

EVALUATION OF LEAF PROTEIN CONTENT, EXTRACTION, AND
PURIFICATION IN NAPIERGRASS (*Pennisetum purpureum* SCHUMACH.)

A Thesis

by

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ABSTRACT

As protein deficiency is still a major health issue in the less developed portion of the world, novel sources of protein need to be identified. Grasses in the genus *Pennisetum*, such as napiergrass, can be a potential alternative protein source. The robust growth nature of grass provide abundant biomass, as well as enough protein to supply both livestock and human diets. However, plants are difficult to utilize as protein sources due to both non-digestible fiber fractions and antiquality factors (phytates, tannins, etc.). Therefore, feasible approaches are needed to further estimate and purify the leaf proteins. The objectives of this research are, first, to identify the potential relationship between leaf proteins and chlorophyll content index (CCI) for developing indirect tools for leaf protein quantification; and secondly, to further evaluate methods of leaf protein extraction and purification techniques, including heat coagulation (HC), mild alkali extraction (AL), and mild alkali extraction with the novel utilization of activated carbon (ALC). The percentage of crude protein in napiergrass was found to decrease sequentially at 30, 60, and 90 days of growth in this study. Trends of decreasing crude protein content between napiergrass parent group and both its self-pollinated progeny group and its F₁ hybrid progeny group were also identified. Two spectroscopic methods were used in this experiment, and both have reported low r² values (the highest r² = 0.477) for the correlation between crude protein content of napiergrass and CCI at three harvest dates. Neither heat coagulation nor mild alkali

extraction method improved the extractability of leaf protein. Activated carbon column treatment effectively removed anti-quality factors (tannins) detected for this experiment. However, the utilization of activated carbon also caused lower protein yield in the final purified product.

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NOMENCLATURE

dta	Dry Ton Per Acre
CP	Crude Protein
CCI	Chlorophyll Content Index
CCI-LP	Chlorophyll Content Index (Standard Leaf Press)
CCI-LP	Chlorophyll Content Index (Leaf Homogenate)
LJ	Leaf Juice
HC	Heat Coagulation
AL	Mild Alkali Extraction
ALC	Mild Alkali Extraction (with Activated Carbon Column)
FC	Folin Ciocalteu

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INTRODUCTION

Protein deficiency remains one of the major nutritional problems worldwide, especially in developing nations (Latharn, 1997; FAO et al., 2015). Leaf biomass is an abundant protein source with potential to supply essential amino acids to not only livestock but also human diets. However, plants are difficult to utilize as protein sources for most non-ruminant animals due to both non-digestible fiber fractions and antiquality factors (phytates, tannins, etc.). One strategy to increase utilization of leaf proteins is to isolate and purify high-quality leaf protein concentrate (LPC). While previous attempts to develop LPC for human nutrition have been limited, interest has increased recently in extracting LPC as a value-added coproduct in biorefineries utilizing lignocellulosic feedstocks such as perennial grasses.

Napiergrass (*Pennisetum purpureum* Schumach.), also known as elephant grass, is a high-biomass, perennial grass native to Africa that has been cultivated and well-adopted in tropical areas in Asia, Oceania, and the Americas, as forage, fodder, and silage. Napiergrass has further potential as an integrated biorefinery feedstock, possessing one of the highest protein contents among plants and lignocellulosic composition suitable for both high quality forage and cellulosic ethanol conversion (Carlsson et al., 1984; Urribarrí et al., 2005).

In order to facilitate development of improved LPCs, less expensive methods are needed for: 1) phenotyping feedstocks for leaf protein content, and 2) isolation and purification of food quality leaf protein isolates.

LITERATURE REVIEW

Approximately 815 million to one billion people worldwide, predominantly in developing countries, suffer from malnutrition or protein deficiency (FAO et al., 2015). In 2000 alone, hunger or the result of hunger caused the premature death of around 36 million undernourished people (Gasperini and Maguire, 2002; Ghaly and Alkoaik, 2010). With current average birth rates, it is estimated that the global population will reach 9.8 billion by the year of 2050 (United Nations, 2017). As a result, malnutrition and food security will continue to be a critical concern.

The majority of protein used by the world population is derived from seeds (including cereals, legumes and various dicotyledonous non-legumes), especially in less advanced regions of the world (Eggum and Beames, 1983). In order to solve protein deficiency and related malnutrition problems, novel sources of protein need to be identified. Leaf protein from many grass species are considered to be edible (Lim, 2016) and an underutilized fraction of biomass. LPCs are further abundant and available potentially for human diets (Tenorio et al., 2017). Numerous studies on the isolation of leaf proteins have been conducted, and related technologies have been developed to varied extents (Fiorentini and Galoppini, 1983; Castellanos et al., 1994; Rao et al., 2007). The nutritional value of LPCs has further been found to be comparable to animal protein isolates and superior or similar to seed proteins (Badar and Kulkarni, 2011).

Researchers have evaluated hundreds of species for leaf protein content and quality (Telek, 1983; Mulder, 2010). With superior performing plant materials and suitable extraction techniques, leaf protein yield per hectare can exceed four times that of seed-derived protein (Telek, 1983). LPC has been successfully utilized for animal feed with alfalfa in Europe, and the strategy has more recently begun to be adopted by the United States (Telek, 1983). The protein isolates extracted from alfalfa also have comparable nutritional value as that of a soybean meal (Kuzmicky and Kohler, 1977). In addition to alfalfa, many other tropical legumes have also been reported to produce LPCs with similar amino acid profile to soybean meal (Telek, 1983).

Certain members of genus *Amaranthus* have also been documented to have comparatively high leaf protein content, with crude protein content ranging from 17.92% (dry weight) for *Amaranthus hybridus* (Akubugwo et al., 2007) to 30% crude protein for *A. mantegazzianus* (Telek, 1983). Among plant species with high biomass potential in addition to protein content, perennial grasses have potential as LPC feedstocks yielding the highest protein per acre. Typical tropical grasses such as napiergrass (cv. Mott) have protein content of 10-15% depending on the growth stage of the plant (Urribarrí et al., 2005). While the percentage of protein is lower compared to the previous legumes examples, the higher biomass potential in napiergrass (20+ dta) results in significantly higher protein yield per acre.

Napiergrass

Napiergrass, is a C4 perennial grass native to Africa with high biomass yield, now widely planted in sub-tropical regions worldwide as forage, fodder, and silage (Rong, 2013). Napiergrass is an allopolyploid ($2n=4x=28$), and it has a genome formula of A'A' BB. The A'A' genome is homeologous to the AA genome of pearl millet (*Pennisetum glaucum* (L.) R. Br.), which allows it to be easily crossed with pearl millet and produce a sterile triploid hybrid progeny. Napiergrass facilitates outcrossing, and it sets little self-pollinated seed due to self-incompatibility (Hanna et al., 2004).

Napiergrass is a hardy grass that can grow in clumps up to seven meters in height, and it is further useful for erosion control, mulch, and a windbreak for other crops (Dussadee, 2016). The robust nature of napiergrass gives it the ability to produce more biomass per unit time than most other grass species (Hanna et al., 2004). According to Schreuder et al. (1993), yields of napiergrass vary between 10-40 tons dry matter per hectare depending on cultivar, location, years since planting, soil fertility levels, climate, and other management factors. Many studies have also reported high dry matter yields for napiergrass at different locations (Vicente-Chandler et al., 1959; Skerman and Riveros, 1990), and the crop's biomass production record of 84,800 kg ha⁻¹ DM per year was achieved when high rates of fertilizers (under 2000 mm natural rainfall yr⁻¹ and 897 kg N ha⁻¹ yr⁻¹ fertilizer) were applied (Skerman and Riveros, 1990).

There are two plant ideotypes in napiergrass: dwarf and tall (Singh et al., 2013), and both form dense, bamboo-like clumps (Hanna et al., 2004; Orodho, 2006). Dwarf

napiiergrass has a maximum height of about 1.6 meters, compared to 4 - 7 meters for tall napiiergrass (Hanna and Monson, 1988).

Depending on cultivars and fertilization levels, the protein yield of napiiergrass is believed to reach as high as 5000 kg ha⁻¹ yr⁻¹ under suitable environments (Gore et al., 1974). Intercropping napiiergrass with herbaceous legumes further increases the dry matter yield and crude protein of the forage (Orodho, 2006). Therefore, Napiiergrass can be considered a superior candidate feedstock for LPC production.

Leaf Protein Concentrates

Leaf proteins consist of two major categories: chloroplastic proteins (green leaf proteins) and cytoplasmic proteins (white leaf proteins). Both types of protein occur at approximately the same amounts in leaves, and cytoplasmic proteins are generally considered to have higher nutritional value than green proteins (Urribarri et al., 2005). Previous research on leaf protein extractions have mainly focused on the soluble protein fraction (green leaf proteins such as RuBisCO) of plant biomass, which could be a limiting factor for the further utilization of leaf proteins (Tamayo, 2016). Methods have been developed to increase the extractability of white leaf proteins. By treating leaf biomass with ammonia, a 4.5 fold increase in protein yield has been achieved in dwarf elephant grass (Urribarri et al., 2005).

Mechanical pressing is the most common method of separating leaf protein from cell wall components, in which fresh biomass material is pulped and pressed to produce a protein rich “juice”. The leaf “juice” extract contains proteins, sugars, salts, lipids,

vitamins, and water (Badar and Kulkarni, 2011). LPCs have traditionally been separated by heat coagulation or acidification to pH 4 (Badar and Kulkarni, 2011). These methods have been studied since the 1940s, when concerns over food supply led to attempts to find alternative protein sources for human use (Bals, 2012). To date, however, large-scale utilization of LPCs for human consumption has not been realized. The lack of development in LPCs is most likely due to limited improvement for removal of anti quality factors (tannins, etc).

An alternative method for LPC isolation yielding significantly lower antiquality factors is ultrafiltration, in which the biomass is processed through an extraction column, filter compression, and ultra-filtration drying (Bals, 2011). This method does not require high energy costs and can be performed on dried material; however, reagent costs are high and column fouling from protein aggregation is common. Activated carbon treatment to protein extracts has been reported to effectively remove phenolic contents and improve flavor of the protein product (How and Morr, 1982; Seo and Morr, 1985). Utilizing activated carbon for differential separation of proteins and tannins requires selection of appropriate pH. Rubisco has an isoelectric point approximately 5.5 to 6 (Kung, 1984; Stone, 2014), therefore, conditioning of the activated carbon to a high pH can help ensure the protein does not adsorb to carbon.

LPCs recovered by traditional heat coagulation methods consist of a green protein rich curd (Badar and Kulkarni, 2011), in which proteins are separated from the fiber of leafy materials, and which has the potential to be a high-value replacement for soy. Depending on plant species and extraction methods, the total protein content in

LPCs varies from 40 to 70% (Badar et al., 2011). Significant levels of minerals and vitamins, including β -carotene (pro-vitamin A) and vitamin E, are also recovered in LPCs (Badar et al., 2011). LPCs can therefore be used as protein-vitamin-mineral supplements in animal or human diets, and studies have demonstrated successful LPC incorporation into poultry (Ameenuddin, 1983), fish (Ngugi, 2017), calf (Odwongo and Mugerwa, 1980) and human nutrition (Pirie, 1978). Previous reports have shown that certain napiergrass varieties developed in Nigeria have appreciable amounts of vitamin C (ascorbic acid) and pro-vitamin A (carotene) (Ukpabi, 2015), which render its leaf protein products high in nutritional value.

Anti-quality Factors

Promising data from previous reports have indicated significant potential of LPCs for animal and human consumption (Sinclair, 2009; Ukpabi, 2015); however, current extraction methods are not able to significantly remove antiquality factors in LPCs. These constituents have different mechanisms and metabolic interactions with crucial chemicals that affect nutrient uptake (Makkar, 1993) or cause unfavorable taste in LPCs (Huisman and Tolman, 1992).

Common antinutritional substances found in plants include phytates, saponins, tannins, lectins, protease inhibitors, oligosaccharides and non-starch polysaccharides, glucosinolates, phytoestrogens, alkaloids, antigenic compounds, gossypols, cyanogens, mimosine, cyclopropenoid fatty acids, canavanine, antivitamins, and phorbol esters (Francis et al., 2001).

Oxalates

Oxalates are also antinutritional factors widely found in plants. Although ruminants tend to have more tolerance to oxalates than non-ruminants, large and sudden consumption of oxalates can induce toxicity in grazing animals (Rahman et al., 2013). Cases of oxalate poisoning have been reported for livestock upon feeding with napiergrass, in which calcium deficiency was caused by oxalate intake (Sidhu et al., 1996).

Phytates

Phytates, most commonly found in plant seed (Francis et al., 2001), cause growth reduction when fed with high phytate diets (Wallace et al., 1998). The ability of phytate to form complexes with minerals (such as Cu^{2+} , Zn^{2+} , Co^{2+} , Mn^{2+} , Fe^{2+}) makes these dietary minerals unavailable for uptake (Harland and Oberleas, 1985) and negatively affects feed digestibility (Nwokolo and Bragg, 1977).

Tannins, Alkaloids and Saponins

Tannins, alkaloids and saponins are all known to have antinutritional effects (Francis et al., 2001). Tannins comprises a diverse group of secondary compounds that have various biological properties in animals (Brouard et al., 1988), which in many cases, have been reported to cause growth depression (Davis, 1981). Alkaloids are also unsuitable for animal consumption, since they are known to interfere with nerve function (Peterson, 1982). Saponins in leaf protein products can also impart hemolytic activities on red blood cell (Jones and Elliot, 1969).

Previous research has shown that while napiergrass contains high level of tannins, it has much lower amounts of alkaloids, saponins, cyanogenic glycosides, and oxalates. It is further almost devoid of phytates (Okaraonye and Ikewuchi, 2009). The significant reduction of tannins, therefore, is the primary goal in developing high quality LPCs from napiergrass.

Protein Purification

Protein purification utilizes methods or processes designed with the intention to isolate the desirable protein fraction from the feedstock source. Protein purification techniques have been performed for over 200 years (Healthcare, 2010). The goal of protein purification processes are not only the removal of unwanted materials, but also to concentrate and transfer the target protein (or proteins) to an environment where it remains stable and ready for the intended application.

The development of protein purification is often related to the latest discovery and studies of protein. Filtration, precipitation and crystallization have been important protein separating methods since the early days of protein chemistry (Scopes, 2013), and are mostly involved with protein relative solubility (Healthcare, 2010; Scopes, 2013). Protein precipitation is considered one of the simplest approaches to obtain protein isolates from unwanted contaminants. Protein precepitation with acetone and TCA are described and widely used in studies for quantification or determination of proteins (Stepanchenko, 2011).

Heat Coagulation

Temperature and pH are often the key factors in protein precipitation procedures (O'Connell and Fox, 2000). Thermal precipitation of protein is also referred to as 'heat coagulation', and has been described as a reaction taking place between protein and water with different temperature coefficients, depending on the types of protein, acidity, and salinity condition of the solution (Chick, 1910). 'Heat coagulation' of protein is generally considered an irreversible process (Anson and Mirsky, 1931; Wright and Humphrey, 2002); however, studies have reported the reversibility of coagulated proteins under certain conditions (Anson and Mirsky, 1931). Thermal precipitation or heat coagulation of protein can rarely be performed in biochemical studies due to denaturation of most proteins upon heating (Neurath et al., 1944). Capture and maintenance of protein isolates with their active status is often a strict requirement for protein purification, especially for functional studies and other analyses that require native structure of the protein (Healthcare, 2010).

Some proteins are more difficult to separate from the complex mixture of cellular contents depending on the type of protein. For example, insoluble proteins such as thylakoid proteins (membrane proteins), unstable protein complexes, and proteins with certain post-translational modifications will be more difficult to maintain in an active and stable form after purification (Kaufmann, M. 1997). In some chemical composition analyses, active protein structure may not be as crucial, and harsher purification methods such as strong denaturants, extreme pH and heat, and salt concentration can be utilized.

Chromatography

At the onset of the twentieth century, the term ‘chromatography’ was introduced by Mikhail Tswett (Lederer and Lederer, 1953). Chromatographic separation has proven to be a very efficient technique and utilized in numerous common protein purification methods (Janson, 2012). These include affinity chromatography, immobilized metal ion affinity chromatography, ion exchange chromatography, size-exclusion chromatography (gel filtration), hydrophobic interaction chromatography, reversed phase chromatography and chromatofocusing (Healthcare, 2010).

The advantage of chromatography is that it can be used to separate the interference of components in a complex-mixture without knowing in advance the nature and quantities of the chemicals presented in the solution (Rocklin and Johnson, 1983). Physical or biochemical properties of proteins are used in different chromatographic purification methods. Differences in size, positive and negative charges, hydrophobicity and other biospecific binding properties are used to purify desirable proteins from other proteins and non-protein contaminants (Karlström and Hober 2006).

Soluble protein extracts can further be concentrated by using ultrafiltration. Removal of anti-quality factors, including phytate and oligosaccharide content, using ultrafiltration have been reported (Omosaiye et al., 1978; Omosaiye and Cheryan, 1979). Centrifugal ultrafiltration has been widely used in laboratory-scale protein purification due to its low energy requirement, high flexibility on the selection of membranes, low contamination potential, and ease of operation(Xu et al., 2005).

Leaf Chlorophyll Content

The measurement of leaf chlorophyll concentration has been utilized in many aspects of crop production, as well as research activities including crop breeding, pest control, irrigation management, environmental stress evaluation, and nutrient analysis (Wood et al., 1992; Rodriguez and Miller, 2000; Yuan et al., 2016; Ravier, 2017).

Therefore, the quantification of leaf chlorophyll concentration has potential to be studied for its association with leaf protein content and used as a phenotyping tool. Leaf chlorophyll content can be most accurately determined by in vitro measurements of leaf extracts using a spectrophotometer. The Kjeldahl method and Dumas method for determining total nitrogen content are also used routinely by analytical laboratories to estimate protein content (Jung et al., 2003). However, faster, cheaper and non-destructive optical techniques would be preferred and are becoming more popular in determining relative indication of leaf chlorophyll concentration (Parry et al., 2014).

Leaf Nitrogen and Proteins

Chlorophyll content meters are also widely used for monitoring the nitrogen (N) levels of crops in agricultural systems (Xiong et al., 2015). According to previous studies, approximately 80% of leaf nitrogen can be found inside chloroplasts, and around half of the total leaf nitrogen is involved in photosynthetic activities by forming chlorophyll-protein complexes or other enzymatic associations (Xiong et al., 2015; Le Roux, 1999). The chloroplast is also known to contain at least 1000 different types of protein (with a concentration as high as 300 mg mL⁻¹) which are associated with

photosynthesis or other critical functions (Robinson et al., 2001). Nitrogen involved in photosynthesis can be divided into two groups: soluble proteins and thylakoid proteins (Evans, 1989). Soluble proteins are mostly Rubisco, along with a smaller fraction of other chloroplast enzymes, carbonic anhydrase, ribosomal proteins and photorespiratory enzymes in the mitochondria and peroxisomes (Evans, 1989).

Rubisco

Ribulose-1,5-bisphosphate carboxylase/ oxygenase, or Rubisco, is an important green leaf protein due to its relationship with photosynthetic productivity in plants. Being essential for atmospheric carbon fixation, Rubisco is the most common protein in the world and constitutes the largest fraction of leaf proteins (Bals, 2012). Approximately one quarter of total leaf nitrogen is contributed by RuBisCO, while other associated photosynthetic enzymes account for another 25% (Chapin et al., 2002). Rubisco consists of comparatively higher amounts of lysine, which is an amino acid limited in non-ruminant diets (Bals, 2012).

Thylakoid proteins

Thylakoid proteins are considered integral membrane proteins, which carry out important light-dependent reactions of photosynthesis on the Thylakoid membrane. These proteins include four major multisubunit membrane proteins: pigment-protein complexes Photosystem I (PSI) and Photosystem II (PSII) (Evans, 1989), Cytochrome b6f complex (cyt b6f) and ATP synthase (FOF1) (Kurisu et al., 2003; Junge and Nelson, 2015).

Chlorophyll Content Meter

Hand-held chlorophyll meters are convenient and can provide rapid results for diagnostic purposes. Widely used chlorophyll content meters for non-destructive, in situ measurement include the Apogee model CCM-200 and Minolta model SPAD-502, which use chlorophyll content index (CCI) and soil-plant analyses development (SPAD) value as their display output unit, respectively. CCI and SPAD values are both relative indications of leaf chlorophyll concentration, which are calculated by the ratio of the optical density of two different wavelengths (Minolta, 1989 ; Parry et al., 2014). Numerous studies in attempt to finding the relationship between readings obtained from chlorophyll content meter and crop nitrogen status have been conducted for various crops, such as common bean (Silveira and Gonzaga, 2017), apple trees (Nielsen et al., 1995), rice (Yuan et al., 2016), corn (Schepers et al., 1992), wheat (Ravier, 2017), and cotton (Wood et al., 1992).

Among most of these studies, close correlations between chlorophyll meter readings and crop nitrogen status were identified. Since nitrogen is one key component of amino acids (Morot-Gaudry, 2001), the relationship between chlorophyll concentration and nitrogen has indicated the potential of using a chlorophyll content meter as a sampling tool for quantification of leaf protein levels. However, there are certain limitation of the utilization of a chlorophyll content meter in terms of the variation of the measurements.

The unevenly distributed chlorophyll content within and between leaves, as well as the light-scattering properties of plant cells, may affect the accuracy of chlorophyll

meters to estimate the actual chlorophyll concentration in leaves (Monje and Bugbee, 1992). Other studies have also reported the factors that can influence chlorophyll meter readings, including genotype, leaf thickness, growth stage, and irradiance (Blackmer and Schepers, 1995; Samborski et al., 2009; Yuan et al., 2016). Therefore, novel adjustments or improvements on the experimental methods will be helpful to increase the reliability of the research data.

OBJECTIVES

The objectives of this project are to:

1) Compare indirect selection tools and methods using a chlorophyll content meter for quantification of leaf proteins, thus to explore and identify the potential relationship between leaf proteins and chlorophyll content index (CCI).

2) Experiment and evaluate methods of leaf protein isolation, including heat coagulation and mild alkali extraction; evaluate and compare leaf protein purification techniques involving the utilization of micro-membrane filtration, ultrafiltration and activated carbon column.

MATERIALS AND METHODS

Experiment 1: Indirect Quantification of Leaf Protein Contents

Plant Material

A total of 109 individual napiergrass (*Pennisetum purpureum*) genotypes were utilized. Plant material used in this experiment was selected randomly and harvested from previously established napiergrass plots located in Texas A&M University research farm in Snook, TX (30°32'N, 96°26'W; elevation 81m). The soil type is Weswood silty clay loam (pH 8.0). Ammonium sulfate (100 lbs N ha⁻¹) was applied to selected plots as a single application in late March 2017. These were randomly selected from eight napiergrass family groups, including one parental group, three S₁ inbred progeny groups, three S₂ inbred progeny groups, and one F₁ hybrid progeny group as described below.

The parental group consisted of four elite accessions:

- 1) Napiergrass cultivar Merkeron,
- 2) Napiergrass accession PEPU 09TX01,
- 3) Napiergrass accession PEPU 09FL02,
- 4) Napiergrass accession PEPU 09FL03.

The S₁ inbred progeny (45 total) included 15 S₁ progeny each derived from parental accessions:

- 1) Napiergrass cultivar Merkeron,
- 2) Napiergrass accession PEPU 09FL02,

3) Napiergrass accession PEPU 09FL03.

The S₂ inbred progeny (45 total) included 15 S₂ progeny each derived from parental accessions:

- 1) Napiergrass cultivar Merkeron,
- 2) Napiergrass accession PEPU 09FL02,
- 3) Napiergrass accession PEPU 09FL03.

The hybrid group included fifteen F₁ napiergrass hybrids from elite x elite crosses.

Phenotyping Methods

Harvesting and Estimation of Protein Content

Plant samples were collected from each individual genotype at 3 dates of April 28, June 2, and June 30 2017, which approximately represented 30, 60, and 90 days of growth, respectively. Samples were collected by cutting one or two culms from plants (top 2-3 ft portion of the plant, with stem and leaves included). Harvested samples were dried in a forced air oven at 55° C (for 1 week) immediately after harvesting and subsequently ground to a 1 mm screen size using a Wiley mill.

Two spectroscopic methods were used to estimate total leaf protein content: 1) a leaf press standard method, and 2) a leaf homogenate method. For the leaf homogenate method, a smaller sub-sample from each genotype was collected and stored (-20 °C) until analyzed.

Leaf Press Standard Method

The leaf press (LP) method utilized non-destructive, field assays of plants using an Apogee CCM-200 Plus leaf spectrometer. Two readings were taken on the approximately same area of one single leaf on each plant, and averaged. Three replicated measurements were taken on the same plants at 3 dates including 30, 60 and 90 days of growth, which represent different stages of maturity within one growing season. The measurements were completed during the harvesting of samples.

Leaf Homogenate Method

The leaf homogenate (LH) method involved destructive sampling of a subset of tillers from field plants, followed by leaf spectrometer assays of leaf extract fractions. The leaf extract fractions were obtained by placing subsamples in cold storage (-20° C) immediately upon collection and then dried in a forced air oven at 55° C for 48 hours. Samples were ground for 60 s utilizing a coffee grinder, with 80 mg of each sample placed into a 1.5 mL micro centrifuge tube. 1000 uL methanol was added before vortexing for 30 seconds. After incubating at room temperature for 24 hours, samples were centrifuged and the extract supernatant collected. A volume of 50 uL supernatant from each sample was then pipetted onto standard #1 Whatman filter paper (1 inch x 1 inch). When the initial samples placed onto filter paper had dried, another 70 uL of supernatant was again pipetted onto each sample on the same filter paper in order to increase pigment concentration. Samples were again allowed to dry, with readings then taken using the leaf chlorometer (Apogee CCM-200 Plus).

Total Crude Protein Determination

As a control treatment, total leaf protein determination for all samples was completed by a service laboratory using a high temperature combustion Dumas process (Leco CHN-600 Determinator) (Sheldrick, 1986; Sweeney, 1989).

Data Analysis and Statistics

Data for crude protein (CP) and the two methods of chlorophyll content index (CCI) readings (CCI-LP and CCI-LH) were analyzed by repeated measure analysis of variance (ANOVA) for 109 plants. A multivariate analysis of variance was also conducted for CP, CCI-LP and CCI-LH with eight napiergrass families as treatment groups, and multiple means were compared using All Pair, Turkey HSD with JMP software (JMP Pro13, Statistical Analysis System, USA). Differences were considered significant at $P \leq 0.05$

Experiment 2: Evaluation of leaf protein isolation methods

Plant Material

Napiergrass cultivar Merkeron was used for protein purification experiments. Previously established Merkeron plants in field were clipped to 3-4 inch long (with one node), and propagated under greenhouse conditions in 75 L pots. A peat-based potting substrate (Sun Gro Professional Growing Mix) was used. Plants were cut back on August 5, 2017 and fertigated weekly with 400 ppm nitrogen fertilizer (Miracle-Gro All Purpose Plant Food) to substrate saturation. Samples are harvested on September 20,

2017 and dried in a forced-air oven at 55° C for 48 hours. Four replications of each treatment were completed.

Experimental Methods

Leaf Juice (LJ)

Oven dried samples were initially ground in a Wiley mill to a screen size of 1 mm and further through a Udy cyclone mill to obtain micronized powder. For each experiential unit, a total of 0.833 grams of ground biomass was mixed with 12.5 mL ddH₂O (1:15 tissue:water) and vortexed for 5 minutes. Samples were then centrifuged for 10 minutes at 2500 g at 5° C. The supernatant was then collected and filtered through standardized #1 Whatman filter paper with mild hand pressing to obtain the leaf juice (LJ). The remaining pulp tissue was collected, dried, and sent to a service lab for determination of the remaining protein content and fiber analysis.

Heat Coagulation (HC)

Heat coagulation followed protocols as previously described (Telek, 1983). Dried tissue (0.833 g) was mixed with distilled water (12.5 mL), and vortexed for 5 minutes. The extract was incubated in 90 °C water for 10 minutes to coagulate the proteins. The extract was then adjusted to the pH 10 by adding NaOH to resuspend the proteins and centrifuged for 10 minutes at 2500 g. The supernatant was collected and filtered through standardized #1 Whatman filter paper to obtain the heat coagulation (HC) extract.

Mild Alkali Extraction (AL)

Dried tissue (0.833 g) was mixed with distilled water (12.5 mL) and vortexed for 5 minutes. The pH of extract was then adjusted to 10 by adding 5 N NaOH. The extract was incubated in 60 °C water for 30 minutes to extract proteins. After centrifuging for 10 minutes at 2500 g, the supernatant was collected and filtered through standardized #1 Whatman filter paper.

Mild Alkali Extraction with Activated Carbon Column (ALC)

Additional samples were initially processed as in the AL treatment. An activated carbon adsorption treatment followed, utilizing coconut shell granular activated carbon (Prominent Systems 'PSC1240'). Carbon granules were mixed in a 1:10 ratio with ddH₂O, boiled for 1 hr, and rinsed with ddH₂O. The granules were then mixed in a 1:10 ratio with 0.1 M NaOH and stirred for 1 hr at room temperature. The pH was adjusted to 10, and the granules were screened (80 mesh) before adding to the 50 mL volume in a 60 mL disposable syringe. The carbon-syringe columns were allowed to drain by gravity for 1 hr before use. Samples were then loaded onto the columns, allowed to flow via gravity for 10 min, and then purged by plunging syringe insert.

The LJ, HC, AL, and ALC treatments were compared for leaf protein yield and antiquality factor removal. Membrane-based ultrafiltration methods utilized Vivaspin-6 centrifuge column spin kits with 10 Kd exclusion pore size following manufacturer guidelines.

Four replications of mild alkali extract were made for membrane ultrafiltration (AL), which mild alkali extracts were processed through a 0.45 µm micro filtration PES

membrane (TISCH SCIENTIFIC) and followed by ultrafiltration. The four ALC replications were processed through activated carbon column, micro filtration membrane and ultrafiltration. Hence, along with four replications of each LJ and HC extract, a total of sixteen samples were subjected to total protein and tannins assays.

Protein Assay

A commercially available protein assay kit (Thermo Scientific™ Pierce™ BCA Protein Assay) was used for the colorimetric detection and quantification of total soluble protein in the sample extracts prepared by the above four methods (LJ, HC, AL and ALC). A series of diluted Albumin (BSA) standards and BCA working reagent were prepared by following manual instructions. A volume of 1.6 mL of working reagent and 0.1 mL of each standard and leaf extract samples were mixed well in separate, labeled microcentrifuge tubes. The tubes were then incubated at 37°C for 30 minutes. Sample solutions were diluted with water (1:1 sample:water) due to the high concentration detected in the original samples. Measurement of absorbance (at 562 nm) for all samples (including standards) were taken by using a BLUE-Wave Miniature Spectrometer (StellaNet Inc.). A calibration curve was constructed by plotting the blank-corrected measurements of each BSA standard versus its concentration ($\mu\text{g/mL}$). The calibration curve was further utilized to determine the protein concentration of all 16 experimental samples.

Tannin Assay

A colorimetric method that utilizes Folin Ciocalteu (FC) reagent was used for tannin assay. Folin Ciocalteu (FC) method is widely used as standardized method for

determination of total phenolic content in research activities (John et al., 2014; Ainsworth and Gillespie, 2007; Makkar et al., 1993). Standard Gallic acid (GA) dilutions were prepared for five concentrations (100, 200, 300, 400 and 500 µg/mL). A volume of 0.2 mL of each standard and samples were mixed with 1.8 mL water in different 15 mL centrifuge tubes. A volume of 0.2 mL of Folin Ciocalteu (FC) reagent was then pipetted into each tube, which was followed by 30 seconds of vortexing to mix the solution thoroughly. A total of 2 mL of Na₂CO₃ was added to each tube. All tubes were incubated in room temperature for 2 hours with lids closed. A calibration curve (for GA standard) was constructed and used to determinate total phenolic content in all 16 samples as the equivalence of tannin levels.

Data Analysis and Statistics

As a baseline for the initial Merkeron biomass, four replicated samples used for purification treatments (LJ, HC, AL and ALC) were sent to a service lab for determination of total crude protein and fiber quality testing. Purification methods were compared based on protein yields and total phenolic content assay as an equivalence of tannin level. Data collected was subjected to analysis of variance and, where appropriate, multiple means separated using All Pair, Turkey HSD with JMP software (JMP Pro13, Statistical Analysis System, USA). Differences will be considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Indirect Quantification of Leaf Protein Contents

Statistical Analysis

The percentage of crude protein (dry weight basis) (Protein%), CCI from the standard leaf press method (CCI-LP), and CCI from the leaf homogenate method (CCI-LH) for the 109 individual napiergrass genotypes all varied between the three harvest times (30, 60 and 90 days of growth) (Table 1). Crude protein, CCI-LP and CCI-LH further showed different patterns of changes over the three dates (Figure 1). Crude protein percentage was highest at 30 days of growth (11.8%), and decreased sequentially at both 60 and 90 days (8.4% and 5.9%, respectively). CCI-LP reading was highest at 60 days of growth and was approximately equivalent at 30 and 90 days. For CCI-LH reading, the value was highest at 30 days of growth, and decreased to equivalent levels at 60 and 90 days.

Similar patterns of decreasing protein content over different growing stages of napiergrass were also reported by other studies (Vicente-Chandler et al., 1959; Clavero and Ferrer, 1995). Vicente-Chandler et al. (1959) reported that under 800 lbs N ac-1 yr-1, napiergrass yielded 12.9, 9.7, and 6.9 percent crude protein when they were harvested at 40, 60, and 90 days intervals, respectively, which is close to the percent crude protein values obtained in this study. Higher percent crude protein can be achieved possibly by applying higher rate of nitrogen fertilizer (Vicente-Chandler et al., 1959). Rengsirikul et

al. (2011) also reported that total nitrogen content of napiergrass decreased from 2.0 to 1.2% as harvesting interval increased from 1 to 12 months. However, different pattern was identified by Kozloski et al. (2005), where the total nitrogen content of napiergrass declined from 30 to 70 days, but increased again at 90 days of regrowth.

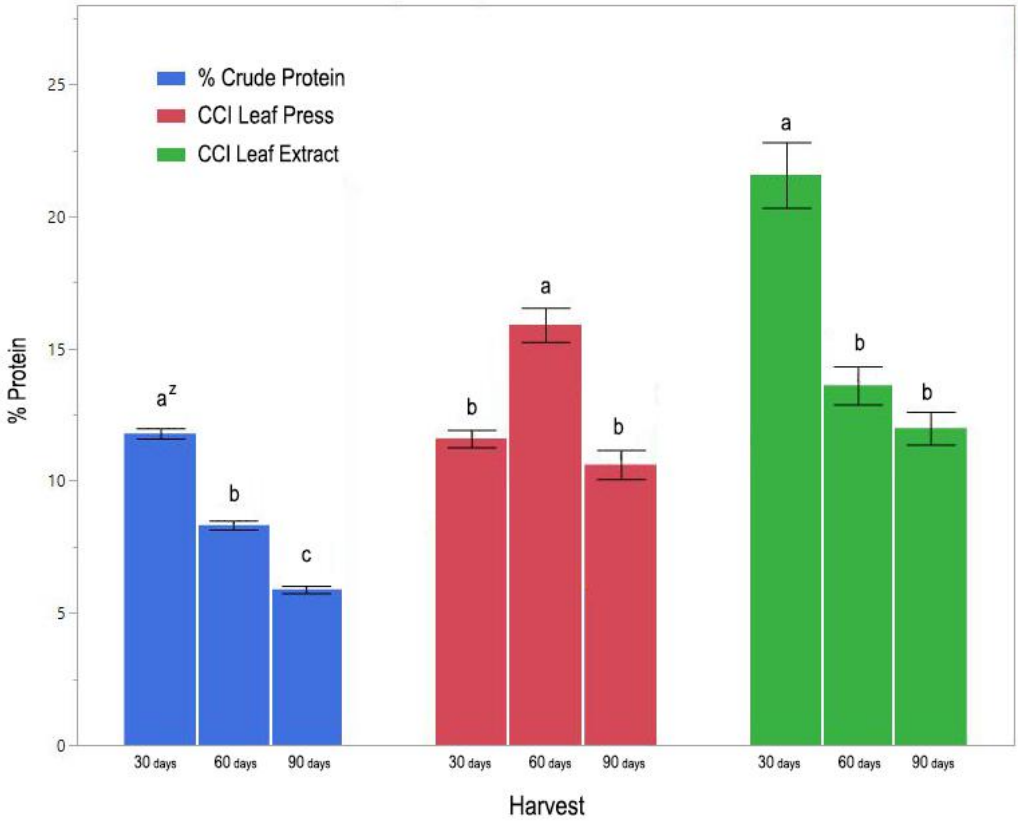
Table 1. One factor repeated measures analysis of variance (ANOVA) of percentage of crude protein (CP) (dry weight basis), chlorophyll meter reading from standard leaf press method (CCI-LP), and chlorophyll meter reading from leaf homogenate method (CCI-LH) of 109 individual napiergrass genotypes over three harvest dates.

	CP	CCI-LP	CCI-LH
	%	Chlorophyll Meter Readings	
Time ^Y	*** ^Z	***	***

^Z NS (nonsignificant) or significant at $P \leq 0.05$ (*), 0.01 (**), or 0.001 (***).

^Y Harvest time: April 28th, June 2th and June 30th in 2017.

Figure 1. Means of percentage of crude protein (protein%) (on dry weight basis), chlorophyll meter reading from standard leaf press method (CCI-LP) and chlorophyll meter reading from leaf homogenate method (CCI-LH) of 109 individual Napiergrass genotypes over three harvest dates (30, 60 and 90 days of growth).



^z Means within the same color bars marked by the same letter are not significantly different according to All Pairs, Turkey HSD.

Comparison of Family Groups

Harvest time and family group effects on crude protein percentage and chlorophyll meter readings (for both CCI-LP and CCI-LH) were also identified using repeated measures ANOVA (Table 2). Interactions between harvest time and family groups were also observed for crude protein, CCI-LP and CCI-LH.

Least square means of crude protein content varied among eight napiergrass family groups, with S1FL02 as the highest and S2MKN the lowest (Table 3). The crude protein percentage of the parent group was not different from most of the progeny groups, with the exception of S2MKN. However, a trend of decreasing crude protein content over selfing generations was observed. All S₁ inbred groups had higher crude protein content than their respective S₂ inbred progeny groups. Similar trends were observed in both standard leaf press and leaf homogenate methods of chlorophyll meter readings. Parent group were the highest for both CCI-LP and CCI-LH. For CCI-LP, S1FL02 and S1FL03 were higher than S2FL02 and S2FL03, respectively. For CCI-LH, S1MKN was higher than S2MKN. S2MKN remained as the lowest for both CCI-LP and CCI-LH, which is consistent the crude protein data.

The F₁ Napiergrass hybrid group was lower than the parent group for both CCI-LP and CCI-LH, and also relatively low in percent crude protein. This may be due to the hybridization between parent Merkeron and PEPU 09TX01. Although parent Merkeron line has higher protein content, parent PEPU 09TX01 (a Texas line that was selected for higher heat and drought tolerance) might have lower protein content, thus this resulted in

the overall lower protein content among randomly selected individuals in the F₁ Napiergrass hybrid group.

Table 2. Multivariate analysis of variance (MANOVA) for repeated measures of percentage of crude protein (Protein%) (on dry weight basis), chlorophyll meter reading from standard leaf press method (CCI-LP) and chlorophyll meter reading from leaf homogenate method (CCI-LH) on eight family groups of 109 Napiergrass genotypes defined as treatments.

	CP	CCI-LP	CCI-LH
	%	Chlorophyll Meter Readings	
Time ^Y	*** ^Z	***	***
Fam ^X	***	***	***
Time*Fam	***	***	***

^Z NS (nonsignificant) or significant at $P \leq 0.05$ (*), 0.01 (**), or 0.001 (***)

^Y Harvest time: April 28th, June 2th and June 30th in 2017

^X Eight Napiergrass family groups (Fam), including one parental group; three S₁ inbred progeny groups: Merkeron, PEPU 09FL02 and PEPU 09FL03; three S₂ inbred progeny groups: Merkeron, PEPU 09FL02 and PEPU 09FL03; one F₁ Napiergrass hybrids group from elite x elite hybridizations

Table 3. Percentage of crude protein (CP) (on dry weight basis), chlorophyll meter reading from standard leaf press method (CCI-LP) and chlorophyll meter reading from leaf homogenate method (CCI-LH) of on eight family groups of 109 Napiergrass genotypes defined as treatment.

Trt Group ^x		Means		
		CP %	CCI-LP Chlorophyll Meter Readings	CCI-LH Chlorophyll Meter Readings
S ₁ Inbred	PARENT	9.76 abc ^Z	18.35 a	23.02 ab
	S1MKN	9.03 bc	13.01 abc	22.03 a
	S1FL02	10.43 a	16.85 a	19.18 ab
	S1FL03	9.20 ab	14.56 ab	15.28 bcd
S ₂ Inbred	S2MKN	7.67 d	9.98 c	11.10 d
	S2FL02	8.05 bcd	11.56 bc	16.45 bc
	S2FL03	7.75 cd	10.37 c	12.96 cd
F ₁ Hybrid	F1HY	8.41 bcd	11.13 bc	11.82 cd

^Z Means within a column under each main factor followed by the same letter are not significantly different according to All Pairs, Turkey HSD.

^x Eight Napiergrass family groups, including one parental group; three S₁ inbred progeny groups: Merkeron, PEPU 09FL02 and PEPU 09FL03; three S₂ inbred progeny groups: Merkeron, PEPU 09FL02 and PEPU 09FL03; one F₁ Napiergrass hybrid group from elite x elite hybridizations

Regression Analysis

Correlation equations were obtained by fitting a linear model for 'crude protein versus CCI-LP' and 'crude protein versus CCI-LH' at each harvest time. Both spectroscopic methods (CCI-LP and CCI-LH) failed to provide a good prediction of the crude protein percentage (Figures). All r^2 values were less than 0.5; however, the P-values were significant for all 6 models. For CCI-LP, there was almost no correlation between CCI reading and crude protein at 30 days of growth ($r^2 = 0.095$). Comparatively, a five-fold increase in r^2 was observed between 30 and 60 days of growth. For CCI-LH, increases in r^2 were also found between 30 and 90 days of growth. This may be due to the early developmental stages of leaves at 30 days of growth, with subsequent changes in photochemical activities upon maturation (Croxdale and Omasa, 1990).

The regression analysis indicated a poor correlation between crude protein and plant chlorophyll content. This is different from the results reported by Kupke (1962), where a strong correlation was identified between the development of a targeted protein and that of chlorophyll in plant seedling. One possible reason of this difference may be that the previous study focused only one certain soluble protein, whereas the percent crude protein in this study was estimated from total nitrogen content (using Dumas method). The estimation of protein content may be less accurate than the previous study because total nitrogen content comprised by both protein and other non-protein compounds (such as nitrate and/or ammonium ions).

Low r^2 value for both spectroscopic methods may also be caused by measuring and sampling methods, since chlorophyll meter readings were only taken twice at approximately the same location on one leaf, while protein content samples were collected and ground with several stalks. Other studies have also reported the poor correlation between chlorophyll meter readings and leaf nitrogen level for field experiments, with lower r^2 value than experiments performed under greenhouse conditions (Rodriguez and Miller, 2000). This may be due to differences in uniformity of crop development between field and greenhouse, or other unaccounted variability in field experiments. Therefore, for future research, improved measuring and sampling methods should be conducted, and greenhouse experiments should be strongly considered in order to further explore the usefulness of chlorophyll meter as an indirect tool for leaf protein quantification.

Figure 2. Relationship between chlorophyll meter reading (CCI-LP) and percentage of crude protein (protein%) for 30 days, 60 days, and 90 days of growth.

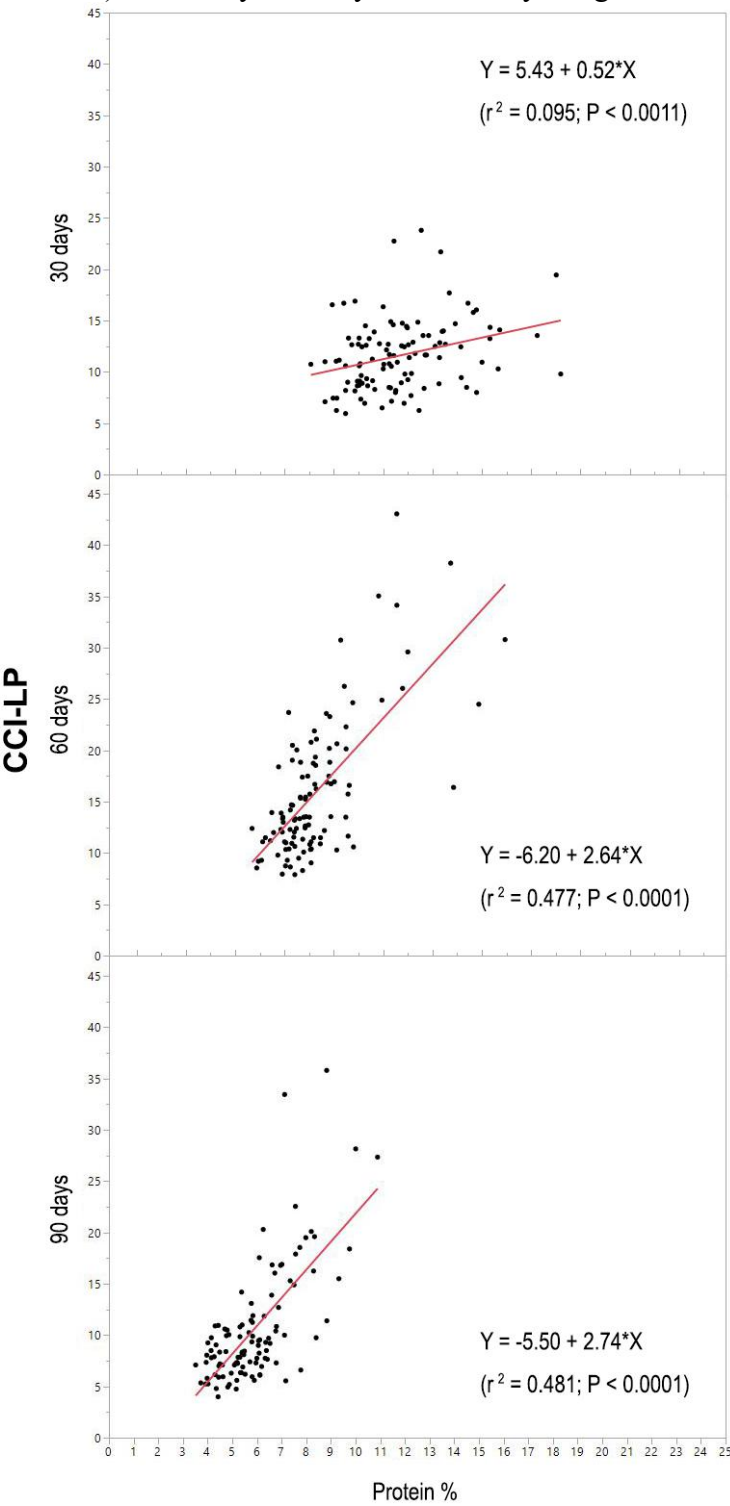
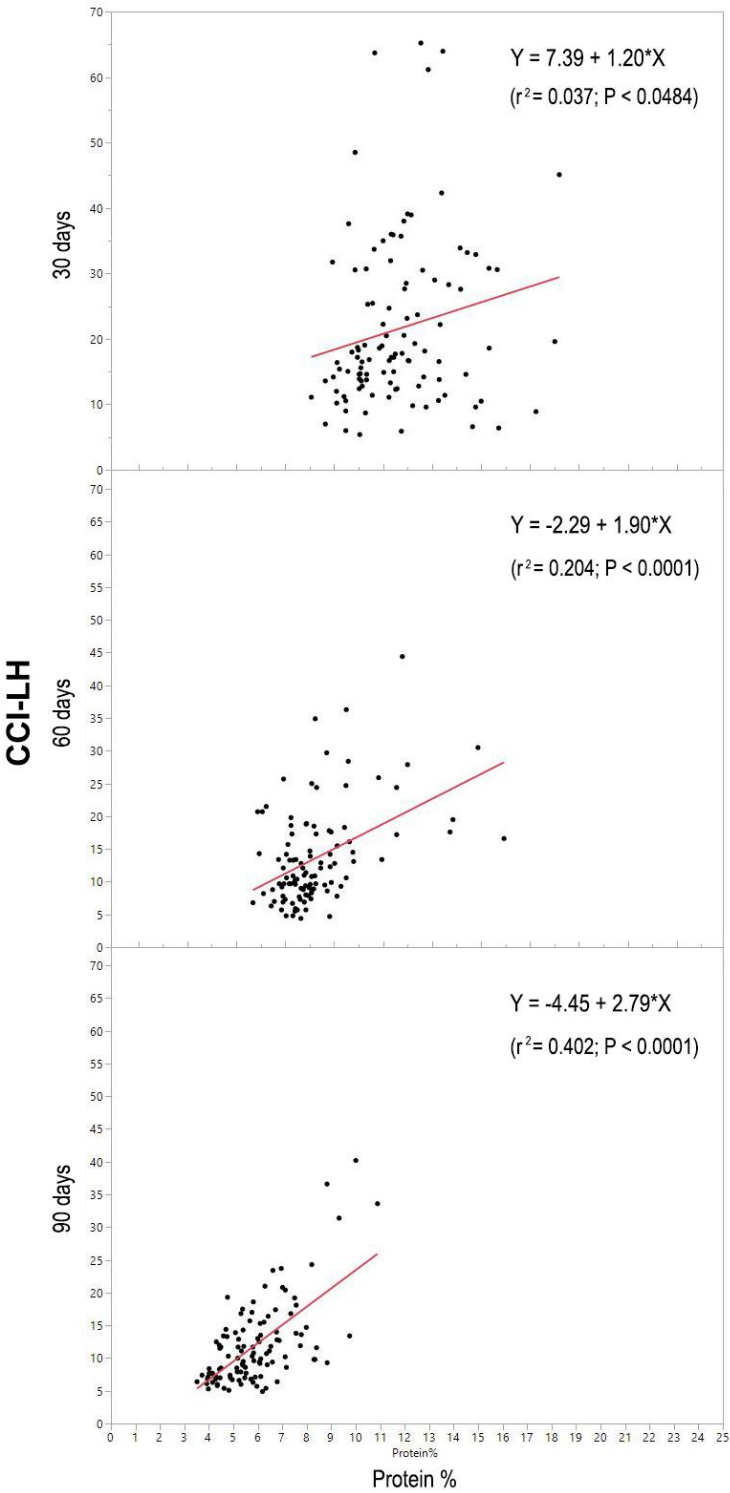


Figure 3. Relationship between chlorophyll meter reading (CCI-LH) and percentage of crude protein (protein%) for 30 days, 60 days, and 90 days of growth.



Evaluation of Leaf Protein Isolation Methods

Protein Assay

A calibration curve for total protein determination was computed from the absorbance values of a series of Albumin Standard solutions (Figure 4). Based on the regression equation ($Y = 0.1167014 + 0.0010733 * X$; $r^2 = 0.986$), the amount of total protein recovered from each sample was obtained. Compared to the total crude protein content of the initial Merkeron sample, a range of 0.086 to 25.58 percent protein were recovered among 4 treatments. There was no difference in protein content for LJ, HC and AL treatments (Table 4). This indicated the heat coagulation and alkali treatments did not provide enough effect to extract more protein than the original leaf juice.

The percent protein recovery of LJ, HC, and AL treatments were within the same range of recovery (22-37%) reported by Gore et al. (1974). However, significantly lower amount of protein was recovered in the ALC treatment. This suggested that the activated carbon column method used may not be adjusted for optimal condition for protein purification. A large amount of protein was lost along with other contaminants due to adherence to the activated carbon. Longer conditioning exposure time of the activated carbon to pH 10 should improve this issue in future experiments.

Figure 4. Diluted Albumin (BSA) standard curve

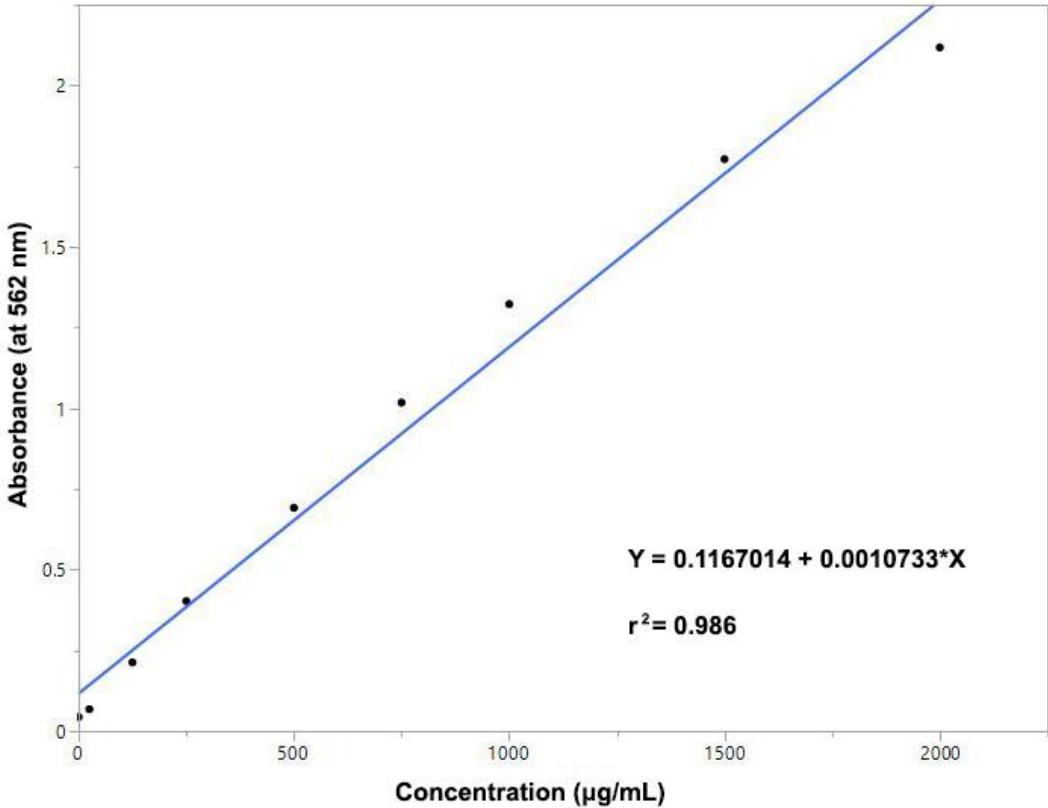


Table 4. Total protein yield (g kg^{-1}) of leaf Juice (LJ), leaf coagulation method (HC), mild alkali extraction method (AL), and mild alkali extraction method (ALC) with activated carbon column.

Treatment	Protein	
	***Z	
	Means	Recovery
	g kg^{-1}	%
LJ	31.9 a ^Y	23.16
HC	30.92 a	22.45
AL	35.23 a	25.58
ALC	0.86 b	0.62

^Z NS (nonsignificant) or significant at $P \leq 0.05$ (*), 0.01 (**), or 0.001 (***).

^Y Means within a column under each main factor followed by the same letter are not significantly different according to All Pairs, Turkey HSD.

Tannins Assay

A calibration curve for total phenolic content determination was computed from the absorbance values of a series of Gallic acid (GA) standard solutions (Figure 5). Based on the regression equation ($Y = -0.029631 + 0.0042184 * X$; $r^2 = 0.99$), the amount of tannins in each sample was determined. Similar results were obtained in the tannins assay across treatments compared to the protein assay. There was no difference in tannins content for LJ, HC and AL treatments (Table 5), and significant reduction of tannins was observed for ALC method. This indicated that the activated carbon column was highly effective in removing tannins and possibly other antiquality factors in the leaf extract. The total phenolic content determined (as tannic acid equivalent) for four treatments is less than the 9.7 g kg^{-1} of total phenolics reported by Dongmeza et al. (2009) in napiergrass. This may be due to the relative young age (around 60 days of regrowth) of plant tissue collected, which may have not yet accumulated higher level of tannins.

Approximately 90% of tannins were removed in the ALC treatment compared to the AL treatment, which is also higher than the 82% removal (of total phenolic content) using activated carbon treatment described by Seo (1985). However, the reduction of protein content due to activated carbon in this study was higher than 90%. Therefore, in order to further improve the efficiency of protein purification, more rigorous confirmation of activated carbon column pH should be attempted.

Figure 5. Gallic acid (GA) standard curve

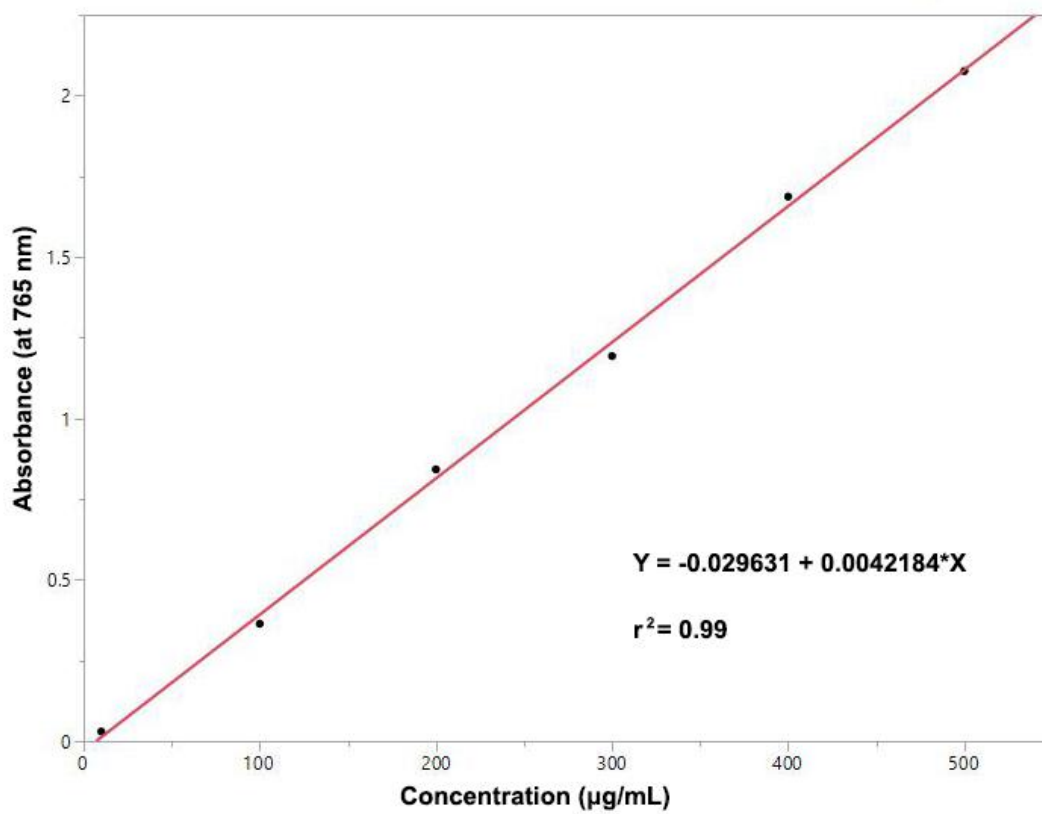


Table 5. Amounts of tannins (g kg^{-1}) of leaf Juice (LJ), leaf coagulation method (HC), mild alkali extraction method (AL), and mild alkali extraction method (ALC) with activated carbon column.

	Tannins
Treatment	***Z
	Means
	g kg^{-1}
LJ	3.21 a ^Y
HC	3.62 a
AL	3.60 a
ALC	0.30 b

^Z NS (nonsignificant) or significant at $P \leq 0.05$ (*), 0.01 (**), or 0.001 (***).

^Y Means within a column under each main factor followed by the same letter are not significantly different according to All Pairs, Turkey HSD.

CONCLUSION

Both spectroscopic methods failed to provide good prediction of crude protein content of napiergrass over 30, 60, and 90 days of growth (all r^2 values were less than 0.5). Large increases in r^2 between 30 and 60 days for CCI-LP, and similarly between 30 and 90 days for CCI-LH, suggested that a later growing stage of plant may be more useful for future research on indirect spectroscopic estimation of leaf protein. The percentage of crude protein in napiergrass decreased in chronological order at three harvest dates. A trend of decreasing crude protein content between napiergrass parent group and self pollinated progeny group and F1 hybrid group was also identified. This indicated that selfing and hybridization of napiergrass parent group might have caused the lower protein content in napiergrass progeny group.

For leaf protein purification experiments, both heat coagulation and mild alkali extraction method did not improve the extractability of leaf protein. Activated carbon column had effectively removed anti-quality factor (tannins) detected for this experiment. However, the utilization of activated carbon had also caused lower protein yield in the final purified product. Therefore, current method of protein extraction and configuration of activated carbon column in this experiment is not ideal for protein purification purposes.

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