. Plant growth and development has also been studied with Infrared Microspectroscopy (IMS) in

combination with Synchrotron Radiation, which can allow a researcher to examine the plant at a cellular level. Dokken et al., (2005) studied the use of IMS for development of the plant cell wall structure and components, such as lignin, cellulose, and polysaccharides.

Non-destructive measurement of the carotenoids in rice bran was developed by Belefant-Miller et al., (2005) and can also be used on live plants. The fluorescence quenching technique can monitor the carotenoid using Synchronous Fluorescence Spectroscopy with good precision and sensitivity. Chlorophyll fluorescence measurements are used for sorting seeds for the assessment of the maturity and quality. Fluorescence of the chlorophyll molecule is induced by irradiating the seeds with electromagnetic radiation with a suitable wavelength and measuring the chlorophyll fluorescence. This patented methodology has been proven to have high sensitivity and fully non-destructive, with a very high throughput (Hendrik, 2000, United States of America Patent 6080950). Though, technology is better suited to oilseed crops, the technology may also be useful for maturity identification of immature cereal crops.

1.3. Green Biomass Refining

Although the use of green biomass for the production of renewable chemicals and fuels by fermentation is an exciting and viable area of research, biomass utilization and fractionation is by no means a new concept. Today's concept of green biomass refining initiated in 2000 when the utilization of waste juice from pressing grass, clover and alfalfa into pellets from the commercial fodder industry was examined (Anderson and

Kiel, 2000). Anderson and Kiel (2000) proposed that the juice could be converted into a direct fermentation medium for lactic acid production to avoid potential economic and energy demands created by pelleting or drying. Recent efforts in this area involve using green biomass as a raw material for the production of bio-based products, including protein, lactic acid, succinic acid, lysine, fibres, and energy (Hernandez and Hernandez, 1994; De et al., 1997; Starke et al., 2001; Cukalovic and Stevens, 2008; Hossain and Maisuria, 2008; Chenanan et al., 2009). Bio-based products, such as lactic acid and its derivatives, are produced commercially, although the companies do not use green biomass as a feedstock. Further growth in this area continues as bio-industrial drivers identify the need for alternative products and reduced waste through the conversion of green biomass through biorefinery processes.

1.3.1. Bio-industrial Drivers

Bio-industrial drivers are the forces behind the research and technology that present both opportunity and possibility for commercial growth. Since Europe, the United States of America and Canada are global leaders in green biomass refining research, the drivers for these regions were explored. Although this list is not exhaustive, it does illustrate the key reasons for performing research in this area.

In Europe, as well as elsewhere in the world, the benefits of biorefineries are evident not only through product revenues, but also by the creation of employment opportunities and observed carbon dioxide abatement and sequestration potential (Mabee et al., 2005; Reith and de Bont, 2007). As the green biomass biorefinery evolves from

juice extraction during pellet manufacturing to intentional reclamation of land for biomass production, European researchers, as part of the Network for the Implementation of Biorefineries, evaluated sustainable and economical strategies to produce non-food products from green biomass (Kamm et al., 2000; Kromas et al., 2004b). Austria, specifically, has adopted a viable option for the conservation of cultural landscapes, as well as improving the stay option for farmers though grass biorefining (Kromas et al., 2004). Farmers have left their land to reclamate and planted grass as a cover crop to maintain the soil. As a result, approximately 100,000 to 150,000 hectares of pastureland, no longer used for milk production, are available for growing feedstocks for biorefining (Kamm et al., 2000).

In the United States, in addition to meeting Kyoto objectives, reducing climate change, and increasing the awareness of the depletion of fossil fuels, the USA Energy Independence and Security Act of 2007 has encouraged exploring alternative materials for chemicals and fuels to secure an independent supply (Hermann et al., 2007; Hwang et al., 2009). Additionally, the Farm Security and Rural Investment Act of 2002 enabled the use of Conservation Reserve Program to utilize grassland for the production of biorefining feedstocks (Mapemba et al., 2007). This policy act restricts days of harvest per year and harvest frequency, but allows unused grassland crops to be used for green crop bioethanol production.

Canadian drivers are similar to those of the United States with respect to meeting Kyoto objectives, reducing climate change, and increasing the awareness of the depletion of fossil fuels (Hermann et al., 2007). However, an additional key consideration, due to seasonal variations, is the climate conditions in which the biomass is grown. New

concerns have arisen with the mediation of failing crops in periods of drought. In recent years, there have been more incidences of drought and, as a result, farmers had to forfeit their oilseed and cereal crops to silage (Parker, 2006; Ziegler, 2009). These immature and failed crops are perfect candidates for green biomass biorefining, which also provides the benefit of reduced income loss to the farmer.

1.3.2. Green Biomass Refinery Process

The biorefinery process is best described by Kamm and Kamm (2004a) who published a defining document encompassing most of the biorefining literature found today. They described the various types of biorefineries, including green biorefinery using wet biomass such as grass, lucerne, clover and immature cereal. Figure 1.1 provides a schematic of how such green biomass can be used to create value-added bioproducts. Briefly, green biomass can be fractionated into two fractions using a screw press yielding a cake and a juice fraction. This physical separation allows for the further identification, separation and potential use of the resultant components from both the solid and liquid fractions into value-added products (Kamm and Kamm, 2004b). Based on the potential product streams, the main areas of research in green biomass include: the development of continuous fermentation; reduction of expensive supplementation of the green juice for fermentation media; down-stream processing efficiency; new bioconversion processes, and identification of new end products.

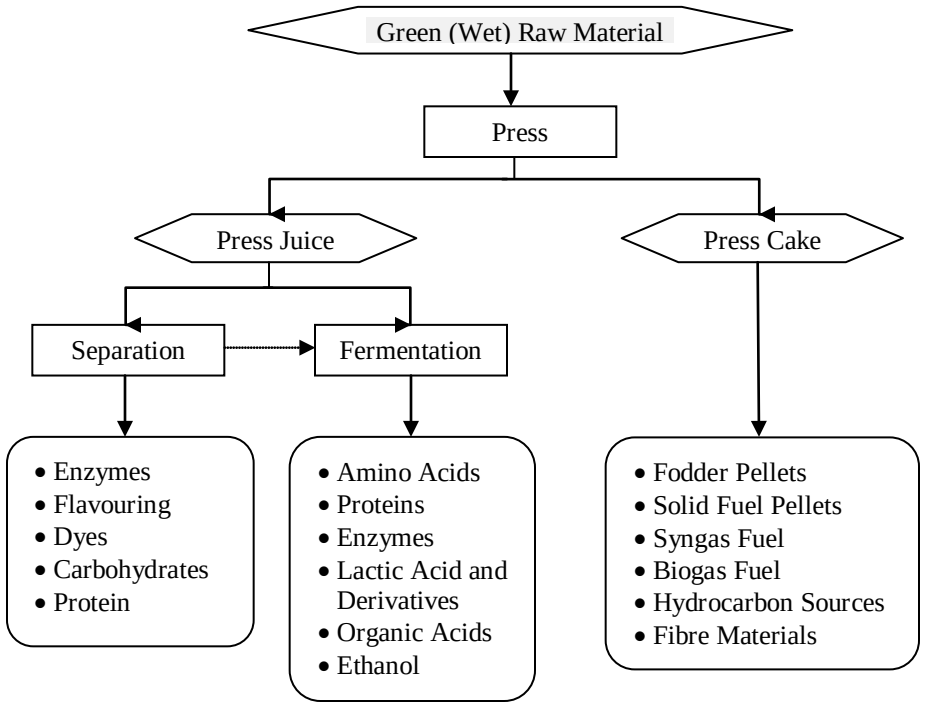

Figure 1.1 Schematic of the general process to product flow of a green biomass biorefinery (adapted with permission from Kamm and Kamm, 2007).

1.3.3. Green Biomass Refinery Projects

There are four major green biomass biorefining projects, each recognizing the sustainable and industrial relevance of the pathway shown in Figure 1.1, in North America and Europe to date.

The Austrian Green Biorefinery project was developed around the concept of using grass silage to produce lactic acid, proteins and amino acids (Kromus et al., 2004). Joanneum, a research institution in Graz, Austria, further focuses on the technical, economic and ecological use of sustainable raw materials such as grass, maize and agro-waste for the production of bulk chemicals, feed products, plant fibers, and biogas

production. Successful research out of the University of Brandenburg, Germany and Forschungsinstitut Bioactive Polymersystem e.V. (BIOPOS) research institute (http://www.biopos.de/) resulted in a green biomass biorefinery demonstration facility located west of Berlin, Germany. The pilot plant is equipped with bioreactors and separation equipment for the purpose of processing renewable resources. It has been commissioned using alfalfa, clover and immature cereals. Recent research at this facility has included both ethanol and lactic acid as identified biorefinery products (Mabee et al., 2005; Reith and de Bont, 2007).

Jurag Separation A/S of Demark developed a continuous separation process using a Reverse Electro-Enhanced Dialysis (REED) technology to produce lactic acid from green biomass (www.jurag.dk). The process, as shown in Figure 1.2, includes a substrate membrane bioreactor, REED apparatus and bipolar electrodialysis module. The fermentation broth from the bioreactor is sent to a REED module, where the alternating current on the diode allow material to migrate back and forth from each membrane allowing the product to be removed before the organic matter builds up. The unique process design eliminates the need for a filtration step prior to the REED. The extracted alkaline stream is then passed through a bipolar electrodialysis module (EDBM) where the solution of lactic acid is 20% concentration. The concentrated solution is then sent to 3 falling film evaporators producing an 88% lactic acid solution, which is further condensed through further purification.

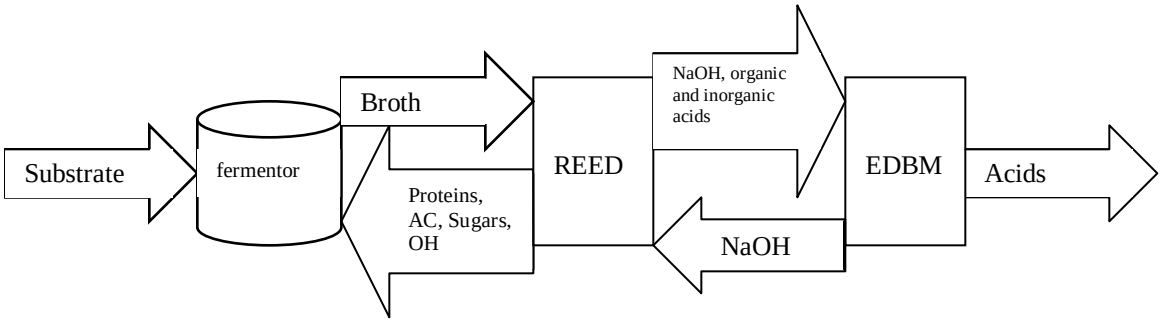

Figure 1.2 Lactic acid production model developed by and reproduced with permission from Jurag Seperation A/S.

In Canada, Alberta Agriculture and Rural Development and the University of Alberta are collaborating on a Green Biomass Fractionation project with the objective of utilizing immature grain to evaluate the most technically feasible process parameters. This work evaluates the use of immature triticale as green material for extraction of compounds for potential use as bio-products. Some of these compounds are identified in this thesis, as well as other extractives that can be found throughout the growth cycle of immature grains.

The green biomass refinery process, although in its infancy in the scientific literature, is based on a rather simple principle: the use of raw material biomass in its entirety to produce bioproducts with no or minimal waste generation. To maximize the potential product streams from green materials, it is important to first understand what potential feedstocks exist coupled with an understanding of what components are present at each stage during the growth cycle.

1.4. Green Biomass Feedstocks

Green biomass feedstocks, although diverse in classification, are relatively similar in overall composition based on the growth cycle of plant material. The plant biochemistry of most mature and forage crops have been described in detail (Bewley and Black, 1978; Vicente-Carbajosa and Carbonero, 2005); however, the contrary is true for immature crops. There is a general lack of understanding of the biochemistry information of many potential green or immature biomass crops (Kamm and Kamm, 2007). The following sections describe feedstock's that could potentially be used in a green biomass biorefinery and their advantages or constraints.

1.4.1. Forages

Forage crops are native pasture plants, crop residues or immature cereal containing the plant leaves and stems generally used as animal feed. The advantage to using forage crops as a bioindustrial crop is that farmers are already familiar with the management, including the capacity to grow, harvest, preserve and transport the biomass (Sanderson and Adler, 2008). Forage crops that have been studied for green biomass biorefining are alfalfa and sweet sorghum.

1.4.1.1. Alfalfa

Alfalfa has been studied since the late 1960s (Oelshlegel et al., 1969a; Oelshlegel et al., 1969b) regarding the utilization of leaf protein concentrates. Leaf protein concentrates provided a protein supplement to countries facing food shortages however for human consumption, the collecting the protein required removing the chlorophyll by a simple coagulation process and solvent extraction, respectively (Hernandez and Hernandez, 1994). Generally, alfalfa was processed by mechanical pulping and separating into a juice and a press cake. The juice was frozen at -25°C, and when allowed to thaw, the liquid was poured off leaving an unpurified protein curd coagulum. Then the coagulum was purified by a 2-propanol wash to remove the chlorophyll. Hernandez et al. (1995) determined that the solvent not only removed the chlorophyll, but also increased the amount of methionine, thereby creating a high quality protein extract. Alfalfa processing technology was also developed as part of the Wet Fractionation System (Schoney and McGuckin, 1983) and was then determined that alfalfa was most profitable as a proteinatous feed when harvested in mid-bud to first flower stage, at which it has a high moisture content to allow for better silage juice extraction. Koschuh et al. (2004) investigated a heat coagulation step with centrifugation as compared to ultrafiltration for both alfalfa and ryegrass at different harvest stages. Purified protein recovery was not as successful as the previous solvent extraction; however, the technology allowed for a greater crude protein recovery despite some protein degradation via shear forces through ultrafiltration (Koschuh et al., 2004). Another method of capturing the protein was continuous decanting of alfalfa juice after heating to 55°C to

remove particulates, insoluble proteins and chlorophyll (Lamsal et al., 2003). Microbial coagulation was also investigated using nine bacterial strains able to coagulate the protein as efficiently as heat coagulation as studied earlier, in alfalfa green juice (Godessart et al., 1987). In general, alfalfa is a good model for protein extraction and purification.

1.4.1.2. Sweet sorghum

Sweet sorghum is an annual forage crop that is high in moisture, but not optimal for hay feeding (Snyman and Joubert, 1996). The crop has received much attention in the United States due to its high sugar and fibre content (Worley et al., 1992). Today, sweet sorghum is juiced for molasses, and the waste fibre is under investigation as a potential medium for fermentation into ethanol and lactic acid production. Although sweet sorghum cannot directly compete with a dried fibre crop, it could be used for the green biomass biorefining concept (Worley et al., 1992). The sweet sorghum by-product can be ensiled, delivered at a reasonable cost, and harvested and stored like hay. Ensiling forage sorghum at the flowering stage with formic acid was found to be an optimal combination to maximize nutrients and quality (Snyman and Joubert, 1996).

1.4.2. Grasses

The use of grasses in green biomass biorefinery has been researched intensively in Europe. Grasses have little available sugar for fermentation; however, once the grass is preserved through an ensiling process, the juice from silage is a great source of lactic and

amino acids. For example, cool season grasses, such as wheatgrass and tall fescue, were tested to increase fructans percentage and were shown to contain an average concentration of glucose (14%), fructose (10%), sucrose (15%), fructans (38%) and starch (23%) (Livingston III, 1991; Zhao et al., 2008). Increasing these components is a target for genetic engineers who are trying to enhance biofuel production through fermentation of carbohydrates (Murray et al., 2008). Hwang, (2009) agrees that the economics only work if juicing operations were performed on farm and coordinated with livestock use. This is demonstrated in Figure 1.3 whereby silage produced on the farm and pressed with a mobile press is optimal if the fibre or press cake was of low value (Halasz et al., 2005). The right side of this diagram indicates that if the fibre were to reach a higher value, sending the entire silage crop to a central facility would have greater economic viability.

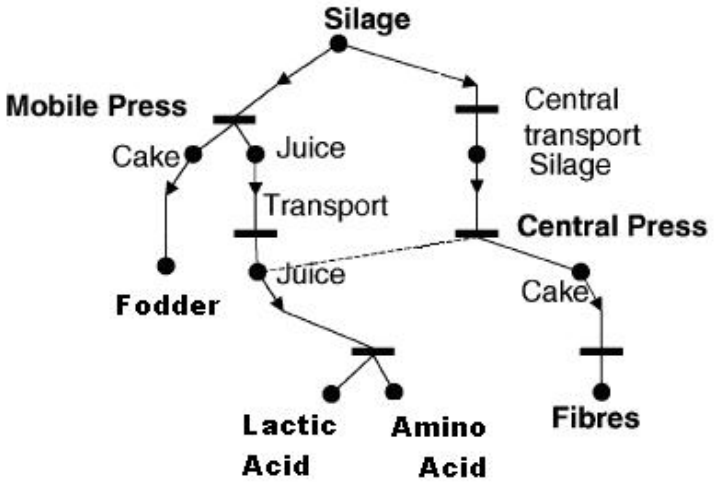

Figure 1.3 Green biomass biorefining concept model for using press cake as fodder as developed by Halasz et al., (2005), (with permission from the publisher).

Although such research on the applicability of grasses for green biomass refining is ongoing, the term “grass” has not yet been well-defined. To some extent, the research published on grasses indicates that the term ‘grass’ refers to immature cereal crops, rather than a true grass. Switchgrass, on the other hand is a perennial native grass, grown in both Canada and the United States, and has been the focus for an industrial green biomass biorefinery crop.

1.4.2.1. Switchgrass

Switchgrass is an interesting crop for green biomass biorefining. Jin et al. (2009) have developed a detailed process design with mass and energy balance equation using switchgrass with up to 20% moisture content to generate biomass power using gasification; however the question of feasibility on a larger scale remains unanswered in regards to harvest and cost. The advantage of switchgrass over cereal crops is the potential wide window of opportunity for harvest. Despite modelling attempts to define the growth pattern of switchgrass, the optimal harvest strategy still needs to be determined (Madakadze et al., 1999; Sokhansanj et al., 2009). Other research evaluating the larger scale harvest, production and logistics of switchgrass processing concluded that transportation of the material to a large scale biorefinery is the most cost effective (Kumar and Sokhansanj, 2007; Sokhansanj et al., 2009). The cost of total direct activity, including fixed costs and operating cost, in farm production and supply is estimated to be $2.76 billion by 2015 (Sokhansanj et al., 2009).

1.4.2.2. Cereals

Rice, corn, wheat, rye, barley, millet, triticale, sorghum and oat are types of cereal grains. The nutrient composition, plant yield and biomass production of cereal grasses are greatly influenced by ecological conditions and production management activities. Such factors include species composition, stage of maturity, climatic conditions, soil fertility status and harvest season (Enoh et al., 2005). For example, it has been demonstrated that the when the harvesting interval was delayed from 8 to 12 weeks, crude protein decreased by 23% and crude fibre content, such as neutral and acid detergent fibres and acid digestible lignin, increased over time (Enoh et al., 2005; Chatterton et al., 2006). Fructans were also reported to be concentrated within the plant at the early to mid endosperm development stages (Chatterton et al., 2006). The plant's metabolites prodcuted due to defence mechanisms can also be exploited for commercial products according to Broberg et al., (2007), who identified 18 metabolites of interest. Secondary metabolites such as Benzoxazinoid hydroxamic acid, can also be induced by a fungus response in younger cereal tissues (Niemeyer, 2009).

Younger, immature cereals, such as rye, barley and triticale, have been demonstrated and suggested to be candidates for feedstock in the biorefinery model but present questions regarding storage of the juice and utilization of the fibre (Campbell et al., 2007). Researchers, Stewart and Houseman (1977) introduced a method of lowering the pH with chemicals and biological yeast fermentation to preserve grass juice to improve storage time. Cereal grass fibres isolated after silage were subjected to chemical treatments such as acid, alkali as well as microbial activity through retting to evaluate the

effect of the treatments to the fibres by Smole et al.(2004). It was observed that the acid treatment disassociated pectin; the morphology of the fibres was damaged by the alkaline treatment and retting enzymatic treatment left the fibres in good mechanical and geometrical quality. Thorsell et al. (2004) demonstrated mature lignocellulosic material created a potential advantage to be used in a biorefinery where gasification-fermentation potentially in the same facility as the biorefinery. Neureiter et al. (2004a) suggest a mild acid hydrolysis of the press cake would release sugars and a cold water wash prior to hydrolysis with a dilute acid, under moderate conditions, significantly increased the yield to recover hemicellulose-derived sugars (Neureiter et al., 2004b). It therefore can be conceived that lignocellulosic fibres have the potential to be used even after harsh pretreatment, for non-woven textiles, insulation applications and gasification technologies.

Application of the lactic acid fermented juices effluent was determined to behave like a heavy metal scavenger for soil remediation (Leidmann et al., 1994). To present a further challenge, the scientific literature fails to report the full chemical composition of the specific varieties of cereal crop grasses, reiterating the lack of information on the biochemical, genomic and metabolic information of many potential green or immature biomass crops (Kamm and Kamm, 2007). Although such information does exist for mixed crops or forage material to be juiced and the fibre as a fodder product, this review singled out the potential cereal varieties such as rye, barley, triticale, switchgrass and others, found in the literature for their contribution to the green biomass biorefinery.

1.4.2.2.1. Rye

Rye varieties offer interesting product recovery opportunities with fatty acids, protein recovery and environment manipulations. High concentrations of fatty acids, C16:0, C18:2 and C18:3 were present in the mature cultivars when twelve cultivars of perennial ryegrass were compared, for fatty acid composition and nutritive value (Palladino et al., 2009). Rye was also investigated for the protein recovery by centrifugation after heat coagulation compared to ultrafiltration at different harvest stages (Koschuh et al., 2004). Other opportunities include rye grass use as alleopathic natural products, such as weed suppression toxins. When the plant is encroached by a weed, the plant releases toxins such as benzenoxazolin-2one, 2,4-dihydrox-1,4-benzoxazin-3-one, 2-hydroxy-1,4-benzoxazin-3-one, 2,4-dihydroxy-1,4-benzoxazin-3-one glucoside, β-hydroxybutyric acid and β-phenyllactic acid (Finny et al., 2005), which could be potential chemicals to recover if these toxins were induced.

1.4.2.2.2. Barley

Barley contains chemical compounds during early and late stages of growth that also provide interesting opportunities. Young barley leaves, as young as two weeks after germination, underwent an ethanol extraction where phenolic compounds and antioxidant activity equivalent to tocopherol in the lipid peroxidation system were detected (Osawa et al., 1992). Paulickova et al. (2007), characterized the barley grass to contain vitamin C, high total phenolics, ferulic acid, monosaccharides and amino acids, concluding that the

plants nutritional value is strongly dependent on the growth phase, variety and planting site. Reimann,(2005) investigated the successful use of shredded barley fractionation as a feedstock for the fermentative production of lactic acid.

1.4.2.2.3. Triticale

Triticale was developed with interbreeding of wheat with rye, which created a high-yielding, drought-tolerant, protein enriched crop species suitable for poor-quality or marginal growing areas (Salmon, 2004). This higher protein biomass was evaluated by Shurkhno et al. (2005) who identified *Lactobacillus plantarum* BS933 as a suitable microorganim for the production of lactic acid and silage quality. Today, breeding programs focuses on manipulating the plant genome for industrial chemical production, such as crops with increased ethanol yield or crops with colourful identification markers. For the Canadian prairies, the growth of triticale is advantageous and, as a result, it has been identified as having potential to create a dedicated industrial crop without interfering with any food production system.

1.4.3. Others

Cereal grains, forages and grasses form the bulk of green biomass feedstocks but other materials evaluated are lupins, slipper flower, trees and algae green biomass. Lupins are an annual crop that fair well in the northern climates. Kamm et al., (2006)

explored different chemical and enzymatic treatments for the hydrolysis of lupins for the looking at ways to biorefine into ethanol. Other energy crops were slipper flower (De et al., 1997) and fifteen laticiferous plant species of *apocyanacea, asclepiadaceae, convolvulaceae, euphorbiaceae* for suitability as energy or phytochemical crops (Sekar and Francis, 1998). Attention has turned to aquatic algae biomass for potential hydrogen generation, and to obtain protein concentrate from the juice and the use of fibrous residue as fodder (Caffrey et al., 1996).

1.5. Development of and Considerations for a Green Biomass Bioindustrial Process

In industry where the raw materials shape the technology (Narodoslawsky et al., 2008) the green biomass biorefinery will need to consider the growth stage of crops of harvest to capitalize on optimal characteristics. Other considerations are the logistics of transporting the feedstock, quality of the feedstock and the engineering parameters of the materials. Briefly, there are three current areas of research of all biomass; agronomy to improve the crop for industrial processing, chemistry of extraction and transformation of the components, and specific process engineering innovations to improve the overall economics (Campbell et al., 2007). The following sections address existing technologies and the above-mentioned challenges in green biomass biorefining.

1.5.1. Engineering Parameters

Engineering parameters assist process engineers to design commercial production facilities. Recently, Chevanan et al., (2009) measured the shear strength and flow properties of switchgrass, wheat straw, and corn stover and suggested that chopped switchgrass would bridge in a hopper due to the angle of internal friction and would not move by gravity alone. They also reported parameters for chopped switchgrass as summarized (Table 1.1).

Table 1.1 Measured parameters for developing material transfer devices for switchgrass. (Source: Chevanan et al., 2009).

Parameter	Value
Coefficient of friction	1.0 ± 0.19
Maximum shear force (displacement)	44 ± 7 mm
Cohesive Strength	0.75 ± 0.18 kPa
Angle of internal friction	43.3 ± 3.1°
Unconfined yield strength	3.38 ± 0.67
Consolidation strength	10.42 ± 0.95
Tensile strength	0.81 ± 0.24 kPa

Energetic considerations were also evaluated on a green biomass biorefinery model for lactic acid and lysine production based on a 40,000 ton per day processing facility offering a demonstration of the mass and energy balances of each unit operation described in the following sections for lactic acid fermentation (Kamm et al., 2009).

1.5.2. Processing Considerations

1.5.2.1. Harvesting

Generally, biomass producers are familiar with the agronomic management for food and feed crops, including: the possibility of reseeding to reduce the time to productive stands, optimizing inputs for maximum biomass yield, and all other aspects of harvest quality (Sanderson and Adler, 2008). For example, the effect of mechanical conditioning on wilting and nutrient losses was investigated to prove that methods of harvesting have a huge effect on the quality (Hwang et al., 2009). The primary goal of forage conservation is to maintain quality with minimal loss during harvest and storage, which are influenced by factors such as the type of forage species, the kind of hay making equipment, storage facilities, the weather conditions at harvest, and the drying conditions (Enoh et al., 2005). The stage of maturity will influence the chemical composition, such as dry matter and crude protein (Borreani, 1999) which indicates the timing of the harvest is also fundamental. Crops that have been contaminated with disease and are unfit for animal feed could be a feedstock for a green biomass biorefinery, provided the disease does not interfere with mechanical pressing or is not toxic for the fermentation organisms effecting yeild. Herbicides and pesticides utilized for growth performance can also interfere with the chemical analysis of the solvent extracted material. Fusarium toxins are especially not good for farm animals by causing bloating (Krska et al., 2007), but they also interfere with the seed development as well as harden and blacken of the seed.

1.5.2.2. Logistical Considerations

Harvesting and transporting high moisture green material to the processing facility to maintain quality, in the heat of the summer months, will create additional considerations for bioindustrial crop production. The logistics of transporting raw material to the biorefining facility was evaluated by Halasz et al., (2005) who concluded that a central biorefinery if the price of fibre is higher, as shown in Figure1.3 will lead to economic success despite the low transport density and high water content of the raw material and silage. Thorsell et al., (2004) estimated the cost of production for a facility with both fermentation and gasification operations, using the economics from the field to facility door. The researchers designed a coordinated harvest with ten labours, nine tractors, three mowers, three rakes, three balers, and one bale transporter at a total cost between $11.26 and $14.01 (US dollars) per tonne of material, depending on the yield but these costs do not include storage, land procurement or other costs associated with the biorefinery.

1.5.3. Chemical perspective

Building platform technologies for biosynthesis of bulk chemicals such as lactic acid, succinic acid, lysine, acetic acid and ethanol provided the research community with the opportunity to research the fundamental carbon metabolic pathway, which could provide a more holistic understanding of the biomass to chemical conversion. Nikolau et al., (2008) explored the environmental performance of potential 15 bulk chemical

production systems and concluded that lactic acid, succinic acid, and lysine offer greenhouse emission savings per ton produced. Hermann, (2007) states that future technologies should offer better performance and production efficiencies on these processes. The process technologies discussed in this section include mechanical and thermal extraction, as well as lactic acid, succinic acid, and lysine fermentation production systems.

1.5.3.1. Extraction and Separation Technology

Extraction techniques and separation technology reviewed for extracting and isolating compounds within the green biomass biorefining include mechanical pressing, membrane separation, solvent extraction, and gasification potential.

1.5.3.1.1. Mechanical pressing

A primary processing step of the green biomass biorefinery is the separation of the liquid fraction from the solid biomass by mechanical pulping or screw press. The mechanical shear liberates the juice for collection as fermentation media to produce chemicals that are discussed later in this chapter (Kamm and Kamm, 2007). The resulting press cake has reduced moisture content for economical transportation, for fodder or nonwoven fibre production. Research in the use of pulsed electric field assisted juice extraction as a technology to rupture the cell membranes leads to an increased

alfalfa juice yield by 37.8% (Gachovska et al., 2006) when combined with mechanical pressing.

1.5.3.1.2. Membrane separations

The membrane processes evaluated with green biomass juice, allow for the separation of valuable substances through pressure driven processes such as microfiltration, ultrafiltration and nanofiltration for the isolation of lactic acid, sugars and proteins (Novalin and Zweckmair, 2009). In addition, a comprehensive review of performance limitations of various types of membrane technologies were evaluated to conclude that electrodialysis filtration, which is discussed in the section of fermentation, was identified as the most suitable technique for purifying fermentation products (Huang et al., 2007); however, further separation was required for higher quality purification (Novalin and Zweckmair, 2009).

1.5.3.1.3. Solvent extraction

Solvent extraction related to green mass biorefinery has not been reported in the literature, however, the process should be considered since it is commonly used in the study of plant volatiles with direct solvent extraction using Soxhlet extraction, developed by F. Soxhlet in 1879 (Letellier and Budzinski., 1999). Solvent selection depends on the characteristics of the target compounds. Using n-pentane extraction was efficient in

removing low boiling point plant volatiles (Xu et al., 2005). Mohsen and Ammar (2009) and Zhao et al. (2006) with similar methodologies evaluated to the polarity of solvents, including water, ethanol, methanol, acetone, hexane, chloroform, butanol, petroleum ether and methylene chloride, for extracting phenolic compounds in corn tassels and barley to find that ethanol exhibited the highest extractability. Zhao et al. (2006) found that acetone showed the highest extraction capacity for (+)-catechin and ferulic, caffeic, vanillic, and p-coumaric acids; methanol had the highest extraction capacity for (-)-epicatechin and syringic acid; and water had the highest extraction capacity for protocatechuic and gallic acids. The selectivity of tris-cationic receptors can bind to the anionic carbohydrate in an aqueous solution, offers an alternate potential extraction techniques of sugars (Schmuck and Schwegmann, 2005). Pigments such as chlorophyll are extracted with diethyl ether by adding distilled water (Schoefs, 2004).

The dispute of solvent extraction as toxic and expensive can be mediated with careful solvent capture and recycling as well as safe processing techniques (Schoefs, 2004; Delhomme et al., 2008) confirming solvent extraction can be a viable and scalable option for the production of renewable chemicals.

1.5.3.1.4. Gasification

Generally, gasification is a thermo-chemical process converting any carbon source, usually a waste stream, into a syngas for energy production. Though a green biomass biorefinery should have minimal waste, this technology provides alternate options for the fibre press cake. Jin et al. (2009) have predicted an increase in efficiency

with a process to convert the green biomass with 15% moisture on a dry basis, into a mixture of light combustible products such as carbon monoxide, hydrogen and methane, and heavy hydrocarbons like tar and oil.

1.5.3.2. Fermentation

Green biomass liquid streams can be used for fermentation, as explained by Thomsen (2005); however, for the best results and higher yields, the biomass material should be pre-treated with enzymes, chemicals or heat prior to fermentation (Kamm and Kamm, 2007). Carboxylic acids such as acetic, acrylic, butyric, citric, fumaric, gluconic, iaconic, lactic, malic, propionic, pyruvic, and succinic acids from green biomass liquids can be produced from fermentation processes (Corma et al., 2007); however, the media requires a pasteurized sterilization for both reducing contamination and storage (Anderson and Kiel, 1999; Thomsen, 2005). Alternate extractive fermentation and chemocatalysis (Yang, 2007; Claus et al., 2008) are believed to replace conventional stirred tank or air lift fermentation processes to compete with petrochemical processes due to low yield, reactor productivity, and end-product inhibition due to the concentration of the product. The dilute fermentation products are accompanied by impurities to be removed by ion-exchange, chromatography, adsorption membrane filters, vaporization, reverse osmosis, or electrodialysis (Yang, 2007). However, the final product will require further processing through chromatography or crystallization methods (Kaghazchi et al., 2006). It is these final purification steps that can increase the cost of production.

1.5.3.2.1. Lactic Acid Production

In 2004, the estimated world production of lactic acid was 50,000 to 80,000 t/a based on microbial fermentation (Richter and Nottlemann, 2004; Kamm and Kamm, 2004). The final product of poly (lactic acid) or PLA exceeds 250,000 tons per year globally (Gandinin, 2008). *Lactobacillus brevis* is a heterofermentative lactic acid bacterium that uses the phosphoketolase pathway to produce a mixture of lactic acid, ethanol, acetic acid and carbon dioxide from an anaerobic fermentation specific for glucose, fructose, and lactose (Teixeira, 1999). Alternately, silage is a preservation technique for forage crops by fermentation with native plant lactic acid bacteria under anaerobic conditions. The lactic acid can be simply mechanically pressed from preserved material and purified while the press cake can be returned to the livestock. When searching for current practices, the optimization of any bacterial fermentation begins with a pure feedstock of starch. The kinetic and reaction rate equations of glucose from starch are well described (Akerberg and Zacchi, 1997) and metabolic pathway of the lactic acid bacteria is reviewed for metabolic engineering to produce industrial chemicals (Teusink and Smid, 2006). The choice of *Lactobacillus* strain has shown that efficiencies can range from 78.4% to 96% lactic acid yield as gram of lactic acid produced per gram of corn flour (Altaf et al. 2007). Barley green juice was used as a successful media, producing 11.8 grams of lactic acid per litre of media in an hour with *Lactobacillus paracasei* (Venus and Richter, 2006). Bacteria selection with proteolytic activity and osmotic stress tolerance from the natural environment around legume crops could increase production rates according to Shurkhno et al. (2005). Lignocellulosic material,

saccharified into hemicellulose and pentose was evaluated for positive pre-treatment effects on lactic acid production by Neureiter et al. (2004).

Fermentation scale-up requires that many bioprocess parameters for the organism are taken into consideration such as nutrient requirement, morphology, immobilization, pH, oxygen requirements and temperature. Commercial production is desired to be economical with a reduction of limitations of nutrient requirement, batch size of the fermentation and downstream processing for final product recovery.

1.5.3.2.1.1. Nutrient Limitations

Additional ingredients of nitrogen and sugars are required to increase fermentation yields from green biomass juice. The use of rye grass, clover and alfalfa waste juice were identified as media for lactic acid fermentation (Thomsen et al., 2007); however, the juice required fortification with wheat starch to increase lactic acid production with *Lactobacillus plantarum* A6. In other systems, the addition of vitamins, in particular B vitamins, to the media enhanced the growth of *Lactobacilli* and production of lactic acid (Yoo et al., 1997; Kwon et al., 2000).

To overcome some of the disadvantages of limited nutrients, tolerant microorganisms and it was shown that the production of lactic acid using *Rhizopus* fungi may have advantages over bacteria (Zhang et al., 2007; Lin et al., 2007). Other considerations to increase the yield include the use of other microorganisms for biorefining applications, such as, *Escherichia coli*, which can withstand lower pH (Warnecke and Gill, 2005) and *Saccharomyces cerevisiae*, that provided an optically pure

form of L(+)-lactic acid and can be genetically modified by synthetic biology to withstand the toxic by-products produced by fermentation (Gao et al., 2009).

1.5.3.2.1.2. Batch Limitations

Batch fermentations are not as functional as continuous fermentation for commercialization because as the organism produces the product, the environment becomes more toxic to the organism halting its growth and production capacity in a batch fermenter. To reduce the toxicity and production costs to provide a bulk product leads to continuous fermentation using a membrane reactor system; however, product inhibition depends on the dilution rate and cell mass formation (Richter and Nottelmann, 2004). Alternate options include the design of a three-phase fluidized bed bioreactor that allowed for an extractive fermentation, alleviated the product inhibition as well as regulated pH (Lin et al., 2007).

1.5.3.2.1.3. Downstream processing

Traditional recovery process of lactic acid from the fermentation broth requires costly purification processing step resulting in an impure product. The production of biodegradable plastics required for human use or controlled drug delivery devices or artificial prostheses require pure lactic acid. Purification processing steps such as bleaching, ion-exchange and bipolar electrodialysis were utilized to collect high-grade

biomaterial for these applications (Madzingaidzo et al., 2002). Lower quality lactic acid can be used as an additive for animal feed or as a road de-icer (Danner et al., 2000). Joglekar et al. (2006) assessed downstream processing options for lactic acid recovery with a result that continuous fermentation, reactive extraction and reactive esterification-hydrolysis would be higher in capital cost, however will reduce waste. Values of the distribution coefficient of the lactic acid with the solvent utilized for physical separation is very important. Hossain and Maisuria (2008) developed a process using less toxic solvents such as oleyl alcohol but the yield resulted in a lower percentage being extracted. It would be desirable to have a less toxic, environmentally friendly process; however, the economics are conflicting and revenue prevails. A review was compiled to discuss the unit operations required: nanofiltration, electrodialysis and reactive extractants.

1.5.3.2.1.3.1. Nanofiltration

Nanofiltration is useful for initial concentration and intermediate clarifying step to remove magnesium and calcium ions as well as in the final purification step to remove the phosphate and sulphate ions after electrodialysis (Bouchoux et al. 2006). A study where six nanofiltration organic and ceramic membranes of different materials with pore sizes below 1 kDa were screened for filtering green biomass, claimed that lower than 1 kDa will degrade the proteins (Koschuh et al., 2005). If the nanofiltration membranes are negatively charged, they become selective for charged essential amino acids (Li et al., 2003). Within the biorefinery process, the 1 kDa cut-off membrane filter was used for

initial filtering prior to electrodialysis or reactive extractants (Thang et al., 2005a; Koschuh et al., 2005).

1.5.3.2.1.3.2. Electrodialysis

Electrodialysis is a separation process whereby two ion exchange membranes, one cation membrane and the other anion membrane, are used to filter ions from a solution using an electrical charge. A diluent is placed outside the cell and the solution flows through the cell based on ions migration across the membrane and into the diluent for further separation. Lee et al. (1998) mathematically modeled an electrodialysis membrane after experimentally determining that the electrical resistance of the membrane was expressed by the inherent resistance of the membrane, as well as the inverse proportional relationship to the lactate concentration in the solution.

Thang et al. (2004a) showed separation of lactic acid as well as amino acids from grass juice, utilizing a two-stage electrodialysis based on a stepwise pH decrease (Thang et al., 2005b). Also the use of a conventional three compartment electrodialyzer and cation exchange membrane was 97% effective in separating lactic acid and 90% effective for amino acids. Researchers were able to successfully purify lactic acid based on a mono-polar and bi-polar electrodialysis (Madzingaidzo et al., 2002) as an in situ acid removal (Li et al., 2004) for continuous fermentation processing.

1.5.3.2.1.3.3. Reactive extractants

Through the improvement of the conventional process for lactic acid production, reactive extraction is suitable for reducing the toxicity during fermentation and for the recovery of lactic acid. Reviews by Wasewar et al. (2004) and Joglekar et al. (2006) discuss the suitable extraction of the lactic acid and the methods of back extraction to recover both the lactic acid and the extractant. At low concentrations of lactic acid in an aqueous solution, Martak and Schlosser (2006) evaluated phosphonium ionic liquids as reactive extractants and identified the mechanism of extraction by causing the water to be released by splitting the reverse micelles. This technology is still under development and researchers continue to investigate the stability of the ionic liquid as a liquid membrane in a spiral channel flat sheet module (Martak et al., 2008). Gao et al. (2009) developed an extractive fermentation processed with a tertiary amine as an extractive proving to produce a high lactic acid concentrated solvent, based on a metabolically engineered *Saccharomyces cerevisiae* able to withstand a pH lower than 3.5.

1.5.3.2.2. Succinic Acid Production

Production of succinic acid is primarily used in the form of succinate which is derived from glucose to later be converted to 1,4 butandiol and tetrahydrofuran (Kamm and Kamm, 2007) or replacing the current maleic anhydride production for the C_4 platform (Delhomme et al., 2009). The technology of the overall production and isolation of the succinate were not reported in the literature however, a review of the chemicals

that can be produced from succinic acid (Cukalovic and Stevens, 2008) and a critical review of the catalytic possibilities for conversion of the chemicals (Delhomme et al., 2009) are available and are outside the scope of this review.

1.5.3.2.3. Lysine Production

The feed pellet industry creates a juice through the pelleting process which is referred to as brown juice, and this juice has been a research focus in Denmark and Germany for fermentation media (Starke et al., 2001; Kamm and Kamm, 2004; Thomsen and Keil, 2008) resulting in a facility in Esberg, Denmark producing lysine (Thomsen, 2005). Researchers have tested several strains of *Lactobacillus* for the production of lactic acid, which will preserve the juice at a lower pH before continuing with fermentation with *Corynebacterium* for the conversion to lysine (Thomsen and Keil, 2004). An extraction step for the isolation and quantification of L-lysine fermented in potato juice was developed and evaluated by Starke et al. (2001); however, Choi et al. (2002) discovered that ion substitution with a solution of hydrochloride, purified the lysine through electrodialysis which was more efficient than conventional dialysis.

1.5.3.2.4. Ethanol Production

Large scale production of ethanol from forage juice was feasible but not with high yield when Alvo et al. (1996) assessed the agronomic aspects of forage production and

the processing aspects of the forage conversion into glucose for fermentation into ethanol. Despite this evaluation, it can be done in small scale and has been proven by Shen et al. (2009) using *Saccharomyces cervici*. Ethanol fermentation has been well described as a by-product of mature, dry lignocellulosic fermentation and is beyond the scope of this thesis on green biomass biorefining.

1.5.3.2.5. Anaerobic Digestion

Anaerobic digestion of agricultural wastes and other municipal organic wastes produces biomethane that can be converted to heat, electricity or transportation fuel. Resch et al. (2008) and Nordberg et al. (2007) demonstrated the feasibility to use both whole corn, grass, clover and alfalfa silage as a biodigestion substrate, modelled with different amounts of recirculated fermentation liquid for increased of efficiency. In summary, fresh water is necessary however improvements were achieved with 50% recirculation of fermentation broth. The design of the digester also affects the efficiencies with the highest methane production being achieved in a leach bed reactor with a two-stage process without pH adjustment; proving that grass silage had a 66% methane potential within 55 days (Lehtomaki et al., 2008).

1.6. Conclusions

Green biomass biorefineries use plant material from immature forages, immature cereal crops or cultivated perennial grasses at various stages of growth. The technologies utilized within green biomass biorefinery integrate green forage preservation, physical separation and biochemical conversions to produce high value products. These high value products include protein, lactic acid, succinic acid, lysine, fibres and energy.

1.7. Research Overview and Objectives

Green biomass biorefineries presents opportunities for biochemical extractions and conversion to higher value products. This project explored immature Western Canadian cereal crops to identify the unique advantage for green biomass biorefining. The purpose of this thesis was to explore the biomass from triticale, wheat and rye, for chemical composition changes as the plant matures through four growth stages. The first objective of this project was to develop rapid methodologies to evaluate levels of target compounds during growth and to assess the potential to use these methodologies for quality control and growth stage verification using test methodologies evaluating total protein, starch and phenol, sterol content and fatty acid profiling. The secondary objective was to develop a solvent extraction method for further characterization of the biomass which included fractionation of the biomass to identify potential chemicals presented at different growth stages for potential extraction or bioconversion.

2. Material and Methods

2.1. Overall Protocols

Characterizing the feedstock for a green biomass biorefinery allows for the comparison of types of immature cereal crop varieties most suitable in a biorefining process. Figure 2.1 illustrates the chapter outline, and project design, which will be further discussed in this chapter.

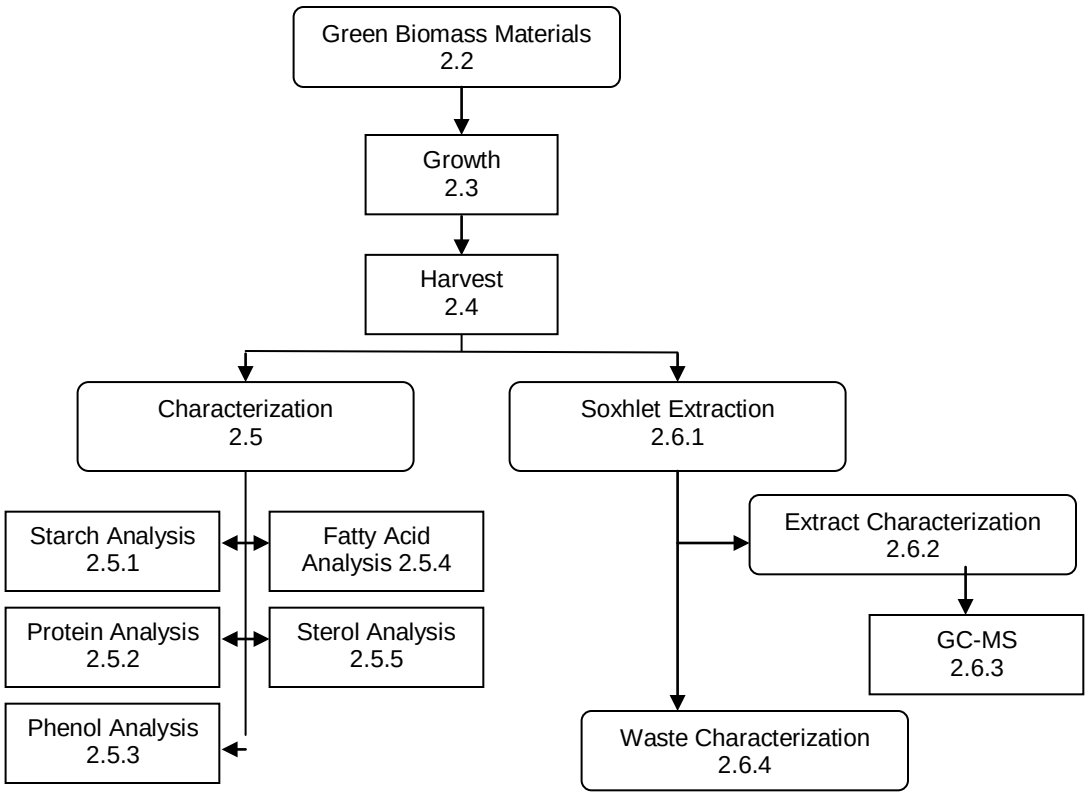

Figure 2.1 This project's experimental protocols depicted in a flowchart.

2.2. Green Biomass Materials

Tables 2.1 and 2.2 outline the cereal crop varieties as well as the chemicals, analytical kits and enzymes utilized throughout the experimental design.

Table 2.1 Cereal crop varieties studied as the biomass feedstock.

Species Common Name (Botanical Name)	Varietal Name	Description
Triticale (*Triticosecale sp.*)	Pronghorn	Spring triticale developed by Alberta Agriculture and Rural Development, Field Crop Development Centre. Registered in 1995. Awned. (University of Saskatchewan, Dept of Plant Science, 2009; Field Crop Development Centre, AAFRD, 2001)
	AC Ultima	Spring triticale developed by Agriculture and Agri-Food Canada Semiarid Prairie Agricultural Research Centre. Registered in 1999. Awned. (University of Saskatchewan, Dept of Plant Science, 2009; Field Crop Development Centre, AAFRD, 2001)
	Bobcat	Winter triticale developed by Alberta Agriculture and Rural Development, Field Crop Development Centre. Registered in 1999. Reduced awn. (University of Saskatchewan, Dept of Plant Science, 2009; Field Crop Development Centre, AAFRD, 2001)
	Fridge	Winter triticale. Excellent winter survival. Awned. (Elmy's Friendly Acres seed farm, 2009)
Wheat (*Triticum sp.*)	AC Pathfinder	CWAD spring wheat, AC Pathfinder durum wheat (*Triticum turgidum* L. var. durum) developed as part of a breeding program in Swift Current, SK. Midlong awn, short growth time and good resistance to leaf rust (Clarke et al., 2001). Registered in 1998. Developed by Agriculture and Agri-Food Canada Semiarid Prairie Agricultural Research Centre. (University of Saskatchewan, Dept of Plant Science, 2009)
	CDC Falcon	CWAD winter wheat (*Triticum aestivum* L.) developed by the University of Saskatchewan, Crop Development Centre. Registered in 1998. Good winter hardiness. (University of Saskatchewan, Dept of Plant Science, 2009)
Rye (*Secale cereale sp.)*	Gazelle	Spring rye with rapid pollination creating a Ergot resistant variety. (Sosulski and Bernier, 1975)
	AC Rifle	Winter rye developed by Agriculture and Agri-Food Canada Semiarid Prairie Agricultural Research Centre. Registered in 1994. (University of Saskatchewan, Dept of Plant Science, 2009)

Table 2.2 Chemicals, enzymes and kits used in this work.

Name	Catalogue number	Supplier
Alpha-amylase, Thermal Stable	KC-TSTA1	Cedarlane Laboratories, Burlington, ON
Amyloglucosidase	KC-TSTA2	Cedarlane Laboratories, Burlington, ON
Brassicasterol	B4936	Sigma-Aldrich, St. Louis, MO
BSTFA containing 1% TMCS	33149-U	Supelco, Bellefonte, PA
Calcium Carbonate	S719222	Fisher Chemicals, Fair Lawn, NJ
Calcium Chloride	C64-500	Fisher Chemicals, Fair Lawn NJ
Campesterol	C5157-5mg	Sigma-Aldrich, St. Louis MO
Chloroform	C607-4	Fisher Chemicals, Fair Lawn NJ
d-Cellobiose	C7252	Sigma-Aldrich, St. Louis, MO
d-Glucose	G5767	Sigma-Aldrich, St. Louis, MO
Diazomethane Diazald® Distillation Kit	prepared in-house	Sigma-Aldrich, St. Louis, MO
Dichloromethane HPLC Grade	AC610050040	Fisher Chemicals, Fair Lawn NJ
Dimethyl Sulphoxide lab grade	D5879	Sigma-Aldrich, St. Louis MO
d-Mannose	M2069	Sigma-Aldrich, St. Louis, MO
d-Xylose	X1075	Sigma-Aldrich, St. Louis, MO
Ethyl Acetate HPLC Grade	E195-4	Fisher Chemicals, Fair Lawn NJ
Fatty acid Internal Std Methyl C17:0	H4515-1G	Sigma-Aldrich, St. Louis MO
Fatty Acid Std 15A	prepared in-house	Sigma-Aldrich, St. Louis MO
Fatty Acid Std 463	GLC-463	Nu-Check Prep, Inc, Elysian, Minnesota
Gallic Acid Solid	A122-500	Fisher Chemicals, Fair Lawn NJ
Glacial acetic acid	BP2401SI212	Fisher Chemicals, Fair Lawn NJ
Glucose Buffer	KC-TSTA2	Cedarlane Laboratories, Burlington, ON
Glucose Determination Reagent	KC-TSTA2	Cedarlane Laboratories, Burlington, ON
HPLC Water HPLC Grade	W5-4	Fisher Chemicals, Fair Lawn NJ
Internal Std (dihydrocholesterol)	D6128-10G	Sigma-Aldrich, St. Louis MO
l-Arabinose	A3256	Sigma-Aldrich, St. Louis, MO
Methanol HPLC Grade	A452SK-4	Fisher Chemicals, Fair Lawn NJ
Methanolic Hydrochloric Acid	33050-U	Supelco, Bellefonte, PA
Methyl heptadecanoate C17:0	H4515	Sigma-Aldrich, St. Louis, MO
MOPS Buffer	M-9381	Sigma-Aldrich, St. Louis MO
Pentane HPLC Grade	P399-4	Fisher Chemicals, Fair Lawn NJ
Potassium Hydroxide	P250-500	Fisher Chemicals, Fair Lawn, NJ
Pyridine, Acros	61099-1000	Fisher Chemicals, Fair Lawn, NJ
Sitosterol	S1270-10mg	Sigma-Aldrich, St. Louis MO
Sodium Azide	S227I-25	Fisher Chemicals, Fair Lawn NJ
Sodium Carbonate, Anhydrous	S495-500	Fisher Chemicals, Fair Lawn NJ
Sodium Chloride	5671-500	Fisher Chemicals, Fair Lawn, NJ
Sodium Hydroxide pellets	RDCS0530100	Fisher Chemicals, Fair Lawn NJ
Sodium Sulfate, Anhydrous	415-1	Fisher Chemicals, Fair Lawn NJ
Sterol STD Mix- Brassicasterol Sigma	B4936-5mg	Sigma-Aldrich, St. Louis MO
Stigmasterol	52424-5g	Sigma-Aldrich, St. Louis MO
Sulfuric Acid, 72%	R8191600-4A	Fisher Chemicals, Fair Lawn NJ
Total Protein Kit, Micro	TP0100-1KT	Sigma-Aldrich, St. Louis MO

2.3. Growth Conditions

Biomass production was conducted in the Agriculture/Forestry Centre greenhouse at the University of Alberta north campus. Three separate trials were initiated in January 2008 (T1), April 2008 (T2) and October 2008 (T3) with a total of nine size 15 cm pots, filled with potting mix and 4 plants per pot. Spring varieties were planted directly into the potting mix, whereas winter variety seeds were subjected to a vernalization procedure before seeding. The vernalization process simulated a cold shock time period that included subjecting the seeds to a holding temperature of -5°C for six weeks after germination, where the seeds were allowed to emerge undisturbed for 5-7 days. After the six weeks of vernalization, seedlings were stored for 2-3 days at room temperature of 23°C until they were transplanted into the 15 cm pots with potting mix and watered.

Once growth was established within pots, they were watered daily and fertilized every two weeks with a general 15-30-15 fertilizer, and the pots were methodically rotated to avoid bench placement effects that could bias growth rate, including space, light, and edge temperatures.

2.4. Harvest Stage

Each crop variety was harvested at 4 different growth stages. The inspection criteria used to assess each growth stage is provided in Table 2.3. Briefly, visual markers indicated harvest readiness at 4-6 leaf (H1), flowering (H2), milk (H3) and soft dough (H4) stages. To ensure adequate material for future analyses, 3 pots of plants were

harvested at the 4-6 leaf stage, and 2 pots of plants were harvested at each of the other three stages.

Harvesting the biomass included the removal of dried growth around the base of the plant. The plants were cut 2 cm above the surface of the potting mix and rinsed under cold water if insects were visible. The biomass was cut into 6 mm inch pieces and stored overnight at -80°C freezer. The next day, the plant material was pulverized by mortar and pestle with the addition of liquid nitrogen (Praxair, Edmonton, AB), then wrapped in aluminum foil and freeze dried (FreeZone®12, Labconco, Kansas City, MO) at 0.520 bar for 48 hr or until dry. Once dry, the materials were stored at –20°C until milling. The materials were milled in a Retsch mill (ZM100, RETSCH Inc., Newtown, PA) with a hammer configuration and 0.5 mm screen and then stored in the dark, in a clear Whirl-Pak® bag at -20°C until used.

Table 2.3 Visual criteria used to define growth stages.

Plant Maturity	Harvest Stage	Inspection Criteria
4-6 Leaf	1	4-6 leaves are present on the stem of the plant with no seed head revealed. First node visible.
Flowering	2	The seed head flowering with yellow flowers.
Milk	3	When the seed pod is removed from the plant and squeezed between the thumb and first finger, a liquid milky white substance is released.
Soft dough	4	When the seed pod is removed from the plant and squeezed, a small solid white substance with the consistency of soft dough is released.

2.5. General Characterization

The changes in chemical compostition of plants over time were explored by using experimental methods to analyze for starch, protein and phenol contents, as well the fatty acid and sterol semi-quantification. All samples were measured in duplicate unless otherwise stated.

2.5.1. Starch Analysis

Total starch was determined according to AOAC method 996.11 (Megazyme, 2008) using a Megazyme kit purchased from Cedarlane Laboratories (Burlington, ON) as per manufacturer's instructions. The sample was hydrated and starch was hydrolyzed with thermostable a-amylase at 95–100 °C for 5 min, followed by hydrolysis with amyloglucosidase at 50 °C for 30 min. The sample was centrifuged, and the supernatant was used to determine digestible starch, using glucose oxidase–peroxidase reagent. Each sample was analyzed in duplicate

2.5.2. Protein Analysis

Protein content was determined using a colorimetric Total Protein Kit purchased from Sigma-Aldrich (St. Louis, MO). This method, commonly referred to as the Bradford assay, is based on the absorption shift from Protein in solution absorbs UV light

at a wavelength 470 nm to 595 nm observed when the brilliant blue G-250 dye binds to protein. The samples were prepared by hydrating 0.1 g of dried material with reverse osmosis water to a total volume of 10 ml. Aliquot samples of 50 µl was used in each assay according to the manufacturer's instructions. Results were derived from a standard curve of the human albumin protein standard provided.

2.5.3. Phenol Analysis

The phenolic content was determined according to the method previously described by Gibreel et al. (2009) and Zhao et al. (2006) using the chemicals listed in Table 2.2. Briefly, 50 mg material was added to 2 ml of 80% methanol (v/v). This mixture was sonicated for 1 hr under nitrogen and centrifuged at 2590 *x g* for 15 min. A 1 ml aliquot of the supernatant was then reacted with 2 ml of 10% Folin Ciocalteu's phenol reagent (v/v) for 5 min. Then 5 ml of deionized water and 2 ml 10% sodium carbonate (w/v) were added to the reaction. After 1 hour of holding the mixture at room temperature, a record to the UV absorbtion at 740nm using a Genesys 10UV spectrophotometer. (Thermo Fisher Scientific, Fair Lawn, NJ). The same solution without the extracted solution was used as a blank. The ccalculation of content of total phenolics, was based on gallic acid standard with equation 2.1.

$$TotalPhenolics = \frac{A_{sample} \times W_{s\,tan\,dard}}{A_{s\,tan\,dard} \times W_{sample}} \quad \text{.....equation 2.1}$$

2.5.4. GC-FID Analysis of Fatty Acids

Fatty acid profile was determined by using GC-FID the standard methodology described by Gibreel et al. (2009) and Outen et al. (1976) using a representative green biomass sample set of: one winter triticale (Bobcat), one spring triticale (Pronghorn), one spring wheat (AC Pathfinder) and one spring rye (Gazelle) variety. Profiled fatty acids were based on calculated methyl esters standards such as 14:0, 16:0, 18:0 18:1 n-9, 18:1 n-7, 18:2, 18:1 n-3, 20:0 and 22:0 using GC-FID. A Varian 3400 Gas Chromatograph equipped with a Varian 8200 autosampler (Varian Inc., Palo Alta, CA) coupled with a flame ionization detector (FID) was used. The inlet heater was set to start at 70°C and then ramp to 230°C, pressure at 25PSI and FID detector at 230°C. Cool-on column injections of 1.0 µl and the column used was a GC Column SGE F10 30m X 0.25mm ID-BP m20 X 0.25, capillary column (SGE Analytical Science, Austin, TX). The column temperature profile was outlined as an initial temperature of 50°C for 0.2 minutes, then a ramp of 20°C per minute to 170°C for a hold time of 5 minutes, ramp 10°C per minute to 230°C and hold for 13 minutes, for a total time of 30.2 minutes. Data was analysed by using Agilent ChemStation for chemical quantification.

2.5.5. CG-FID Analysis of Sterols

The sterol content was determined by GC-FID using a method described by Gibreel et al. (2009) and Mounts et al. (1996) using a representative sample set of one winter triticale (Bobcat), one spring triticale (Pronghorn), one spring wheat (AC

Pathfinder) and one spring rye (Gazelle) variety. Each sample was compared to the sterol standard mixture of brassicasterol, campesterol, stigmasterol and sitosterol using GC-FID analysis. A Varian 3400 Gas Chromatograph equipped with a Varian 8200 autosampler (Varian Inc., Palo Alta, CA) coupled with a flame ionization detector (FID) was used. The inlet heater was set to start at 90°C and then ramp to 280°C, pressure at 23 PSI and FID detector at 300°C. Cool-on column injections of 1.0 µl and the column used was a J&W Scientific DB-5 (30m X 0.25mm ID, 0.25µm film), capillary column (Agilent Technologies Inc.Santa Clara, CL). The column temperature profile was outlined as an initial temperature of 70°C for 0.2 minutes, then a ramp of 20°C per minute to 250°C for a hold time of 5 minutes, ramp 10°C per minute to 280°C and hold for 17 minutes, for a total time of 28.2 minutes. Data was analysed by using Agilent ChemStation for chemical quantification.

2.6. Solvent Extractions

2.6.1. Soxhlet Extraction

Soxhlet extraction was performed with three – 24 hour refluxing steps using consecutive solvents: pentane, petroleum ether and ethyl acetate. This extraction was performed on all varieties at each harvest stage and the extract was analyzed using a GC-MS for comparison. Briefly, the whole plant sample of 1.0 g was placed inside single thickness cellulose Soxhlet extraction thimble. Round bottom flasks were filled with 190ml of solvent and the apparatus was assembled, wrapped with Teflon tape at each

joint to ensure a tight seal and prevent loss of volatile solvents or compounds, and placed in a stand over a heating mantle. The heating temperature was set at the solvent boiling point, and the system was allowed to reflux for 24 h. After 24 h, the apparatus was cooled and disassembled. The cellulose thimble was air dried for at least 30 min before subsequent extractions and procedure was repeated.

Once the reflux extraction was complete, 1 ml of 1 mg/ml methyl ester of C17:0 in methanol solution, as an internal standard, was added to the round bottom flask which contained the solvent and extract. The mixture was concentrated to a 1 ml volume by rotary evaporation using a temperature below the solvent boiling point and variable vacuum sensor (Laborota, Heidolph, Germany). The sample was transferred quantitatively using clean solvent to a 5 ml amber glass drum vial with a Teflon lined lid. This process was repeated with each consecutive solvent. The total extracted amount was determined by the change in mass of the cellulose thimble containing the sample fraction pre- and post- extraction. The residue fibre and thimble was dried and placed in a desiccator for one week to ensure solvent and water evaporation. The residual sample was retained for residual fibre characterization analysis, as described in Section 2.6.4.

2.6.2. Extract Characterization

To assess changes in chemical compounds over harvest time and effectively evaluate the potential use of different solvents for extraction of value-added bio mass components, gas chromatography equipped with chemical ionization and mass spectrometry (GC-MS) detector was used.

2.6.3. Characterization by Gas Chromatography-Mass Spectrometry

2.6.3.1. Derivatization

Prior to GC-MS, the samples were derivatized by a modified method as previously described by Broberg et al. (2007). The extracts were dried under nitrogen (compressed PP 4.8, Praxair, Edmonton, AB) and weighed to determine the percent extract in each solvent. The extract was resuspended in 1 ml of di-chloromethane and 500 µl of Diazomethane in ether (prepared in-house from the Diazald® distillation kit with Clear Seal® joints) and incubated at room temperature for 15 min. The sample was again dried under nitrogen and resuspended with 2 ml of di-chloromethane and 100 µl of N-methyl-N-(triethylsilyl)-trifluoroacetamide, to ensure the detection of all compounds within sample, due to the uncertainty of the bio-chemical make-up. The mixture was vortexed and heated in a 60°C water bath for 1 h with additional mixing after 30 min. The derivatized samples were transferred to 1.5 ml amber glass vials with secured Teflon-lined lids and stored at 4°C until analysis.

2.6.3.2. GC–MS Analysis

All experiments were performed using the model 5975B EI/CI GC-MS with a 7683B series injector and autosampler (Agilent Technologies Inc., Santa Clara, CA) in positive ion mode. The inlet heater was set at 300°C, pressure at 46.5 kPa and detector at 250°C. The total flow was set at 15.9 ml/min with a split ratio of 12.2 ml/min and on-

column injections of 1.0 µl. The column used was a 30 m, Agilent 19091s-433, 0.25 mm ID, 0.5 µm bonded phenyl dimethylpolysiloxane film thickness, capillary column. The column temperature profile consisted of an initial temperature of 100°C for 0.1 min, followed by a ramp of 10°C per min to 210°C for a hold run time of 10 min, ramp 10°C per min to 290°C and hold for 5 min and a final ramp of 10°C per min to 300 and hold for 10 min, for a total time of 45.10 min. Data was analysed by using Agilent ChemStation and the NIST05a.L library for chemical matches over 90%.

2.6.4. Residue Characterization

Residue characterization was performed to identify compounds for potential recovery and use in value added products The reside was underwent a two-step hydrolysis to fractionated the residue into measurable total solids, ash, lignin and carbohydrates in the residue were measured according to Laboratory Analytical Procedures (LAP) established by National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008a). The lignin fraction was measured as both acid-soluble by UV spectroscopy (Genesys 10, Thermo Fisher Scientific, Fair Lawn, CA) and acid-insoluble material during gravimetric analysis of ash. The hydrolysate contains the monomeric carbohydrates measured by high performance liquid chromatography (HPLC, Agilent Technologies Inc.) with a refractive index detector.

2.7. Data analysis

Principle variance component analysis was used to determine the effects of various growth stages and cereal varieties on measured characteristics and test the statistical differences, with the help and expertise of Dr. L. A. Goonewardene of Alberta Agriculture and Rural Development. A 95% confidence level was applied to all analysis. The data presented in this study were analyzed using SAS software, Version 9.1 of the SAS System for Windows (Copyright ™ 2002-2003 SAS Institute Inc. Cary, NC). All treatments in this study were conducted in duplicate and average values were reported.

3. Results

This chapter presents the results that identify potential chemicals at particular growth stages of immature cereal crop utilization for industrial chemical production.

3.1. Green Biomass Growth and Harvest

The overarching objective of this thesis research was to evaluate the green biomass cereal crops as a feedstock for a green biomass biorefinery. It was determined early in the experiments that green biomass was both interesting and a challenge to work with as a feedstock. Concerns developed and were resolved around plant growth to selected maturity, harvesting and transporting to maintain attributes.

In the greenhouse, the plant growth regime was designed minimize any yearly changes that can occur by using the three time trials. The plant pots on the bench moved in a methodical pattern to give equal bench locations to avoid any bench effect. The plants grew quickly to the 5-6 leaf stage as shown in Table 3.1 and the stages were easily assessed by visual markers as to when the next stage had been reached. An example summary to illustrate agronomic data for winter triticale, Bobcat, suggests a benchmark of growth time 177 days to heading and 224 days to full maturity (Alberta Agriculture Food and Rural Development 2008). It was observed that within the greenhouse, the growth was accelerated.

Table 3.1 Days of growth after planting as harvested the time blocks of trials (T) for each cereal variety grown to predetermined harvest stages.

Variety	Trial (T)	Harvest 1 (H1) 4-6 Leaf Stage	Harvest 2 (H2) Flowering Stage	Harvest 3 (H3) Milk Stage	Harvest 4 (H4) Soft Dough Stage
Pronghorn	T1	43	50	78	86
	T2	62	127	141	153
	T3	40	50	61	58
AC Ultima	T1	43	48	Missing[1]	78
	T2	62	105	127	169
	T3	40	50	74	74
Bobcat	T1	43	No growth[2]	No growth[2]	No growth[2]
	T2	105	Missing[1]	141	186
	T3	61	124	145	162
Fridge	T1	43	No growth[2]	No growth[2]	No growth[2]
	T2	105	135	141	186
	T3	61	124	124	162
AC Pathfinder	T1	43	Missing[1]	78	86
	T2	62	105	117	135
	T3	40	50	61	58
Gazelle	T1	43	43	64	78
	T2	62	91	105	127
	T3	40	40	61	58
AC Rifle	T1	43	No growth[2]	No growth[2]	No growth[2]
	T2	66	118	135	186
	T3	Seed n/a[3]	Seed n/a[3]	Seed n/a[3]	Seed n/a[3]

[1] Missing: samples were not found after transporting them from University of Alberta, main campus, Ag/For Building to University of Alberta, south campus, Agri-Food Discovery Place.
[2]No growth: the winter variety required a vernalization period as discussed in section 2.3.
[3]Seed n/a. AC Rifle (rye) in T3 was used for vernalization trials therefore the seed depleted and the variety was no longer available from the supplier.

For data analysis, the time trial samples were pooled by harvest stages to be evaluated as independent variable to reduce the effects of time of year or greenhouse bench effect.

3.1.1. Chemical characterization

Plant chemical characterization was designed to evaluate traditional laboratory practices for grain analysis that could identify differences in harvest stage for chemical recovery. The identification and verification of a laboratory technique or evaluation of multiple techniques for green biomass will assist evaluating harvested material growth stage as it enters a processing facility. In this study, the samples were first harvested after visual inspection with subjective human abilities to recognize the plant maturity indicators. As such, it was hypothesized that harvest growth could be identified by simple laboratory tests of starch, protein, phenol, sterol and fatty acid.

3.1.1.1. Analysis of Starch

Starch content of the seed head would be an ideal indicator of full maturity because the starch accumulates within the seed head; however, it is not necessarily a good indicator for the whole plant, including the stalks of the proposed immature cereal feedstock. The expected trend for the starch content was an increase as the plant matures to the soft-dough stage (H4). The observed trends in Figure 3.1, were not as conclusive when examining the interactions of the variety and harvest stage.

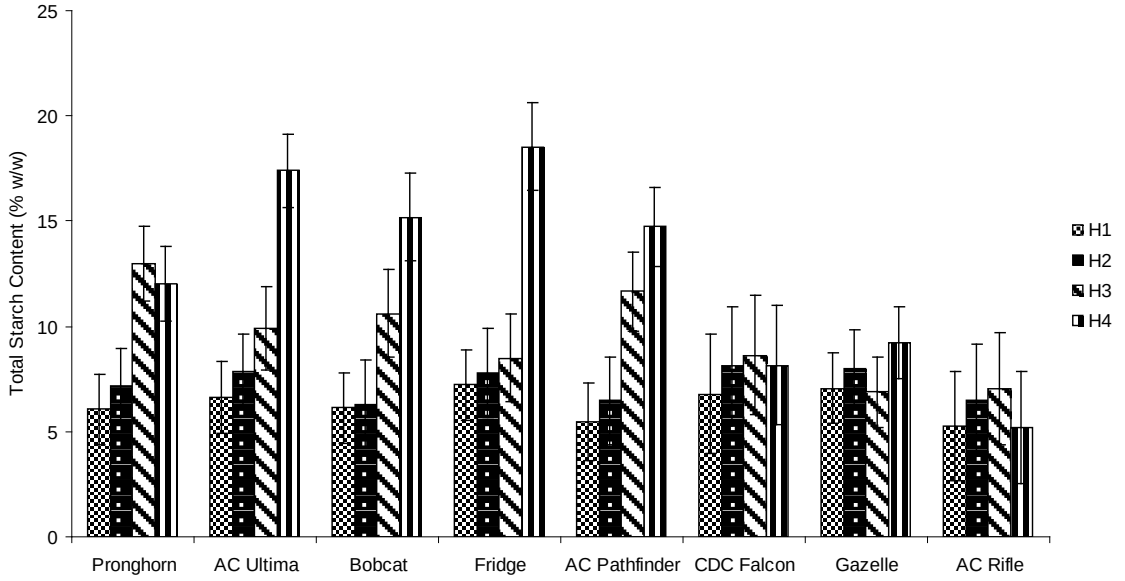

Figure 3. Starch content (% w/w) at each harvest stage of all the immature cereal varieties.

The triticale varieties of Pronghorn, AC Ultima, Bobcat and Fridge as well as AC Pathfinder (wheat), present a trend of increasing starch content as the plant matures but CDC Falcon (wheat), Gazelle (rye) and AC Rifle (rye) do not show the same trend. All varieties had 5.5 to 7.0% starch content in the first harvest stage (H1) with a slight increase to 6.5 to 8.0% starch content, when measured at the flower stage (H2). The final milk (H3) and soft dough (H4) stages resulted in higher values of percent starch in the Pronghorn (triticale), AC Ultima (triticale), Bobcat (triticale) and Fridge (triticale) compared to Gazelle (rye), AC Rifle (rye) and CDC Falcon (wheat). The differences shown in the crop varieties indicate a difficulty in conclusively utilizing this method as an maturity indicator alone. Varietal correlations were also noted as the spring and winter triticale varieties values closely relate to AC Pathfinder (wheat) than any other variety. AC Rifle (rye) exhibited low values at each harvest stage.

3.1.1.2. Analysis of Protein

Protein estimation was considered as an indicator to growth stage with an expected trend of increased protein concentration in young grasses as indicated by Kohler (1943). Data acquired from the samples using the Total Protein Kit (Sigma-Aldrich, St. Louis MO) in Table 3.2, indicating there wasn't a significant difference between harvest stages. It was also notes that protein values were not different from variety type to variety type.

Table 3.2 Protein content (mg/g) at each harvest stage, irrespective of cereal varieties.

Harvest Stage	Protein (mg/g) (mean± SE)
H1	20.76±3.45b
H2	13.81±4.01a
H3	19.62±3.69ab
H4	12.42±3.89ab

n=21

a,b: means followed by the same letter in a column are not significantly different (P>0.05).

3.1.1.3. Analysis of Phenolic Groups

Total phenols content was measured as one more method of determination of the maturity of the feedstock. Phenolic compounds present in plant material serve many roles in the plant biochemistry, and are induced by environmental factors such as cold or insect infestation. There was no supporting literature to indicate the use of total phenol

content as a quick technique for maturity verification other than the Folin – Ciocalteu reagent being used to determine the total phenolic content of whole grains (Anson et al., 2008). Figure 3.2 is the comparison of the entire data set that was evaluated by assessing the interactions of the variety and harvest stage.

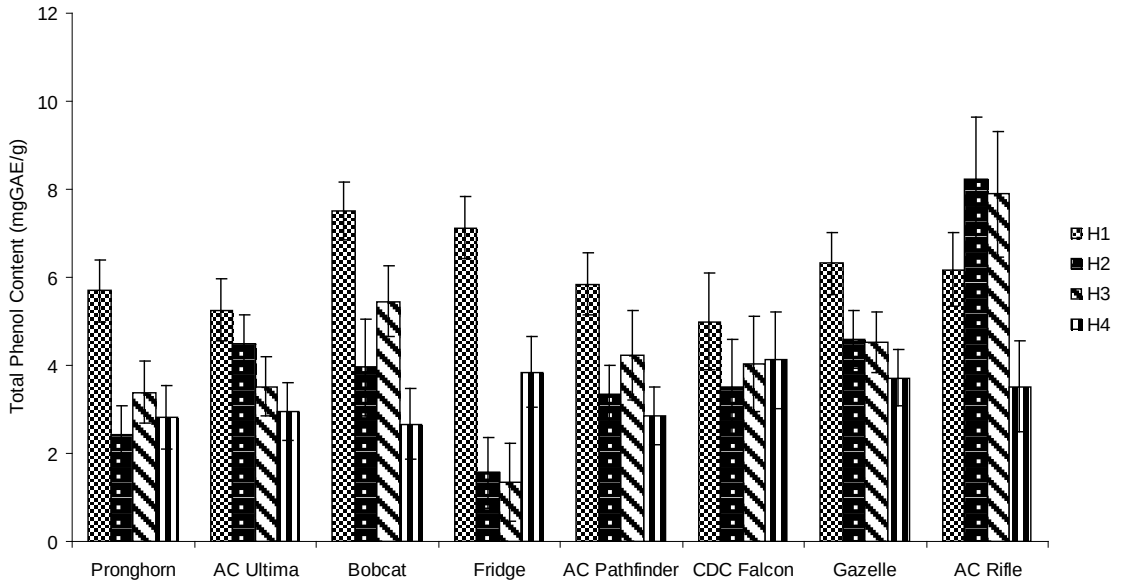

Figure 3.2 Total phenolic content (mg GAE/g) at four harvest stages; 4-6 leaf (H1), flower (H2), milk (H3) and soft dough (H4), of the immature cereal varieties.

There was no consistent trend for phenolic content as affected by variety or harvest stage. Pronghorn (triticale) and Bobcat (triticale) have similar total content trends to the AC Pathfinder (wheat); however, Bobcat (triticale)'s phenolic content was higher in the leaf (H1), flower (H2) and milk stage (H3). AC Rifle (rye) presented the higher values of phenolics during the flower (H2) and milk stage (H3) with a significant decreased by soft dough (H4) to a value similar to the all other plant varieties. Fridge (triticale) had an initial content that significantly decrease below the average of the

flower stage (H2) of all the varieties; however, by the soft dough stage (H4) it returned to average plant values.

3.1.1.4. Analysis of Fatty Acids using GC-FID

Fatty acids chosen for analysis were identified through literature (Ryan et al., 2007) as commonly found in fully mature cereal grains. Fatty acid concentrations of myristic C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids were also reported for ryegrass (Palladino et al., 2009). The GC-FID response was quantified using the raw material, prior to extraction, of the cereal varieties at four harvest stages; 4-6 leaf (H1), flower (H2), milk (H3) and soft dough (H4). The results of fatty acid quantity comparison in each harvest stage of the cereal varieties is presented in Figures 3.3, Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7, Figure 3.8, Tables 3.3 and Table 3.4.

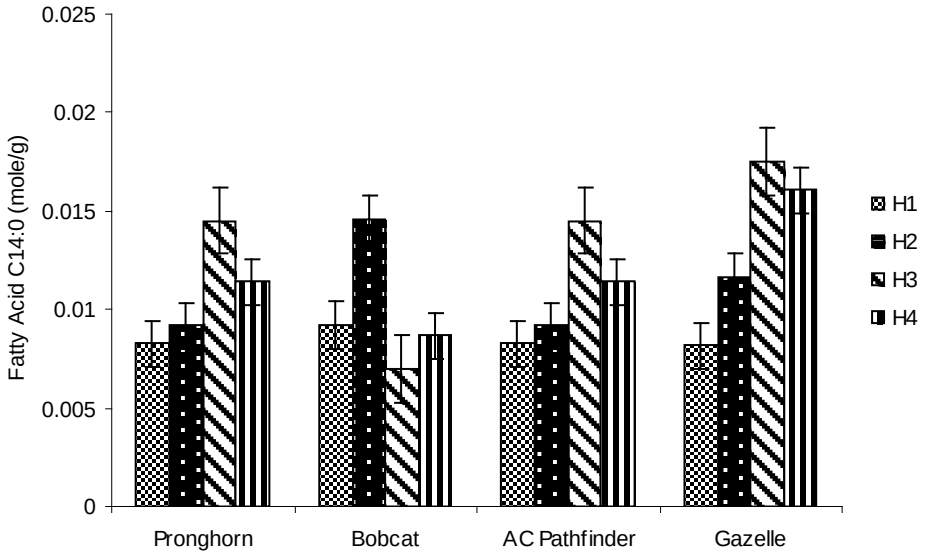

Figure 3.3 Myristic acid C14:0 content (mole/g) of raw material samples Pronghorn (triticale), Bobcat (triticale), AC Pathfinder (wheat) and Gazelle (rye) at four harvest stages.

Table 3.3 Palmitic acid C16:0 content (mole/g) of raw material samples Pronghorn (triticale), Bobcat (triticale), AC Pathfinder (wheat) and Gazelle (rye).

Variety	Fatty Acid C16:0 (mole/g) (mean±SE)
Pronghorn	2.59±0.29a
Bobcat	1.92±0.29a
AC Pathfinder	0.79±0.29
Gazelle	1.49±0.29a

n=8

a: means followed by the same letter in a column are not significantly different (P>0.05).

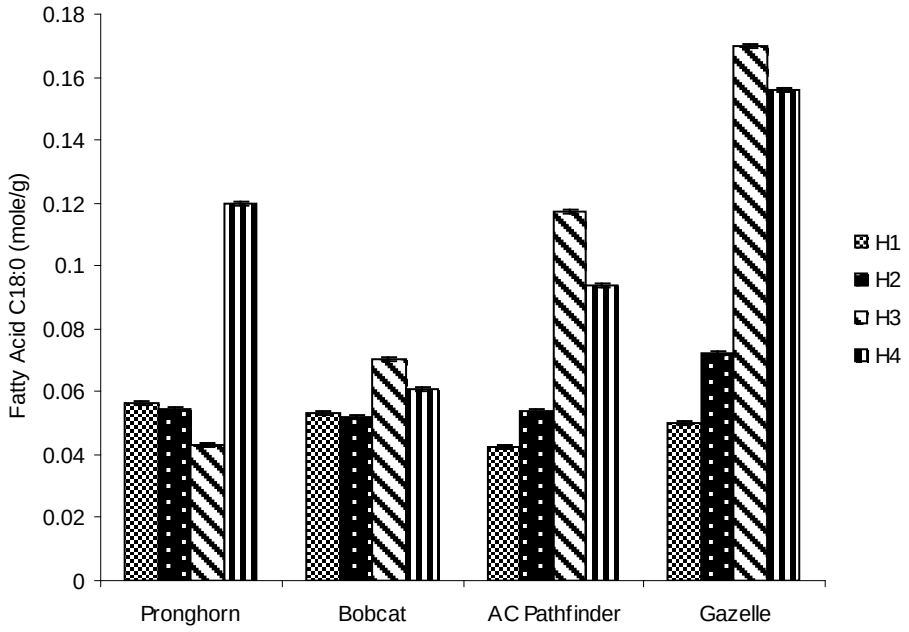

Figure 3.4 Stearic acid C18:0 content (mole/g) of raw material samples Pronghorn (triticale), Bobcat (triticale), AC Pathfinder (wheat) and Gazelle (rye), at four harvest stages.

Table 3.4 Oleic acid C18:1 n-7 content (mole/g) of raw material samples Pronghorn (triticale), Bobcat (triticale), AC Pathfinder (wheat) and Gazelle (rye).

Variety	Fatty Acid C18:1 n-7 (mole/g) (mean±SE)
Pronghorn	0.0019±0.0003a
Bobcat	0.0015±0.0003a
AC Pathfinder	0.0008±0.0003
Gazelle	0.0026±0.0003a

n=8

a: means followed by the same letter in a column are not significantly different (P>0.05).

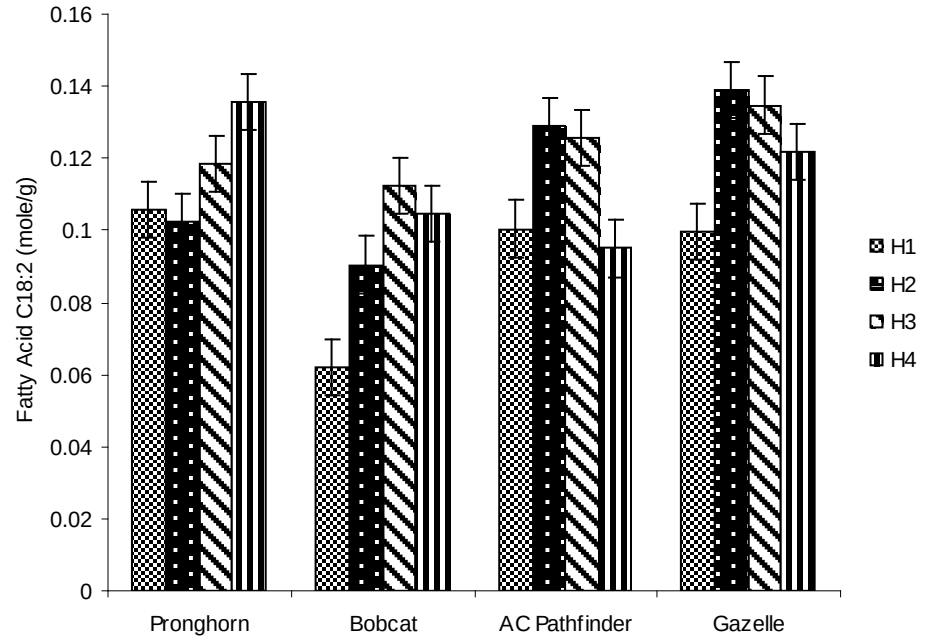

Figure 3.5 Linoleic acid C18:2 content (mole/g) of raw material samples Pronghorn (triticale), Bobcat (triticale), AC Pathfinder (wheat) and Gazelle (rye), at four harvest stages.

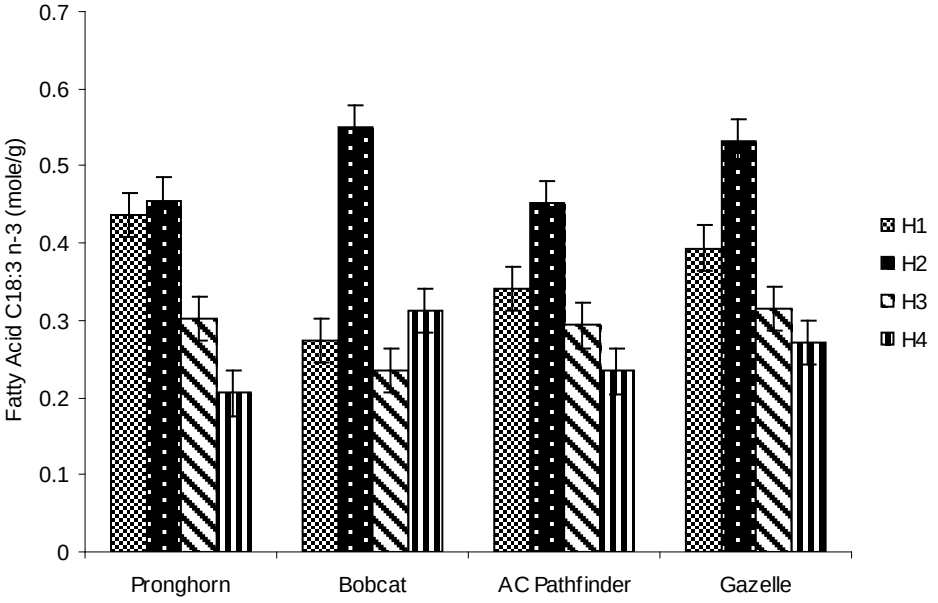

Figure 3.6 Linolenic acid C18:3 n-3 content (mole/g) of raw material samples Pronghorn (triticale), Bobcat (triticale), AC Pathfinder (wheat) and Gazelle (rye), at four harvest stages.

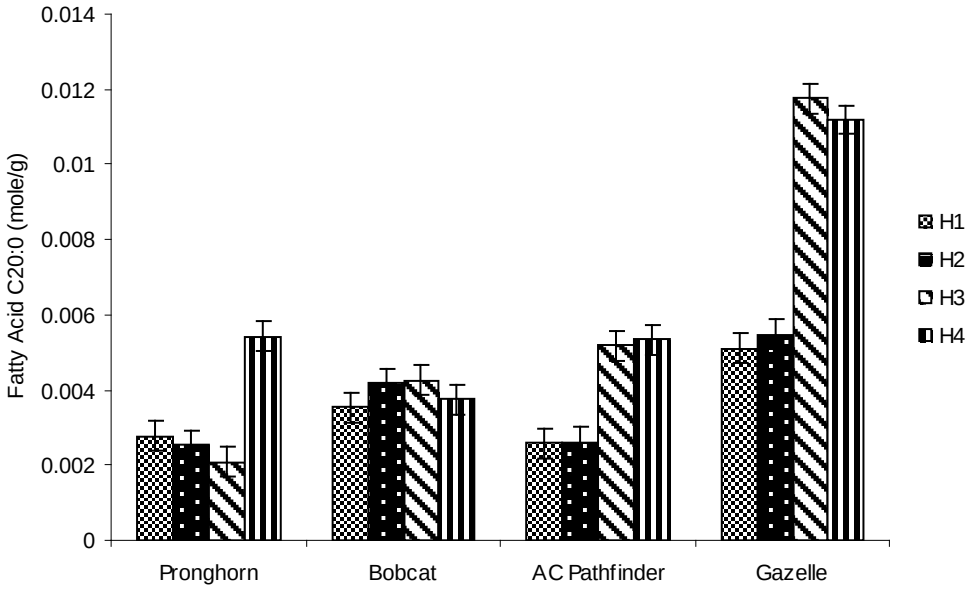

Figure 3.7 Arachidic acid C20:0 content (mole/g) of raw material samples Pronghorn (triticale), Bobcat (triticale), AC Pathfinder (wheat) and Gazelle (rye), at four harvest stages.

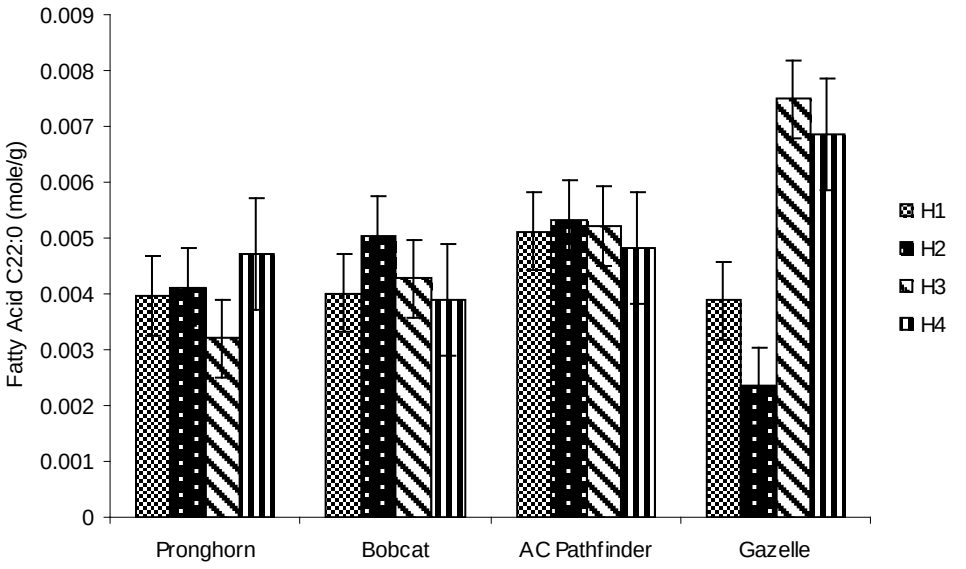

Figure 3.8 Behenic acid C22:0 content (mole/g) of raw material samples Pronghorn (triticale), Bobcat (triticale), AC Pathfinder (wheat) and Gazelle (rye), at four harvest stages.

The fatty acid concentrations were profiled in the whole raw material, which indicates the quantity of each fatty acid available prior to any treatment or processing. Mystic acid trace amounts were consistent for stage of growth in each variety which increased at the milk stage (H3) for Pronghorn (triticale), AC Pathfinder (wheat) and Gazelle (rye) with the exception of Bobcat (triticale) where the increase was in the flowering stage (H2) as shown in Figure 3.3. Palmitic acid concentration remained consistent though each harvest stage but was significantly lower in AC Pathfinder (wheat) (Table 3.3). Stearic acid content was measurable in each variety at each harvest stage without a consistent trend of concentration available as shown in Figure 3.4. Gazelle (rye) presented the strongest concentration at milk (H3) and soft dough (H4) stage for the cereal varieties tested. Oleic acid trace amount were consistent from growth stage to growth stage of each variety (Table 3.4). All varieties were found to have a concentration of approximately 0.1 to 0.14 mole/g of Linoleic acid (Figure 3.5). Linolenic acid was concentration were presented as highest at the flowering stage and decrease as the plant matures, as shown in Figure 3.6. Trace amount were found at all stages of growth for both Arachidic (Figure 3.7) and Behenic (Figure 3.8) acid.

3.1.1.5. Analysis of Sterols using GC-FID

Compounds chosen to be quantified were brassicasterol, campesterol, stigmasterol and stitosterol, which have been used in previous evaluations of whole cereal grains (Nystom et al., 2007; Dunford et al., 2009; Gabriel et al., 2009; and Leguizamon et al., 2009). The GC-FID response was quantified using four cereal varieties at four harvest

stages at four harvest stages; 4-6 leaf (H1), flower (H2), milk (H3) and soft dough (H4). The results of sterol quantity comparison in each harvest stage of the cereal varieties is presented in Tables 3.5, Table 3.6, Figures 3.9 and Figure 3.10.

Table 3.5 Brassicasterol content (mole/g) of raw material samples.

Harvest Stage	Brassicasterol (mole/g) (mean±SE)
H1	0.008±0.002
H2	0.001±0.002
H3	trace
H4	BTL

n=8

Table 3.6 Campesterol content (mole/g) of raw material samples Pronghorn (triticale), Bobcat (triticale), AC Pathfinder (wheat) and Gazelle (rye), irrespective of harvest stage.

Variety	Campesterol (mole/g) (mean±SE)
Pronghorn	0.24±0.31a
Bobcat	0.13±0.31a
AC Pathfinder	0.13±0.31a
Gazelle	4.59±0.31

n=8

a: means followed by the same letter in a column are not significantly different (P>0.05).

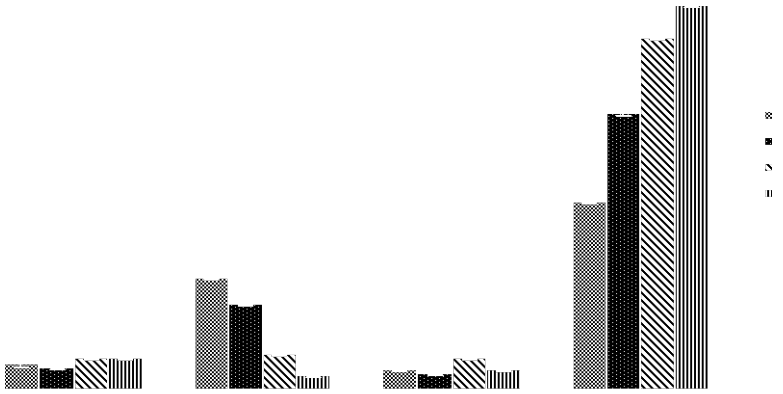

Figure 3.9 Stigmasterol content (mole/g) of raw material samples Pronghorn (triticale), Bobcat (triticale), AC Pathfinder (wheat) and Gazelle (rye), at four harvest stages.

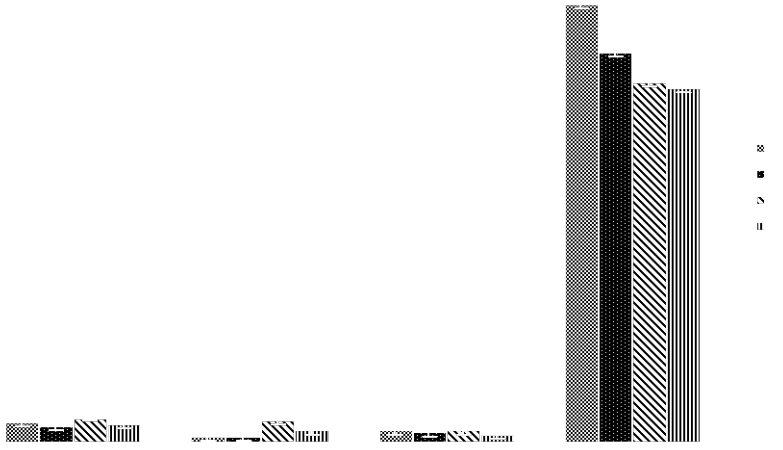

Figure 3.10 Stitosterol content (mole/g) of raw material samples Pronghorn (triticale), Bobcat (triticale), AC Pathfinder (wheat) and Gazelle (rye), at four harvest stages.

The fatty acid concentrations were profiled in the whole raw material, which indicates the quantity of each fatty acid available prior to treatment or processing. Mystic acid trace amounts were consistent for stage of growth in each variety which increased at the milk stage (H3) for Pronghorn (triticale), AC Pathfinder (wheat) and Gazelle (rye) with the exception of Bobcat (triticale) where the increase was in the flowering stage (H2) as shown in Figure 3.3. Palmitic acid concentration remained consistent though each harvest stage but was significantly lower in AC Pathfinder (wheat) (Table 3.3). Stearic acid content was measurable in each variety at each harvest stage without a consistent trend of concentration available as shown in Figure 3.4. Gazelle (rye) presented the strongest concentration at milk (H3) and soft

The sterol concentrations were evaluated from the whole raw material which indicated for each sterol, the quantified available value prior to treatment or processing. Brssicasterol trace amounts decreased to undetectable limits as the plant matured (Table 3.5). Campesterol content was consistent for stage of growth in each variety as shown in Table 3.6. Stigmasterol concentration was low in the cereal varieties with the exception of Gazelle (rye), where the concentration was higher in all stages and increased as the plant matured (Figure 3.9). Stitosterol content was consistent at each stage of growth for Pronghorn (triticale), Bobcat (triticale), and AC Pathfinder (wheat) except Gazelle (rye) (rye) were the concentration decrease as the plant matured as shown in Figure 3.10.

3.1.2. Solvent Extraction

The solvent extraction procedure performed included a three step sequential extraction to evaluate the chemical differences between the growth stages and varieties of cereal crops. Solvent selection was critical for the selective extraction of compounds from the plant material. The solvent extraction experimental protocol was developed utilizing a series of solvents from non-polar to polar. Solvent selection was based on evaluation of GC-MS chromatograms of detection peaks. Pentane, petroleum ether and ethyl acetate solvents were utilized.

3.1.2.1. Soxhlet extraction

The percent extracted was tracked as an indication of efficiency throughout the year as presented in Figure 3.12. The extracted amount, which was determined by a weight loss, indicates a trend of decreasing in percentages as the plant material was stored due to oxidation or possibly the change in weather influenced the moisture in the air.

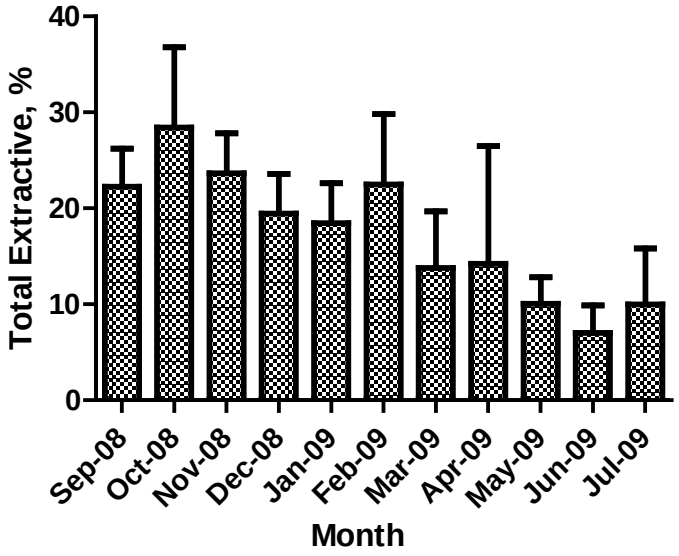

Figure 3.11 The percentage of extracted material as a function of time of all varieties.

Solvent extraction with Soxhlet extractors was used because of their longstanding history of usability for heated extraction. Soxhlet extractors allow the green biomass solids to be extracted with recycled but fresh, heated solvents at increased rates over a 24 hour reflux time.

3.1.3. Extractive Sample Chemical Identification

3.1.3.1. Analysis of Extractives using GC-MS

Most organic compounds are volatile enough for direct GC analysis (Zenkevich, 2005) but compounds that are polar and non-volatile are not well suited for GC analysis in their native form. In order to make these compounds more amenable to GC-MS analysis, they must first be derivatized to increase their volatility and reduce polarity.

Derivatizing also creates sensitivity to detect all fatty acids (Casal and Oliveira, 2006), amino acids (Zenkevich, 2005), carbohydrates and others. The derivatization used in this thesis was chosen from literature as a recommendation to use TMS, acetyl or TFA derivatives (Zenkevich, 2005) for their stability to hydrolysis and reduction compounds. The analytical results show that the TMS derivatization of the very complex carbohydrate mixtures from extraction of the green biomass is amenable for GC-MS identification. Each product gives a characteristic pattern of peaks which could be reproduced by a combination of their retention times and electron-ionization mass spectra.

All samples were examined on the GC-MS and all the chromatograms were compared both against the replicates as well as against varieties. Results are found to be similar of the various cereal crops and growth stages. The MS Chemstation™ software library (Agilent Technologies Inc.) was used to match the chemical identity. A quality of 90 or higher was used to generate a list of potential chemical compounds to measure over time.

3.1.3.1.1. Pentane

Results for potentially identified compounds are presented in Figure 3.12, Table 3.7 and Table 3.8 for the pentane extracts. There were 15 extracted compounds that were commonly matched at all stages for all varieties, where approximately 8 of those compounds were potentially fatty acids. There was proof that there were chemical changes from the young grass to the later stage of seed head formations when comparing the GC-MS data for differences. Table 3.8 presents a compilation of the changes in

chemical composition observed with respect to harvest stages. Some of matches indicated that aromatic di-carboxylic acid, carboxylic acid, and organic acids were identified in H3 and H4. The results also indicate that potentially, carbohydrates and waxes are in greater abundance at earlier growth stages.

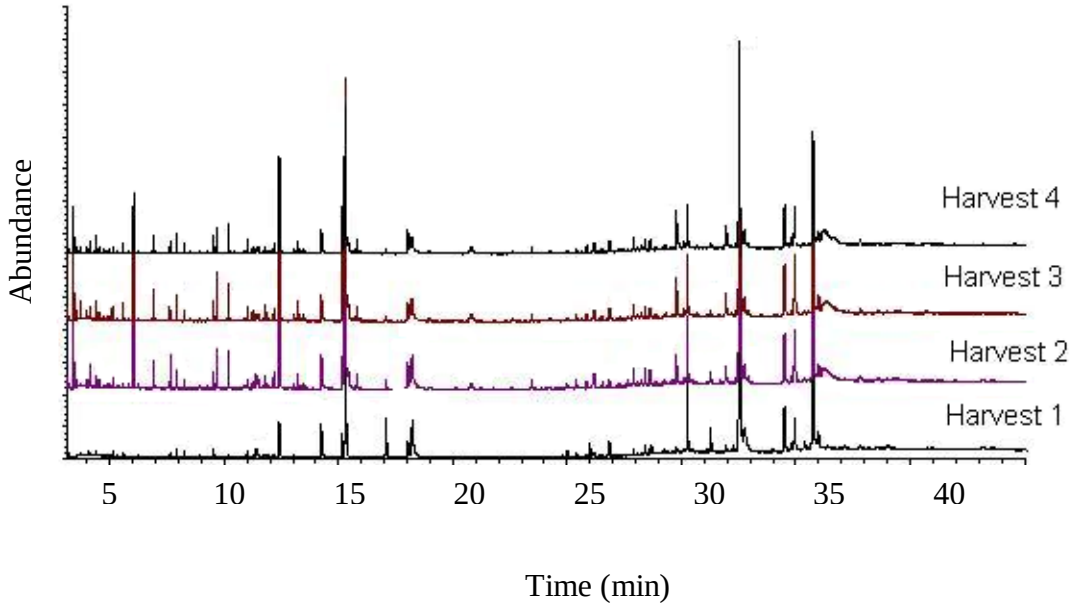

Figure 3.12 CG-MS chromatogram of pentane extraction of Pronghorn (triticale) at each harvest stage.

The GC-MS chromatograms confirmed that chemical changes are detected from harvest stage to harvest stage. In Figure 3.12 Pronghorn (triticale) samples extracted with pentane exhibits chemical composition changing from harvest 1 (H1) to that harvest 2 (H2) to harvest 4 (H4).

Table 3.7 Pentane extracts: GC-MS library quality match of 90% or higher of the compound identified to be common at all stages of growth in all the samples.

Compound identified (confidence of 90% or higher)	**Family name or symbol**
Nonanal	Alkyl Aldehyde
Silanol, trimethyl-, phosphate (3:1)	Phosphate
Nonanoic acid, trimethylsilyl ester	Carboxylic acid
Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	Fatty acid C4
Malic acid, tris(trimethylsilyl) ester	Carboxylic acid
Butylated Hydroxytoluene	BHT fat soluble
Methyl tetradecanoate	Fatty acid 14:0
Hexadecanoic acid, methyl ester or Pentadecanoic acid, 14-methyl-, methyl ester (no overlap of these two but between the two of them at the same retention time, they are found in all the samples)	Fatty acid 16:0
Hexadecanoic acid, trimethylsilyl ester	Fatty acid 16:0
9,12-Octadecadienoic acid (Z,Z)-, methyl ester or 8,11-Octadecadienoic acid, methyl ester or 11,14-Octadecadienoic acid, methyl ester (no overlap of these two but between the three of them at the same retention time, they are found in all the samples)	Fatty acid 18:2
Octadecanoic acid, methyl ester	Fatty acid 18:0
Silane, [(3,7,11,15-tetramethyl-2-hexadecenyl)oxy]trimethyl-	Saturated hydrocarbon
9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	Fatty acid 18:2
Linolenic acid, trimethylsilyl ester or .alpha.-Linolenic acid, trimethylsilyl ester	Organic acid

Pentane exaction profiled groups of biochemical compounds within the family of fatty acids and organic acids as shown in Table 3.7 and Table 3.8 which is expected according to Pavlikova et al. (2005) who also identified hydrocarbons, free sterols, esters of fatty acids and phospholipids.

Table 3.8 Pentane extraction, GC-MS library quality match of 90% or higher of the compound identified to change with growth stages, of all samples.

Changes observed	Compound identified (confidence of 90% or higher)	Family name or symbol
In all sample of H1	Acetophenone	Aromatic ketone
Common in H1 and H2	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-	Carbohydrate
	2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	Olefin wax
Common in H1 and H2 in AC Ultima	3,5-Di-tert-butyl-4-trimethylsiloxytoluene	Petroleum product
Found in AC Ultima H1	11-Tetradecyn-1-ol acetate	Carboxylic acid
Found in AC Ultima H2	13-Borabicyclo[7.3.0]tridecane, 13-propoxy-, (Z)- or (E)-	Alkane hydrocarbon
Common to H2, H3 and H4	Nonanoic acid, 9-oxo-, methyl ester	Carboxylic acid
	Butanedioic acid, [(dimethoxyphosphinothioyl)thio]-, diethyl ester	Organic acid
Only in H3 and H4 mostly in Pronghorn, Fridge and AC Rifle	Dodecanoic acid, methyl ester	Fatty acid C22:0 wax
Common in H3 and H4	Phthalic acid, butyl isohexyl ester	Aromatic dicarboxylic acid
	Phthalic acid, isobutyl octadecyl ester	Aromatic dicarboxylic acid
Only identified in Gazelle	Azelaic acid, bis(trimethylsilyl) ester	Fatty acid
Only found in Pronghorn	Cyclohexasiloxane, dodecamethyl-	
Common in all the samples but does not have the same retention time in each stage.	Phosphoric acid, bis(trimethylsilyl)monomethyl ester	Inorganic acid

3.1.3.1.2. Petroleum Ether

Petroleum ether was the second solvent used in the consecutive solvent extraction method. Results for potentially identified compounds in the extracts are presented in Figure 3.13 and Table 3.8. There were only 2 extracted compounds that were commonly matched at all stages of all varieties. It was the intent of this step to accentuate the difference in compounds available for removal as the solvent progressively increase in polarity. It was successful based on the observation that the extract chromatograms demonstrated peaks smaller and less abundant compared to the pentane extracts.

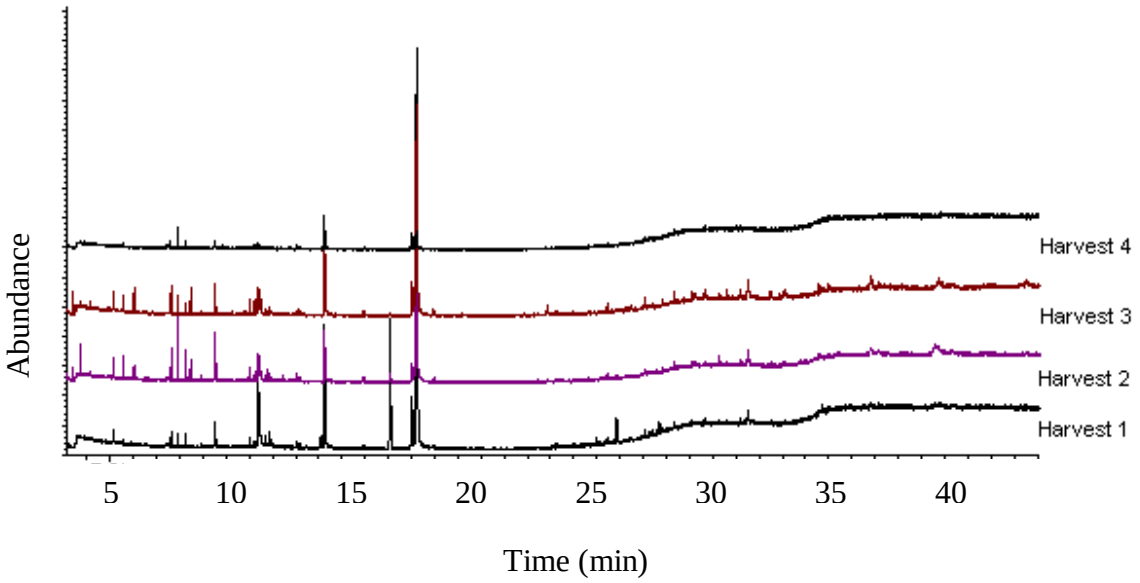

Figure 3.13 CG-MS chromatogram of petroleum ether extraction of Pronghorn (triticale) at each harvest stage.

Petroleum ether extraction results were similar to pentane when comparing the identified fatty acid compounds (Table 3.9) but the abundance of the GC-MS chromatograms peaks were lower at the same harvest stages (Figure 3.13).

Table 3.9 Petroleum ether extract: GC-MS library quality match of 90% or higher of the compound identified to be common at all stages of growth in all the samples.

Compound identified (confidence of 90% or higher)	**Family name or symbol**
Hexadecanoic acid, trimethylsilyl ester	Fatty acid 16:0
alpha-Linolenic acid, trimethylsilyl ester	Fatty acid

3.1.3.1.3. Ethyl Acetate

Ethyl acetate was the most polar solvent that was observed to produce the most vivid and opaque green extract. The GC-MS chromatograms displayed verification of different compounds in the extract by the shift of elution as a result of shortened retention times compared to the pentane extract chromatograms. Results for potentially identified compounds are presented in Figure 3.14, Table 3.10 and Table 3.11. Ethyl acetate extraction was expected to separate saturated and unsaturated fatty acids, phospholipids, phytosterols, free phenylpropanoid acids as well as flavonoid aglycones (Pavlikova et al., 2005).

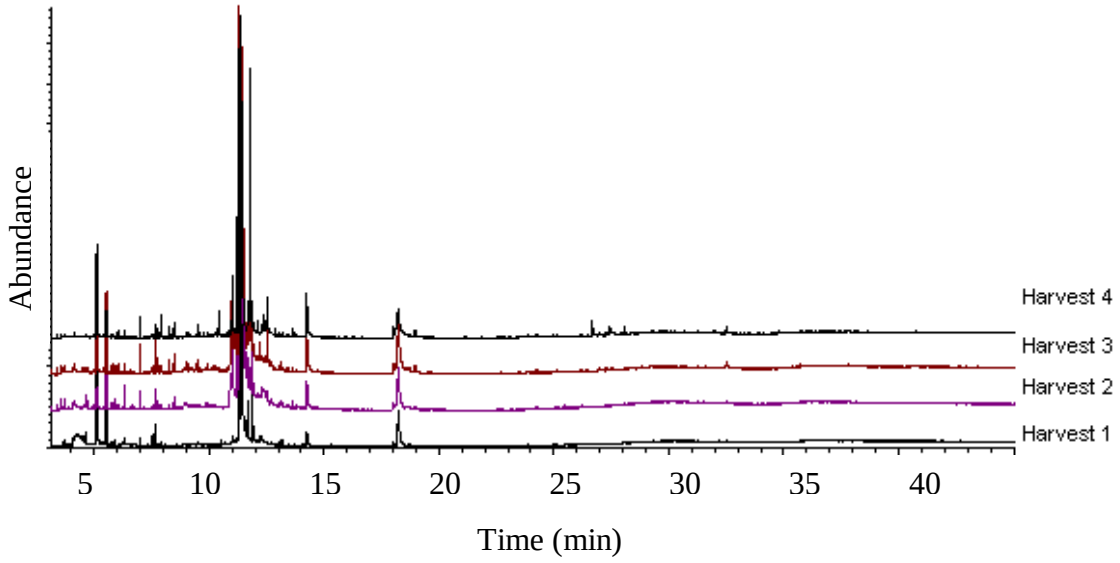

Figure 3.14 CG-MS chromatogram of ethyl acetate extraction of Pronghorn (triticale) at each harvest stage.

Table 3.10 Ethyl acetate extract; GC-MS library quality match of 90% or higher of the compound identified to be common at all stages of growth in all the samples.

Compound identified (confidence of 90% or higher)	**Family name or symbol**
Trimethylsilyl ether of glycerol	Plant oil
Silanol, trimethyl-, phosphate (3:1)	Phosphate
Butanedioic acid, bis(trimethylsilyl) ester	Organic acid
2(3H)-Furanone, dihydro-3,4-bis[(trimethylsilyl)oxy]-, trans-	
Malic acid, O-(trimethylsilyl)-, bis(trimethylsilyl)ester or Malic acid, tris(trimethylsilyl) ester or Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	Carboxylic acid
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-	Carbohydrate
Arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-	Carbohydrate
Hexadecanoic acid, trimethylsilyl ester	Fatty acid C16:0
.alpha.-Linolenic acid, trimethylsilyl ester	Fatty acid

Results of this ethyl acetate extracted samples presented GC-MS chromatograms (Figure 3.14) that were considerably different than pentane and petroleum ether chromatograms as the peak cluster was an earlier time. Ethyl acetate exaction profiled groups of biochemical compounds within the family of carbohydrates as shown in Table 3.10 and Table 3.11. Overall, more compounds were extracted out of the variety, Gazelle (rye).

Table 3.11 Ethyl acetate extraction, GC-MS library quality match of 90% or higher of the compound identified to change with growth stages, of all samples.

Comments	**Compound identified (confidence of 90% or higher)**	**Family name or symbol**
Only in H1 of Bobcat and Gazelle	Ricinoleic acid, trimethylsiloxy, trimethylsilyl ester	Hydroxy fatty acid
Found in H2, H3 and H4	Cinnamic acid, p-(trimethylsiloxy)-, trimethylsilyl ester	Carboxylic acid
Found in H3 and H4	Butane, 1,2,3,4-tetrakis[(trimethylsilyl)oxy]-	Hydrocarbon
Found in spring triticale	Erythrose, O-methyloxime, tris-O-(trimethylsilyl)-	Carbohydrate
Found in Rye varieties	Dibutyl phthalate	
Found in Gazelle H4	L-Proline, 5-oxo-1-(trimethylsilyl)-, trimethylsilyl ester	Amino Acid
In most samples but no trends or correlations observed	Benzene, 1-(trimethylsilyloxy)-2-(trimethylsilyloxymethyl)-	Organic chemical

3.1.4. Residue characterization

The residue material was gathered from the Soxhlet extraction thimble after the consecutive solvent extractions. The material, at the bottom of the thimble, looked similar to the unprocessed sample as fibrous material but was a faint yellow tinted compared to the green raw material.

3.1.4.1. Analysis of Cellulose, Lignin and Structural Carbohydrates

The NREL method is a standard method used in the forestry industry to evaluate the percent of lignin and the concentrated polymeric sugars present after acid hydrolysis from the structural carbohydrates. The data in Table 3.12 are the percentage of lignin found in the residual material after consecutive solvent extractions. The percent of the material that was soluble after this hydrolysis is presented in Table 3.13.

Table 3.12 Percent lignin of the resulting residue after consecutive solvent extractions at four harvest stages, irrespective of varieties.

Harvest Stage	Percent lignin remaining in extraction residue (%) (mean±SE)
H1	10.5±2.8a
H2	5.74±2.6
H3	12.5±2.8a
H4	12.7±2.7a

n=16

a: means followed by the same letter in a column are not significantly different (P>0.05).

Lignin was preserved though the consecutive solvent extraction leaving 5 to 13 % of the weight (Table 3.12). As expected, the lignin content was higher in more mature plants compared to younger plants.

Table 3.13 Percent acid solubility of the residue after consecutive solvent extractions at four harvest stages, irrespective of varieties.

Harvest Stage	Acid solubility of extraction residue (%) (mean±SE)
H1	32.0±1.9bc
H2	29.7±2.1a
H3	25.3±2.1ab
H4	27.5±2.0c

n=8

a: means followed by the same letter in a column are not significantly different (P>0.05).

The acid solubility of the extracted residue is a value that could be useful when deciding on a particular residue utilization technique. In this case to evaluate the cellulose carbohydrates, the waste residue was subjected to a 72% sulphuric acid solution which dissociated 25.3 to 32.0% of the solid material as lignin and complex sugars.

Table 3.14 Carbohydrates concentration of the residual fibre following solvent extraction.

Variety	Carbohydrate (mg/ml) (mean±SE)				
	Glucose	Galactose	Arabinose	Xylose	Mannose
Pronghorn	4.22±0.45b	1.75±0.22	0.09±0.06b	0.66±0.16c	1.27±0.20a
AC Ultima	5.67±0.46a	2.64±0.23b	0.12±0.06b	1.30±0.17a	0.51±0.21c
Bobcat	5.63±0.57a	3.17±0.28a	0.01±0.07	1.39±0.20a	0.97±0.25b
Fridge	4.51±0.50b	2.48±0.24b	0.04±0.06c	0.99±0.18b	0.37±0.22
AC Pathfinder	5.76±0.50a	3.13±0.24a	0.22±0.06a	1.08±0.18b	1.15±0.22b
CDC Falcon	4.72±0.74b	2.74±0.36a	0.38±0.09a	1.03±0.26b	0.52±0.33c
Gazelle	4.29±0.44b	2.39±0.21b	0.04±0.06c	0.71±0.16c	1.61±0.19a
AC Rifle	3.12±0.74	2.06±0.36b	0.06±0.07b	0.84±0.25c	1.48±0.33a

n=8

a,b,c: means followed by the same letter in a column are not significantly different (P>0.05).

Table 3.15 Glucose and galactose concentrations in the residue after solvent extraction with respect to the harvest stages.

Harvest Stage	Carbohydrate (mg/ml) (mean±SE)	
	Glucose	Galactose
H1	3.71±0.39	1.81±0.19
H2	4.96±0.41a	2.75±0.20a
H3	5.01±0.40a	2.80±0.20a
H4	5.28±0.39a	2.81±0.19a

n=16

a: means followed by the same letter in a column are not significantly different (P>0.05).

Carbohydrates such as: glucose, galactose, arabinose, xylose and mannose were measured in the resulting hydrolysate solution as shown in Table 3.14 and Table 3.15. Of

all the sugars measured in the residue, glucose and galactose concentrations were higher than the arabinose, xylose and mannose. Glucose and galactose concentration evaluated at each harvest stage show that these carbohydrate increase as plant matures in all the varieties. All other sugars measured values didn't have a significant difference between the harvest stages.

4. Discussion

Green biomass biorefineries are designed to use processes that will utilize immature forages, immature cereal crops or cultivated perennial grasses for use in bio-chemical production. These biorefining processing facilities are generally conceived from making use of waste streams from the livestock feed industry through fermentation into products such as lactic acid. When evaluating this technology for the Canadian, more specifically central Alberta's industrial sector, the use of agricultural materials is a feasible option. This work was intended to identify the uniqueness of utilizing whole cereal crops as the green biomass as a feedstock for a biorefinery. The first objective of this project was to develop rapid methodologies to evaluate levels of target compounds during growth and to assess the potential to use these methodologies for quality control and growth stage verification using test methodologies evaluating total protein, starch and phenol, sterol content and fatty acid profiling. The secondary objective was to develop a solvent extraction method for further characterization of the biomass which included fractionation of the biomass to identify potential chemicals presented at different growth stages for potential extraction or bioconversion. Plants do change in chemical composition over growth stages, and have been studied as early as 1943. Kohler's (1943) research findings were confirmed that proteins and soluble carbohydrates decrease as the plant enters the flowering stage. The changes in measured chemicals at specific growth stages were examined through this chapter.

The experiments within this work included the greenhouse growth of immature cereal crops including varieties such as: Pronghorn (triticale), AC Ultima (triticale), Bobcat (triticale), Fridge (triticale), AC Pathfinder (wheat), Gazelle (rye), and AC Rifle (rye), which were harvested and evaluated at four stages: 4-6 leaf (H1), flower (H2), milk (H3) and soft dough (H4). Early in the growth and harvest of the material, it was evident that the easiest method of predicting the stage of growth was by visual inspection. Field application of this technique is considered to be appropriate while the crop is standing in the field; however, the stands will most likely be chopped by a forage harvester, destroying distinguishing features such as the number of leaves or yellow flowers or seed head development. When evaluating testing methodologies of the chemical composition of the whole plant at various harvest stages, the evaluations historically have been characterized by the livestock nutritional value and is well known. These tests are lengthy and can be considered more cost prohibitive than the proposed test kits that are readily available via lab suppliers, which are ordinarily used for grain analysis.

In cases where the processing parameters revolve around the maturity of the plant, a plant stage verification step will be necessary. The initial assessments were performed on the whole plant material with easily accessible test kits to evaluate starch, protein and phenolic contents. Once the overall changes were evaluated, it was noted that more analysis would be necessary for identifying any unique characteristics expected to change over the growth stages of the various cereal crops with the selection of fatty acids and sterols. These subsequent tests on the whole plant were conducted with GC-FID for the semi-quantification of selected fatty acids and sterols.

The green biorefinery processes are generally focused on mechanical separation followed by fermentation bioconversions to produce chemicals from green biomass. In this thesis, solvent extraction was explored as an alternative chemical recovery system of the potential production for the plant derived chemicals. The solvent extraction method, using Soxhlet, previously explored by Pavlikova et al. (2005) who evaluated seven step extraction process from non-heated, non-polar to polar solvent system, including petroleum ether, ethyl acetate, butanol, methanol, methanol with water, water, methanol, and water with hydrochloric acid to fully fractionate the chemical composition of providing valuable insight to plant chemical affinity to solvent. To fulfill a biorefinery model, the final testing identified the residue fibre of the solvent extraction as a potential source of cellulose, carbohydrates and lignin for recovery.

There are many ways to examine the extracted samples but since volatile solvents were used for extraction and no water was present, the GC-MS seemed to be an appropriate choice. Chemical ionization was indentified by Starke et al., (2000) to be a very useful for identification of carbohydrates in alfafa and mixed grasses. Sobolevsky et al., (2004) found that using GC-MS in positive ion mode and isobutane as a reagent gas, if the samples were derivatized with isobutyl chloroformate within a chloroform solution, fatty, dicarboxylic and amino acids were identifiable. This approach is useful for more complex mixtures, such as the extracts obtained in this work, and future studies would benefit from this technique. Alternatives explored included the use of an HPLC to recover carboxylic acids from silage extract as it was evaluated by Canale et al. (1984). This method allowed volatile carboxylic acids to be detected and recovered using a aminex HPX-87H column similar to the one used for the structural carbohydrates

analysis. Lipid mixtures were extracted using organic solvents such as chloroform/methanol or 2-propanol/hexane mixtures, which were also analyzed using a HPLC (Emrani, 2005). The HPLC methods seemed useful only when there were known products and therefore were not attempted. Thin layer chromatography was also used to monitor the qualitative composition of samples after GC-MS in order to find the terpenes n-alkanes, n-alkenes, ketones, fatty acids, esters, acids, sterols and vitamins as well as other (Markovic et al., 2008).

Solvents have selectivity and capacity for extracting plant chemicals. The main factor for choosing the pentane, petroleum ether and ethyl acetate, in that sequential order, was based on their polarity and the selectivity, creating distinctive, non overlapping GC-MS chromatogram peaks from solvent to solvent. These solvents were used to extract and identify the plant chemicals as the plant grew over time. An interesting aspect that was presented by Ritchie (2006), showing he effect of pH of the solvents by the identification of auto oxidation effects on plant material where future work may focus on the elucidation effect of pH of 7.49 for pentane, 9.02 for petroleum ether and 3.70 for ethyl acetate and its effect on plant chemical extraction. The Soxhlet extraction technique is a standard technique but alternate potential extraction techniques could utilize binding agents to create larger molecules of the compound of interest, especially in an aqueous solution. Tris-cationic receptors can bind to the anionic carbohydrate in an aqueous solution, which allows for selectivity of different sugars (Schmuck and Schwegmann, 2005). Soxhlet extractions are long and labour intensive but other methods are available such as the Accelerated Solvent Extraction System (Dionex Corp., Sunnyvale, California, USA). Dunford et al. (2009) evaluated using the

Accelerated Solvent Extraction system with solvents such as n-pentane, ethanol, petroleum ether, and chloroform at five different temperatures for phytosterol extraction of wheat straw, bran and germ.

The extractions and chemical analysis created possibilities of immature cereal crops in a biorefinery with respect to growth stage and plant variety. The early growth stage (H1); phenols and protein, C16:0 (myristic acid), C18:3 n-3 (linolenic acid) were highest with very little starch, lignin, carboxylic acid or organic acids. This combination of biochemistry would allow for less complex processing as complex carbohydrates and proteins bonding with structural components have not been formed. Harvesting the plants early also provides an opportunity for agricultural producers to grow multiple crops through the growing season, even in Canada. As the plant matures, the longer chain fatty acids develop and aromatic dicarboxlic acids accumulate, as well as the potential for organic acid and hydrocarbon contamination. Starch accumulates, protein decreases and phenol content is generally decreased. Interestingly, the variety that showed the most sterols at any growth stage was Gazelle (rye), with a total phytosterol content of collectively 20 moles per gram at the soft dough stage. Gazelle (rye) would therefore also be considered as a feedstock to use for the recovery of sterols.

The test kits were not as successful in producing a verifiable check for the stage of growth as anticipated but still maybe useful for a potential cross reference model for future work in a background data for a near infrared (NIR) instrument. Some unexpected results will require more analysis or an examination of the sampling procedure. When assessing the use of each test kit, unexpected trends or unpredicted results, may indicate that the size of the sample was too small or tests that were only designed for the cereal

grain, not the stalk. Starch analysis was according to the Megazyme Total Starch Assay Procedure K-TSTA 05/2008 (Cedarlane Laboratories, Burlington ON) that used amylocosidase to partially hydrolyzed and totally solubilised the starch and the starch dextrins hydrolyzed to D-glucose with α – amylase as a method to examine the free glucose from starch in the samples, but this method can measure lower than alternate hydrolysis methods for the bound, less available, glucose, indicating that the value for percent starch content could be lower than expected. The protein kit proved to show the expected trend and outcome however the kit was not designed for plant protein but was suitable for aqueous samples. In a biorefinery, the material would most likely be mechanically pulped prior to fermentation and the juice sample would be water based, resulting in the belief that this protein kit is suitable. A Kjeldahl method, is generally supported by researchers such as Chapman et al. (2005) with crude protein measurements of triticale and wheat, which proved that at anthesis stage, the protein percentage is 11% and 12% followed by the soft dough stage at 9% and 10%, respectively. Chapman et al. (2005) also recommends that to maintain cereal crops with a higher protein content, the best approach would be to implement good management practices on the field and harvest the plants earlier than mid-dough stage. Phenolics analysis results were generally higher in the initial, 4-6 leaf growth stage with the exception of AC Rifle (rye). Cereal crops also have the potential to be a source of dietary phenolics as determined by Hosseinian and Mazza, (2009) examining triticale to find that the straw and leaves are good sources of proanthocyanidins. The data collected in this work did not present any trends or similarities within the varieties or growth stages that could allow for conclusive statements.

Characteristics of the various cereal varieties were also evaluated with fatty acids and sterols using a semi-quantitative approach. The statistical analysis showed a significant effect of variety and harvest stage. Gazelle (rye) had the highest content with an increase at the flowering stage (H2) followed by a sharp decrease as the plant matures. This is also verified by Nystrom et al. (2007) who found that in rye kernel, the total sterol content was higher. Sitosterols were expected to be the most abundant of the sterols tested since Nystrom et al., (2008) showed that they are about 40-60% of the total sterols in mature rye and wheat grain.

C16:0 (palmitic) decreased and C18:1 n-9 (oleic) increased as the plant matured and remarkable was highest in H3 (milk stage). Linoleic acid, C18:2 remained constant through the growth stages with no differences found between varieties. This finding was supported by Palladino et al. (2009) who discussed that the differences in ryegrass varieties was only found in dry matter and gross energy, neutral detergent fibre and ash content when examining the fatty acid profile changes in 12 cultivars. There was no significant difference found when looking at the 12 varieties at different heading dates (Palladino et al., 2009).

The solid waste from the Soxhlet extraction was examined for lignin and structural carbohydrates using acid hydrolysis procedure. The procedure used was developed to be used with wood based materials that do not contain extractives. It was discussed that the traditional way of measuring lignin was incorrect because in the plant material, the lignin is structurally different and is bound to phenolics and carbohydrate complexes (Buranov and Mazza, 2008). Perhaps the solvent extraction was helpful in removing the phenolics and carbohydrate complex. Linin content was lower at the 3-5

leaf stage than the mature plants, as well higher in the winter varieties, observed to grow for longer periods in the greenhouse. Lignin extraction would create an area of research to determine the most economical process. The concentrated polymeric sugars were measured as per the NREL procedure. The remarkable aspect to the data collected of the solvent washed residue, includes the amount of available sugar after acid hydrolysis suggesting that after the lignin is extracted, then remaining neutralized liquid can be used as fermentation media for ethanol production. Other methods to determine the structural carbohydrates could have been similar to the methods described by Kamm et al. (2006) who tried different chemical and enzymatic methods for hydrolysis from the main stem of a lupin variety. The amount of non-soluble carbohydrates depend on several factors including date of planting, season of harvest, ambient temperatures and plant maturity, according to Chatterton et al. (2006) who studied fructan concentrations and discovered they were highest in plants at the milk and soft dough stages. The highest quantity of carbohydrate in this study was glucose, where wheat and rye were significantly different than spring triticale. Trends in cereal varietal were difficult to comment but concluded that hydrolysis of the fibrous residue can liberate carbohydrates and sugars.

5. Conclusions

The overall project design was developed as a feasibility scan for immature cereal crop utilization for industrial chemical production. Future consideration for commercial production was enhanced with the use of harvest verification tool as part of extraction of potential chemicals at particular growth stages. The following concept biorefining model has been enhanced to include the solvent extraction. The process seemed feasible but much more work is required before implementation.

The feedstock material selection determines the process. When determining a model of the fermentation biorefinery, the mid (H3) to late harvest (H4) stages suggest fewer proteins and phenolics, which could potentially reduce microbial growth inhibition and increased starch will provide more substrate conversion for organic acid production. The plant material at this stage provides a nutritionally rich feed source. Based on all the research of identification and quantification, Gazelle (rye) would be the choice for a biorefinery of extracting sterols. For fermentation into organic acids, the choice would be the soft dough stage of any of the triticale varieties investigated.

5.1. Biorefining model

Green biomass biorefineries use plant material from immature forages, immature cereal crops or cultivated perennial grasses for use in chemical production. This model fractionates of the biomass into high value products reducing or eliminating waste material. The biorefining model presented in Figure 5.1 integrates green forage

preservation, physical separation, biochemical conversions and now, solvent extraction. Alternate chemical production from renewable resources has focused the research community to develop new technologies and renew well known fermentations. This biorefining model fractionate silage and immature grain crops into liquid juices and solid fractions, which could potentially exhibit unique opportunities for biochemical conversion to higher value products and solvent extraction could be added. Forage can be processed as either whole fresh material right off the field, or silage material. The solid press cake can be further processed for crude fibre in animal feed or into technical fibres for products like insulation or used as a feedstock for a biodigester for methane production. The liquid portion can be used as an ideal fermentation substrate to produce organic acids or ethanol. Solvent extraction would be introduced as a treatment of the dried press cake, which also offers lignin and fermentable sugars. Many variations of this model could be employed to produce a green biomass biorefinery.

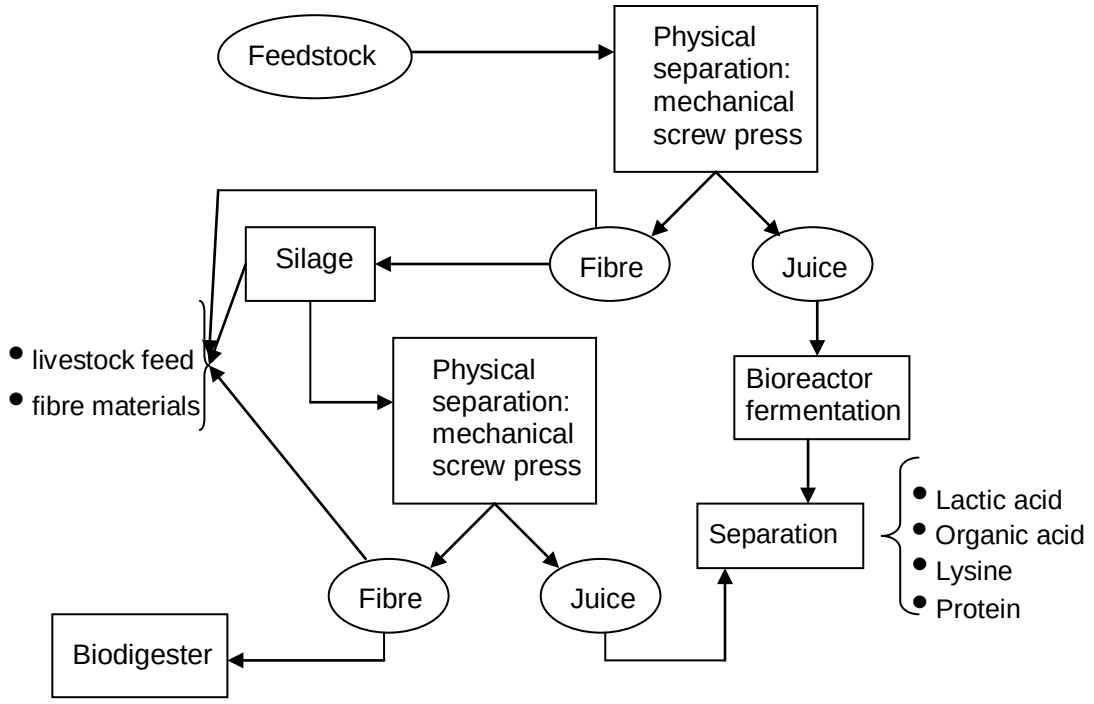

Figure 5.1 Whole cereal green biomass biorefinery concept model.

5.2. Recommendations

This project identified the physical growth and solvent extraction potential for various cereal crop materials. It was determined that plant growth stage at harvest impacts composition and processing characteristics of the green biomass. Further investigations are required to determine, the field optimal growth and harvest conditions to benefit both the producer and chemical producer.

Overall conclusions of the best method of quick determination of the growth stage of a plant was by visual inspection, but this may not always be possible if that material arrives as a chopped forage crop. Therefore based on the data gathered on starch, protein and phenolics, using the test kit used alone is not recommended but data could be used to calibrate a NIR apparatus which would be more suitable.

Recommendation for further research includes optimization of the process. Highly volatile compounds may have been lost in the process of consecutive extractions even though care was taken to reduce the boiling point with vacuum when evaporating the solvents, but some of the volatile components could have been lost at any point during the process. Other processing technologies have been introduced to reduce the amount of volatile compounds lost such as extraction/distillation/evaporation method presented by Xu et al. (2005) where the researchers found that this method was able to reduce the sample size and produce well resolved GC peaks in a shorter time.

Microwave assisted extraction of organic compounds will reduce the time of extraction and reduce the amount of solvent required as opposed to the flow through solvent extraction and direct comparison to soxhlet extraction but requires more energy

input. Microwave heating has been applied to extracting polycyclic aromatic hydrocarbons, polychlorobiphenyls, pesticides, herbicides, phenolics, and soil pollutants (Letellier and Budzinski, 1999).

Future work relating to extraction, purification and quantification has been explored to find jasmonic acid, abscisic acid and indole-3-acetic acid of young maze plants with methanol and ethyl acetate and C18 cartidges (Zhang et al., 2008) which could be a low-cost alternative. Werpy and Petersen (2004) also identified compounds as naturally occurring in plants for chemical production but acknowledge that the technology for isolation of the chemicals requires careful planning.

The logistics, process design and downstream separation for the extraction of a few chemicals are critical for profit. Scale up of the greed biorefinery proposed as a process presents issues with handling of wet, immature green crops and the logistics of moving the material. The process design of a green biomass biorefinery to accommodate fermentation and solvent extraction systems will require a feasibility study. The characteristics of the material flow with respect to the rheological properties of a slurry would present issue to determine the wall slip and settling of particulates in dilute solutions (Knutsen and Libertore, 2009).

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