Correlation of lipid content and phenotypic markers of Canadian field peas (*Pisum sativum*)

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ABSTRACT

Bio-lipid products are extensively used in the production of biofuels, bio-surfactants, bio-lubricants and the oleo-chemical industry, which has the potential to replace many of the petrochemical based products. Growing demand for bio-oils in various industries has increased the importance of vegetable oil production globally. Over 30 % of daily calories in the human diet are supplied by edible oil, which accounts for 80 % of the total vegetable oil produced in the world. In order to meet the demand, oilseed production has increased through improvements in breeding, extending the cultivation area and by producing genetically modified plants. Pea (*Pisum sativum* L.) is one of the most world's important crops and a significant increase in the lipid content of the field pea seeds could facilitate increased vegetable oil production around the world. Previous research has reported that peas are a valuable source of protein and starch, but the lipid concentration in their seeds has been undervalued. Although the pathways for lipid biosynthesis in higher plants have been uncovered, our understanding of the regulatory mechanism controlling lipid accumulation is still limited. Therefore this study investigated the correlation between the lipid content and other field pea phenotypic markers. Seeds of eight pea accessions were screened for lipid content and other phenotypic markers such as content of carbohydrate, proteins, carotenoids, flavonoids, chlorophyll, moisture, ash, phenols, starch and antioxidant activity. The lipid content in field pea seeds was low and ranges from 1.3 to 2.6 %, whereas protein and carbohydrate content was comparatively high and varies from 155 to 232 mg of BSA / g of sample (BSA, Bovine serum albumin) and 357 to 453 mg / g of sample, respectively. Statistical analysis revealed that lipid content was correlated to the variety, seed shape, seed colour, ash content and starch content, but the correlation to protein was insignificant. Lipid content was found to have a strong positive correlation with high ash content, brown color seeds and green color seeds, and negative correlation with smooth surface, yellow colour, high starch content and larger seed volume. On the basis of statistical analysis of phenotypic markers, desired pea variety can be easily selected and significant modification in the field peas can be further performed to improve the nutritional quality.

RÉSUMÉ

Les biolipides sont largement utilisé dans l'industrie des produits oléochimiques ainsi que dans la production de biocarburants, de tensioactifs biologiques et de biolubrifiants. Ces lipides biologiques ont le potentiel d'éventuellement remplacer plusieurs produits pétrochimiques. La demande croissante de biolipides dans plusieurs secteurs industriels intensifie l'importance d'huiles végétales sur une échelle mondiale. Au delà de 30% du régime quotidien calorifique humain est comblé par des huiles comestibles, ce qui représente 80% de la production mondiale d'huiles végétales. Afin de répondre à la demande, la production d'oléagineux a augmenté grâce à une amélioration en termes de la culture sélective et de l'extension des zones de culture et par la production de plantes génétiquement modifiées. Le pois (*Pisum sativum* L.) est l'une des cultures les plus rependues et importantes au monde. Ainsi, une augmentation significative de la teneur en lipides des graines du pois cultivé pourrait faciliter la production d'huile végétale. Les pois possèdent une concentration importante de protéines et d'amidon. Cependant, la concentration de lipides présentes dans le pois cultivé doit accroitre pour éventuellement considérer cette légumineuse comme source importante de biolipides. Bien que les voies de biosynthèse de lipides chez les plantes supérieures ont été découverts, notre compréhension du mécanisme de régulation de l'accumulation de lipides est encore limitée. Par conséquent, cette étude examine la corrélation entre la teneur en lipides et d'autres marqueurs phénotypiques du pois cultivé. Des semences provenant de huit accessions de pois ont été dépistées pour leur teneur en lipides ainsi que pour d'autres marqueurs phénotypiques tels que leur teneur en glucides, protéines, caroténoïdes, flavonoïdes, chlorophylle, humidité, cendres, phénols, amidon et finalement en terme d'activité antioxydante. La teneur en lipides des semences de pois cultivés était faible, se situant entre 1,3 à 2,6%, alors que la teneur en protéines et en glucides était relativement élevée, variant de 155 à 232 mg d'ASB par gramme d'échantillon (ASB, albumine de sérum bovin) et de 357 à 453 mg par g d'échantillon, respectivement. L'analyse statistique révèle qu'il existe une corrélation entre la teneur en lipides et la variété, la forme des graines, la couleur des graines, la teneur en cendres et la teneur en amidon, mais la corrélation entre la teneur en lipides et la teneur en protéine était négligeable. Il existe une forte corrélation positive entre la teneur en lipides et une haute teneur en cendres, et les graines de couleur brune et verte. Il existe une corrélation négative entre la teneur

en lipides et une surface de graine lisse, la couleur de graine jaune, une haute teneur en amidon et un volume de graine plus élevé. En utilisant l'analyse statistique des marqueurs phénotypiques, la variété de pois souhaitée peut être facilement sélectionné et la modification significative peut encore être effectué pour améliorer la qualité nutritionnelle.

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
AACC	American Association of Cereal Chemists
ADPG	Adenosine diphosphate glucose
ADPGP	Adenosine diphosphate glucose pyrophosphorylase
AMG	Amyloglucosidase
AOAC	Association of Analytical Communities
BCA	Bicinchoninic acid
BHA	Butylated hydroxyl anisole
BSA	Bovine serum albumin
BHT	Butylated hydroxyrotoluene
CE	Catechin equivalent
Da	Dalton
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
DPPH	2, 2-diphenyl-l-picrylhydrazyl
EST	Expressed sequence tag
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organisation
GA	Gallic acid
GAE	Gallic acid equivalents
GC	Gas chromatography
GLC	Gas liquid chromatography
GOPOD	Glucose oxidase/ peroxidase
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IEC	Ion exchange chromatography
IMS	Industrial methylated spirits
KOH	Potassium hydroxide
LG	Linkage group

M	Molarity (Molar)
m/m	Mass/mass
MS	Mass spectrometry
Mw	Molecular weight
NMR	Nuclear magnetic resonance
N	Normality
PG	Propyl gallate
QTL	Quantitative trait locus
RAPD	Random amplified polymorphic DNA
RS	Resistant starch
SBE	Starch branching enzyme
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SFC	Supercritical fluid chromatography
SSR	Simple sequence repeat
TIU	Trypsin inhibitor units
TLC	Thin layer chromatography
TPC	Total phenolic content
USA	United States of America
v/v	Volume/volume

Chapter 1

INTRODUCTION

1.1 Background

Legume seeds represents a rich source of dietary protein, carbohydrates, minerals, vitamins, and antioxidants, which offers great potential for human and animal nutrition (Friedman, 1996). Field pea (*Pisum sativum* L.) is one of the most important ancient vegetable and ranks second worldwide among food legume after common bean (*Phaseolus vulgaris* L.) (Kumari et al., 2013). Field pea is an annual cold season crop that is in the family Leguminosae (Singh et al., 2010a; Shereena et al, 2006; Ratnayake et al., 2000). Field pea is a legume because they possess pods with a single cavity ovary that splits along two margins when dry. Field pea is also called common pea, dry pea, green pea (light green seedcoat and dark green cotyledon), yellow pea (light yellow seedcoat and deep yellow cotyledon) and garden pea (Ratnayake et al., 2000). There are two main types of peas (1) smooth seeded and (2) wrinkled seeded. Both green and yellow smooth seeded peas are commonly known as field pea, dry peas or feed peas (Sell, 1993). The smooth seeded peas are mainly used for food and feed whereas wrinkled seeded peas are harvested at an immature stage and are primarily used for freezing and canning. Field pea is an herbaceous crop that has short leaves, climbs with the help of leaf-let tendrils and reaches up to 35 - 60 cm in length. The stem is usually slender, weak and circular. Unlike the stem, roots are small and not strongly developed. Field peas grow well on all soil types from light sandy to heavy clay but they have specific requirements with respect to seasonal changes in temperature during their growth cycle (Singh and Joshi, 1970).

The nutritional profile of field peas is well documented. The basic nutrient composition of peas is illustrated in Figure 1.1. Field pea consists of 21 to 25 % of proteins that are comprised of high levels of essential amino acids, lysine and threonine (7.3 %, and 3.7 % of total N, respectively, as mentioned in Figure 1.1), which are usually low in cereal grains (GL-Pro, 2005; Sosulski et al., 1983). Protein content of field peas often vary with the influence of variety and environment (GL-Pro, 2005; McKay et al., 2003; Hickling, 2003; Anderson et al., 2002). The carbohydrate content of peas is generally high with starch at 54 % and has a high level of digestible fiber (hemicellulose

fraction 7 %) (Anderson et al., 2002). Protein content accounts for 25 % (m/m) of the total mass and is found to show a strong inverse correlation to starch (Hickling, 2003). A major portion of fiber in field pea is derived from the cell walls, although cellulose and lignin levels are comparatively low. The lipid content of field peas is relatively low when compared to starch and proteins (Hickling, 2003; Anderson et al., 2002) and ranges from 1.5 to 2.5 % (m/m) of dry matter (Pryor, 2008; Ryan et al., 2007; Hickling, 2003; Anderson et al., 2002; El-Refai et al., 1987; Welch et al., 1984). However, Letzelter et al. (1995) found that some varieties of field pea contained high levels of total lipid content at 9.7 %. Smooth peas possesses less lipid content than wrinkled varieties (Bastianelli et al., 1998; Welch et al., 1984). Like cereals, pea lipids are mainly composed of polyunsaturated fatty acid, with the amount of unsaturated fatty acids generally higher (79.2 - 86.2 %) than saturated fatty acids (15 %) (Kosson et al., 1994b; Hickling, 2003). Linoleic (50 %), oleic (20 %) and linolenic (12 %) acids are the main unsaturated fatty acids in pea seed lipids (Hickling, 2003; Kosson et al., 1994b).

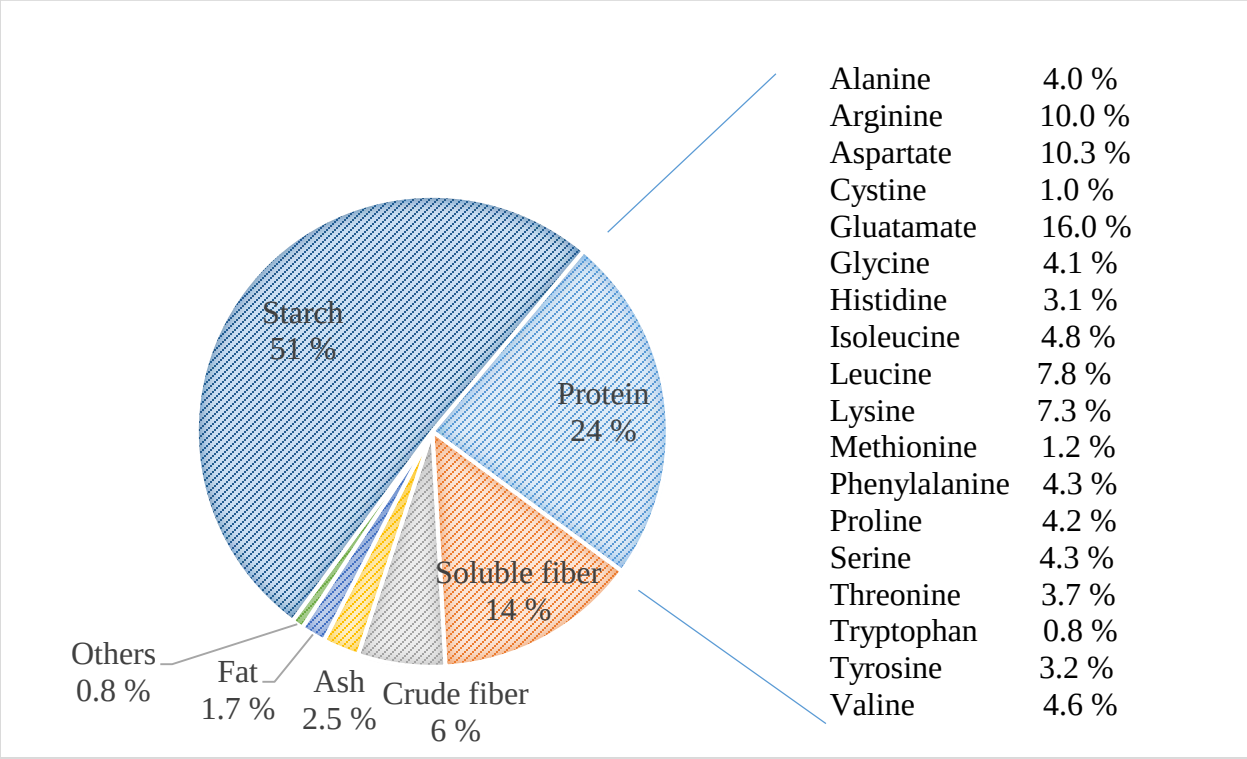


Figure 1.1: Nutritional composition of field pea in terms of per cent of total dry matter (Tosh et al., 2013; GL-Pro, 2005; Hickling, 2003).

Field pea have relatively high amounts of protein, carbohydrates, fiber and amino acids that accounts for 86 – 87 % of total digestible nutrients, making it an excellent source of these nutrients in most diets (Anderson et al., 2002). Various legume seeds such as soybean (*Glycine max*), beans (*Phaseolus* spp.), peas (*P. sativum*), lupins (*Lupinus* spp.) and lentils (*Lens culinaris*) are generally used by humans as a source of protein, carbohydrate, several water-soluble vitamins and minerals (Friedman, 1996). Due to an increase in the demand for protein rich plant material, this crop has great demand worldwide (Santalla et al., 2001). In Europe, field peas have been increasing in consumption rate and are considered as an alternative to soybean (Hickling, 2003; Anderson et al., 2002). A large quantity of this crop is used for various animal feeds including dogs (DeOliveira et al., 2008; Bednar et al., 2001), cats (DeOliveira et al., 2008; Bednar et al., 2001), pigs (Petersen et al., 2006; Stein et al., 2004; Brand et al., 2000), poultry (Nalle et al., 2011; Wiryawan et al., 1999) and for concentrated feed for aquaculture (Adamidou et al., 2009; Allan et al., 2004; Thiessen et al., 2003; Cruz-Suarez et al., 2001). Pea proteins are widely accepted as they have manifold qualities, good functional properties, availability, high nutritional value, and relatively low cost. Utilisation of field pea protein concentrate is increasing as a functional ingredient in the food industry (Nunes et al., 2006). In addition to pea protein, field pea starch is used as an important functional ingredient in noodle production and the fiber is also used in the food industry (Qi et al., 2004a). Thus, field pea has huge potential in the food industry due to its value-added components namely protein, starch and fiber. Moreover, pea pods as well as their products are an important source of biologically active components that have many therapeutic effects and health benefits (Roy et al., 2010).

Production of field pea has been rapidly increasing throughout the world, with many different varieties of peas grown worldwide (Cousin, 1997). On the basis of production and sowing area, field pea ranks fourth amongst legumes after peanut (34,856,007 tons), soybean (216,144,262 tons) and dry bean (28,322,024 tons) (Varshney et al., 2009; Zong et al., 2008; Farrington, 1974). Based on FAO 2004 data, 12.2 million tonnes of field pea production was achieved worldwide on 6.3 million ha of agricultural lands with an average yield of 1.93 tonnes / ha (Duzdemir et al., 2009). Field pea has its origin from Southeast Asia and the major pea producers are Canada, Russian Federation, United States, India, France and Ethiopia (Food and Agriculture Organization, 2012). Among them, Canada, Europe, Australia and the USA are major exporters of peas (McKay et al., 2003). Canada is a world leader in peas and ranks second in production 3.96 kilotonnes (20 % of

total world production) and is the largest exporter at 2.78 kilotonnes (40 % of total world exports) (Agriculture and Agri-Food Canada, 2015; Statistics Canada, 2011; Thiessen, 2004). In Western Canada field peas have been cultivated since farmers started farming the prairies over 100 years ago. Since 1977 there has been a consistent increase in production of field pea (Agriculture and Agri-Food Canada, 2005a). After the opening of the European pea feed market in 1985, pea farming increased by almost 18 fold from 74,400 ha in 1985 to 1,345,000 ha in 2014 (Agriculture and Agri-Food Canada, 2015). Within Canada, 79 % of field peas are grown in Saskatchewan, 18 % in Alberta and 2 % in Manitoba.

Field pea is a high yield crop in temperate regions (Corre-Hellou et al., 2005) and is suited for the Canadian climate as the Canadian temperature varies quite a bit. The ideal temperature range for growing peas is 7 - 24 ^{O}C, however they grow best at 12 – 21 ^{O}C (Duke, 1981). In Canada, canola and soybean are the most commonly used oilseed crops accounting for 40 % and 20 % lipid content respectively (Steffanson, 2013). The lipid content of field pea is comparatively low when compared to these crops and is typically less than 2.5 %, however it is hoped that developing an oilseed pea could result in a crop that can also produce vegetable oil (Sarwar et al., 2013; Solis et al., 2013). Although field pea has never been considered as an oilseed crop (Yoshida et al., 2007), these early results were promising for the development of a novel oilseed crop for Canada.

To the best of our knowledge and extensive literature search, no study has been published that has worked on increasing the lipid content in field peas. Due to the low lipid concentration in field pea, pea lipids have not been considered a valuable commodity. In higher plants the pathways for lipid synthesis has been discovered, but our understanding regarding the regulatory mechanism responsible for its accumulation is still being determined. Therefore this research study will investigate the correlation between lipid content and other phenotypic markers in field pea. Various phenotypic markers considered were the content of carbohydrate, protein, carotenoid, flavonoid, chlorophyll, moisture, ash, phenols, starch and antioxidant activity. Pathways involved in lipid biosynthesis or other phenotypic markers can be correlated with each other, as certain genes responsible for these phenotypic marker might be linked to lipid production. If any phenotypic marker can be found that correlates to the lipid content, then it can be used as a quick screening method and marker for breeding. This research should allow for selection and breeding of a pea plant with improved lipid production.

1.2 Research Objectives

1. To quantify the nutritional composition of field pea.
2. To measure variability of field pea metabolite production over two different years
3. To determine if any correlations exist between lipid content and other metabolite accumulation in field pea.

1.3 Outline of Thesis

The objectives of this research are outlined in Chapter 1. Chapter 2 reviews the literature concerning the general properties of field pea, its genetics, nutritional significance and applications. Chapter 3 outlines the experimental conditions, materials used, and equipment used in this study. Chapter 4 presents and summarizes the results obtained from this research. Chapter 4 also provides detailed discussions of the results. Chapter 5 highlights the conclusions of this research.

Chapter 2

LITERATURE REVIEW

2.1 Field Pea

Field pea (*Pisum sativum* L.) is an annual, herbaceous, climbing plant belonging to the family leguminosae and sub-family papillonaceae, a group named for the butterfly-like appearance of their flowers. Field pea is a diploid (2n = 14) (Hancock, 2004) and normally a self-pollinating crop with both male and female organs in the same flower (Gill et al., 1980). The ovary, contains between 5 - 12 ovules (egg cells). The style is somewhat flat and cylindrical and is at a right angle to the ovary. The pistil is usually surrounded by 9 + 1 stamens, out of which the filaments of 9 stamens are joined together while the 10th stamen is free. There are typically 5 petals in a 2 + 2 + 1 arrangement having 1 standard, 2 wings, and 2 keels that are fused except at their base. The petals cover the pistil and stamens. Flowers of field pea can be white, purple or pink, with petals of different sizes. The fruit is a closed pod, which is 2 to 10 cm long that often has a rough inner membrane. Seeds are primarily round, either smooth or wrinkled, and can be yellow, green, beige, reddish orange, brown, reddish blue, dark violet to almost black, or spotted (Pavek, 2012). The stem is usually hollow and weak, and climbs mostly with support, especially taller cultivars (Elzebroek et al., 2008). Field pea has pinnate, compound and alternate leaves, comprising of stipules (mostly two), leaflets (one to several pairs) and terminal tendrils (McGee, 2012).

Field pea is a cool-season crop and grow well in all soil types from sand to heavy clay (Oelke et al., 2015). It is strongly recommended to grow peas in crop rotations with other crops to break up the disease and pest cycles and contribute nitrogen to the soil (Chen et al., 2006; Biederbeck et al., 2005; Lupwayi et al., 1998). Field pea have a symbiotic relation with a bacteria (*Rhizobium leguminosarum)*, housed in nodules that convert atmospheric nitrogen (N_2) to ammonia (NH_3) that can be used by the plant or added to the soil (Clark, 2007; Ingels et al., 1994). Field pea can grow almost everywhere in the world, including the tropics where it is grown at high elevations and the seeds (grain) are harvested at maturity similar to the cereals crops (European Association for Grain Legume Research, 2007).

Field pea is one of the oldest crops in the world with its utilization traced back to the Neolithic times (Zohary et al., 1988). It is native to Syria, Iraq, Iran, Turkey, Israel, Jordan, and Lebanon, and began cultivation in Europe several thousand years ago (Slinkard, 2000). Gradually it has spread around the world and is now grown in all climate zones (Agriculture and Agri-Food Canada, 2008).

Peas are normally classified according to their uses. All categories of peas, whether they are harvested as immature grains for canning, as dry grains for human consumption or animal feed, or as the entire plant for forage, belong to the same botanical species called *P. sativum* L. (European Association for Grain Legume Research, 2007). Sugar snap peas, snow peas (McGee, 2012) and garden or green peas (Elzebroek et al., 2008) are harvested immature for the fresh or fresh-pack market. The seeds which have been harvested after they have matured and have been allowed to dry on the vine are referred to as dry peas. Field peas, including fall-sown Austrian winter peas, are dry peas, and are primarily used as livestock feed. The seed of field peas, whole, split or ground dried peas are mainly consumed by human (Elzebroek et al., 2008). Whole or parts of the pea plant, such as seeds, pods and other plant remnants, may be used for silage (Davies et al., 1985). Two other major types of peas are smooth seeded and wrinkled seeded. Both green and yellow smooth seeded peas are commonly known as field peas or dry peas or feed peas (Sell, 1993) and are used primarily for food and feed, whereas wrinkled seeded are usually harvested when immature and used for freezing and canning. Other peas such as colored seeded and marrowfat peas are also categorized under smooth seeded pea (Heuze et al., 2015; Slinkard, 2000). The colored seeded pea (Austrian winter pea and maple pea) are not used for human food purposes but for forage or animal feed (Heuze et al., 2015; Slinkard, 2000). In addition, marrowfat pea is a distinguished category with large, angular, green seeds, and used primarily in snack foods and other specialized foods in Asian countries (Slinkard, 2000). For the purpose of this thesis, field pea will refer to the dried mature seeds of *P. sativum*.

Field pea seeds are a valuable source of starch, protein and lipid. However the composition varies in different types of the pea seed. It is reported that smooth pea cultivars contained less crude protein, free lipid, ash, glucose, and sucrose and more starch as compared to wrinkled pea cultivars (Ryszard et al., 1994). Total lipid content is higher in wrinkled than in smooth peas (Colonna et al., 1980; Coxon et al., 1982). In addition, variation is seen in different parts of the seed, for

example the protein content ranges from 3.1- 3.8% in the hull to 14.5 - 34.1 5% in the cotyledon, and the lipid content can range between 0.4 - 0.6% in the hull and between 1.1 - 3.3 % in the cotyledon (Savage and Deo, 1989; Singh et al., 1968). The cotyledon contributes approximately 95 % of the seed protein and 90 % of the seed lipid (Adsule et al., 1989). Pea lipid content in the seed can impact the stability of seeds and pea flour during storage and processing (Colonna et al., 1983). However, no research has reported that green and yellow peas differ in their nutritional content but small differences in nutrient contents has been reported for some pea varieties, which is attributed to differences in the size of the pea grain and the thickness of the hull (Hickling, 2003).

2.2 Genetics of Peas

Pea is a cool-season, self-pollinated and diploid plant. The genome size of pea is 5000Mbp arranged with 7 pairs of chromosomes (2n = 14, n = 7) (Sato et al., 2010). Several studies have reported the complete characteristics of each chromosome of field pea including its relative length, centromereric location, secondary constructions, presence of satellites and other chromosomal rearrangements (McPhee, 2007; Hall et al., 1997a; Hall et al. 1997b; Ben Ze'en et al., 1973; Blixt 1958). Ghulam et al. (2005) carried out a karyotype analysis and found it has two metacentric and five sub-metacentric chromomsome pairs. Two out of the five sub metacentric chromosomes possess satellites of varying size on their long arms. It was reported that the chromosome length of the haploid set (n) was 112 µm, for the diploid set (2n) the total chromosomal length was 224 µm and the average chromosomal length is 16 µm (Ghulam et al., 2005).

Research on *P. sativum* genomics can be traced back to the pioneering work of Gregor Mendel in the 19th century (Reid et al., 2011; Allen, 2003; White, 1917; Bateson, 1901; Mendel, 1865). Mendel's experiment on seven qualitative characters in peas maintain it as a major focus of modern genetic studies (Reid et al., 2011) and has been continuously studied since Mendel (Samatadze et al., 2008). Recent advances in the molecular biology has led to the identification of four of the seven genes reported by Mendel (Table 2.1). Gene responsible for flower color (*A)*, stem length (*LE)*, cotyledon color (*I*) and seed shape (*R)* have been sequenced and their function is known (Reid et al., 2011). However, less information is available for the genes related to fasciation (FA), pod color (GP) and for controlling pod sclerification (V) (Reid et al., 2011).

Table 2.1: Characteristics of the genes responsible for seven qualitative characters in *P. sativum* selected by Mendel (Reid et al., 2011).

Trait	**Dominant Phenotype**	**Recessive phenotype**	**Symbol**	**Linkage group**	**Cloned**	**Gene function**	**Molecular nature of mutation**
Seed shape	Round	Wrinkled	*R*	V	Yes	Starch branching enzyme 1	0.8-kb insertion
Stem length	Tall	Dwarf	*LE*	III	Yes	GA 3-oxidase 1	G-to-A subsitution
Cotyledon color	Yellow	Green	*I*	I	Yes	Stay-green gene	6-bp insertion
Seed coat/flower color	Purple	White	*A*	II	Yes	bHLH transcription factor	G-to-A at splice site
Pod color	Green	Yellow	*GP*	V	No	Chloroplast structure in pod wall	Unknown
Pod form	Inflated	Constricted	*V*?	III	No	Sclerenchyma formation in pods	Unknown
Position of flowers	Axial	Terminal	*FA*	IV	No	Meristem function	Unknown

Seed shape, one of the characteristics examined by Mendel, can be either round or wrinkled (irregular) (Neil, 1997; Fairbanks et al., 2001). White. (1917) denoted round seeds with R and wrinkled seeds with r. The r (rugosus) locus mainly controls the shape of dry seed. Mature seeds are either homozygous dominant (RR) or heterozygous (Rr) for genes with either or both resulting in a round seed, whereas those containing genes that are homozygous recessive (rr) are wrinkled (Kooistra, 1962). Later, several genes were found to be responsible for the wrinkled or round phenotype (Reid et al., 2011). Coxon et al. (1982) described that r_b locus determines the shape of seed and also affects its lipid content. The lipid content ranges from about 2.4 % for a round seeded line (RRRbRb) to about 5.6 % for a wrinkle seeded line (rrrbrb). In addition, recessive genes at two other loci lacunosus (di) and minute-foveatus (mifo) have been described and impact the shape of the dry field pea seed (Blixt, 1972). Beside morphology of pea seeds, r locus has a profound

effects on storage product. The r locus consists of a gene that encodes the starch-branching enzyme. In rr lines (wrinkled) of peas, one of the major isoforms of this enzyme is missing, which prevents the production of this enzyme and hence reduced activity of this enzyme, as a consequence there is less branched starch and a lower overall starch synthesis. This variation of enzyme activity influenced the quantity of sugar as well as the fresh mass of the developing seeds (Stickland et al., 1983). Wrinkled seeds have higher water content in immature seeds due to the presence of a high amount of sucrose, fructose, and glucose, which causes increased osmotic pressure and hence water uptake (Ellis et al. 2011; Smith 1988; Coxon et al., 1982). As a consequence, this results in enlarged embryo volume during development. During drying, the loss of water from the embryo appears to result in wrinkled seed (Wang et al., 1987). In addition, the wrinkled seeds have high lipid content (Coxon et al., 1982) but low level of storage proteins such as legumin (Davies, 1980; Domoney et al., 1985).

A major breakthrough came with the demonstration that starch synthesis in pea embryos is directly impacted by mutant alleles at two separate loci, r and rb, r on chromosome 7 and rb on chromosome 3 (Blixt, 1972). A mutant allele at the r locus lacks one of the major isoforms of a starch-branching enzyme, SBE1, which impacts the activity of the enzyme and results in wrinkled seed (Smith et al, 1988). This variation further led to the cloning of *R* gene and found that the mutant *SBE1* gene is interrupted by a 0.8-kb insertion (Bhattacharyya et al., 1990). A mutant allele at the rb locus affects one of the subunits of another enzyme, namely, ADPG pyrophosphorylase which is also involved in starch synthesis. As a result, the enzyme is less sensitive to allosteric regulation (Hylton et al., 1992). Failure by wrinkled seeds to produce any of the enzyme due to mutation in SBE1 or ADPGP led to complex metabolic changes in starch, lipid, and protein biosynthesis in the seed (Wang et al., 1991).

Other quantitative traits such as winter hardiness, tolerance to fungal diseases and seed yield have been shown to be controlled by multiple genes (Krajewski et al., 2012). Recent advances in the molecular biology have the potential to unveil the identification of various genes. Like other crops, genetic maps have been constructed in pea (Katoch et al., 2010: Loridon et al., 2005). A linkage map of field pea mainly consist of 228 simple sequence repeat (SSR), 231 other markers primarily comprising of random amplified polymorphic DNA (RADP) and 18552 expressed sequence tag (EST) (Sato et al., 2010).

2.3 Oilseed and Biofuels

2.3.1 Biofuels

Vegetable oil and animal fat have been extensively used to produce biodiesel (usually methyl esters derived from oils and fats) and bioethanol (Bender, 1999). The environmental advantage of biofuel has reinforced this trend during the last few decades (Demirbas, 2002). It has been shown that petroleum and biodiesel varies in their chemical structures (Gurr et al., 2002). Diesel fuel consist only of carbon and hydrogen atoms that are arranged in a straight chain or branched chain structures along with aromatic configurations. In contrast, the biodiesel structure is based on triglycerides, which contains up to three fatty acids linked to a glycerine molecule with ester linkages (Demirbas, 2002). Biodiesel is produced from vegetable oil or animal fat by a chemical process called transesterification (Figure 2.1), in which triglycerides are converted into methyl/ethyl esters by reacting with methanol/ethanol (Barnwal et al., 2004). It is carried out in the presence of an alkali, acidic or enzymatic catalyst (Zhang et al., 2003; Gunstone et al., 1994), resulting in methyl ester (biodiesel), a co-product (crude glycerin), and some waste. Sodium hydroxide (NaOH) or potassium hydroxide (KOH) is commonly used as the catalyst.

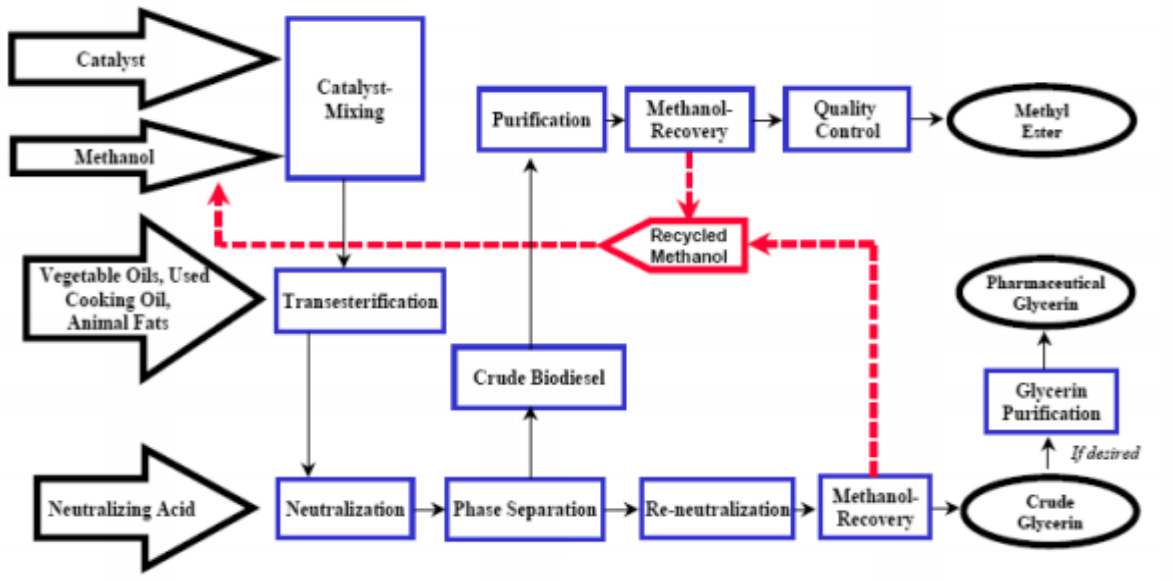


Figure 2.1: Biodiesel Production Process (Yeboaha et al., 2013).

The transesterification reaction process can be summarized as, where NaOH is the catalyst:

$$\underset{(100\ \text{parts})}{\text{Methanol}} + \underset{(1015\ \text{parts})}{\text{Vegetable oil}} \xrightarrow{\text{NaOH}} \underset{(1000\ \text{parts})}{\text{Methyl ester}} + \underset{(100\ \text{parts})}{\text{Glycerine}} + \text{Free Fatty Acid} + \text{Waste}$$

2.3.2 Oilseeds

Oilseed crops are a valuable source of high quality vegetable oils and excellence nutritional composition, which are used for the production of enriched nutrient products, animal feed, natural food and snack food worldwide (Sarwar et al., 2013). Oilseeds are an excellent source of protein, phenolic compounds, phytic acid, fibre, lipids, tannins and have high caloric value (Sarwar et al., 2013).

Their seed store energy for germination, predominantly in the embryo mainly as oil, irrespective with cereals that contain energy in the form of starch (McKevith, 2005). Oil from oilseed is divided into two types on the basis of its uses: (1) Edible oils, extracted mainly from soybean, rapeseed, sunflower, peanut and cotton seed, are extensively used for frying, healthy oil and in various food products (Sarwar et al., 2013). (2) Industrial oils are used for some industrial processes such as lubricants, coating applications (such as paints, inks and varnishes), bio-oil, etc (Savage, 2008; Cahoon, 2003) and are usually extracted from flax (linseed) and castor bean (Sarwar et al., 2013).

Vegetable oils can be divided into three major groups (Gunstone, 2002): 1) Vegetable oil derived from annual plants, such as canola (*Brassica napus* L. or *Brassica rapa* subsp. *oleifera*, syn. *B. campestris* L), sunflower (*Helianthus anuus*) and flax (*Linum usitatissimum*). 2) Vegetable oil extracted from trees, such as coconut (*Cocos nucifera*) and olive (*Olea europaea*). 3) Vegetable oil obtained as by-products in crops such as cotton (*Genus gossipium*) and corn (*Zea mays*). This implies that the production of biodiesel is potentially possible from all extractable bio-lipids (Kemp, 2006).

Oilseed production has gradually increased in the last few decades in order to meet the demand of vegetable oil (Gunstone et al., 2007). Different approaches have been used to increase production, either by increasing the yield per unit area, decreasing disease and stresses, increasing seeded area or expanding the possible cultivation areas and climatic regions (Vollmann et al., 2009). Genetic engineering and breeding has played a vital role in improving the lipid content in oilseed crops (Maheshwari et al., 2014; Murphy, 2014; Seyis et al., 2003, 2005; Friedt et al., 1998). Various

agronomic traits have been developed through transgenetic research including: modification in cell wall composition, herbicide resistance, pest resistance, abiotic stress resistance, drought tolerance, cold and salt tolerance, enriched nutrient use efficiency, alteration in the fatty acid composition and improved processing ability (Kausch et al., 2010). Research has occurred on crops for enhanced lipid content, biomass yield and modification of fatty acid composition (Gunstone et al., 2007).

Agricultural crops that are commonly grown for oil extraction are corn, oat, cotton, soybean, mustard, camelina, crambe, safflower, sunflower, peanut, rapeseed, coconut, oil palm and olives (Sarwar et al., 2013). The oil content in oilseeds ranges from about 20 % for soybean, 40 % for sunflower and 45 % for canola (Sarwar et al., 2013, 2004, 2003). Beside these, some of the plants such as *Lesquerella* and *Pennycress* are currently being researched as future oilseed crop for the development of biodiesel feedstocks. (Friedman et al., 2014). In 2009, 151 varieties of field pea were screened of lipid content at the Bioresource Engineering Department of McGill University and found the mean contents in the range of 0.9 – 5% (Khodapanahi et al., 2012). The fatty acid profile from the oil of pea seed samples reported that it contained saturated fatty acids (THE majority being palmitic and stearic acids), unsaturated fatty acids (primarily oleic, linoleic and linolenic acids) and a small percentages of other long chain fatty acids (Solis et al., 2013). This fatty acid profile proves that this crop holds potential to become a new oilseed crop for the food industry, and subsequently compete with other Canadian oilseeds, namely, canola (>35 % oil) and soybean (>20 % oil). Furthermore, field pea as an oilseed could be used commercially for human consumption and the production of biodiesel or other industrial applications such as lubricants (due to high oleic acid content) or as paints, inks and varnishes (due to high linolenic acid content) (Cahoon, 2003). To the best of our knowledge and extensive literature search, no study has been published that has indicated any problem with the consumption of oil from field peas however the level of trypsin inhibitor is an issue for the animal feed (Hickling, 2003).

2.4 Pigment Analysis

Colour and appearance of food products are widely accepted traits by consumers (Nemeskéri, 2006). Seed color, seed shape (round or angular shape) and seed coat texture (smooth or wrinkled) are important considerations by pulse traders. Visual quality of field pea seeds determine the end use and the market value (Official Grain Grading Guide of the Canadian Grain Commission,

2015). Yellow and green field peas have a large cultivation area in Canada, along with smaller amounts of Austrian winter, maple and marrowfat peas. Field peas with yellow cotyledons and green cotyledons account for 80 % and 20 % respectively of Canadian field pea production. Austrian winter, marrowfat and maple peas account for the remaining 2% pea production (Agriculture and Agri-Food Canada, 2008). According to Official Grain Grading Guide of the Canadian Grain Commission (2008), the natural color of pea seed is the major factor determining grade. Seeds having a natural green color with less than 2 % bleached seeds are among the top grades of green pea. To qualify for the highest grade of yellow pea, seed should have natural yellow color with less than 1 % of other cotyledon color, such as green or orange (Canada No.1). Yellow field pea production is preferred over green field pea production since yellow field pea yield 1 – 5 % higher and are less susceptible to bleaching (which can downgrade their quality and value) than green peas (Saskatchewan Pulse Growers, 2000).

Variation in color characteristics of pea cultivars is controlled by genetics (Goodwin, 1986) but environmental factors, such as temperature can have a significant role (Helyes et al., 2002). McCallum et al. (1997) investigated biochemical changes during development of the seed pigments (chlorophyll a and b, violaxanthin, neoxanthin, β-carotene and lutein) in pea and assessed the genetic linkage analysis of the green seed color. Chlorophylls, carotenoids and xanthophylls are major chloroplast photosynthetic pigments which results in the green color of pea seeds (Steet et al., 1996; Edelenbos et al. 2001). Edelenbos et al. (2001) reported 17 pigments comprising of eight xanthophylls, four chlorophyll b related compounds, four chlorophyll a related compounds and one type of carotene in the processed peas grown under two light regimes. Violaxanthin, zeaxanthin, lutein and β-carotene are the key carotenoids in the field peas. Lutein is the major carotenoid in the field pea with mean lutein concentration ranging from 7.2 µg / g to 17.6 µg / g (Kaliyaperumal et al., 2013). Green cotyledon pea cultivars had been reported to have more total carotenoids than yellow cotyledon pea cultivars. Similar results were obtained by Kaliyaperumal et al. (2013) and Holasová et al. (2009). The deep-red colour in pea plants is typically due to high levels of lycopene, while the orange colour is associated with high β-carotene content (Davis, 1976). Yellow seeds in pea are found to have a low content of carotene (0.32 mg/kg) but a high level of xanthophyll content (10.20 mg / kg) (Nemeskéri, 2006). The *I* gene, reported by White. (1917), is responsible for cotyledon color in peas and has been sequenced recently (Reid et al., 2011). In addition, multiple dominant alleles of the Orc locus in field peas contribute to the orange

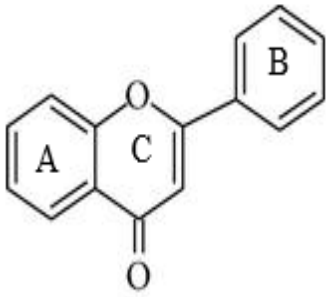
colour found in the cotyledons, whereas the recessive orc allele produces yellow cotyledons (Swiecicki et al., 2000; Swiecicki, 1998). Temperature and water stress plays an important role in the production of carotenoids, by activating specific genes and protein synthesis required in the accumulation of β carotene (Brandt et al., 2003; Iturbe et al., 1998; Terjung et al., 1998; Koskitalo et al., 1972). In addition, β carotene synthesis has been linked to an increase in light irradiance on the plant (Orset et al., 2000). With the advent of molecular techniques, several QTLs (Quantitative trait locus) on LG (Linkage group) III, IV, V and VII were found associated with seed color. QTLs on LG II, III and VII were found related to the color space U and V chrominance (McCallum et al., 1997). Bleaching of cotyledon has been reported in studies and have indicated that I, pa, gla and vim loci have great impact on chlorophyll retention of the cotyledons of green pea cultivars at the time of seed maturation (Weeden et al., 1990; Blixt, 1962). Beside genetic factors, degradation of chlorophyll pigments from the green cotyledon tissues have been shown to be influenced by environmental factors during seed maturation (Maguire et al., 1973) as well as the presence of carotenoids (Griffiths et al. 1955; Anderson et al., 1960).

2.5. Polyphenols in Field Peas

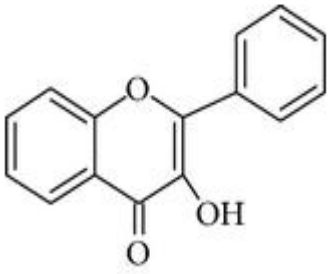
Phenolic compounds are the substances that contain an aromatic ring with one or more substituents group such as hydroxyl, carboxyl and methoxy group and often non-aromatic ring structures (Srivastava et al., 2013). Phenolic compounds are the secondary metabolites in plants, acting as a defensive system against pathogens, parasites and predators, damages by ultraviolet radiation, as well as contributing bright coloured hues of plants (Manach et al., 2004; Shahidi et al., 1995). Phenolic acids and flavonoids are two main types of phenolic components occurring in pulses, a term used for dry seeds of leguminous crops comprising of drybeans, chickpeas, peas and lentils (World Health Organisation and Food and Agriculture Organization, 2007). The phenolic acids are divided into two categories: the benzoic acids such as gallic acid (GA) and the cinnamic acids such as coumaric, caffeic and ferulic acid (Manach et al., 2004) (Figure 2.1). Both benzoic acids and cinnamic acids comprise of an aromatic ring and carboxylic acid but differ in their backbone. The phenolic acids have R groups located at the 3-, 4- and 5- positions of the ring structure.

Flavonoids are ubiquitous in nature and comprises of a vast array of biologically active compounds, commonly found in fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks). Flavonoids occur as aglycones, glycosides and methylated derivatives (Harborne,

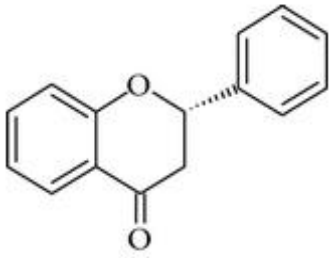
1988). According to the chemical structure, they are categorized into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. The basic flavonoid structure is the flavan nucleus of 15 carbon atoms which consists of two six membered rings linked with three carbon chain (C6-C3-C6) (Middleton, 1984). Rings are often labeled as A, B, and C (Figure 2.1). Each of the ring structures usually consists of hydrogen, hydroxyl, methoxyl or rhamnoglucoside R groups. The hydroxyl groups on the ring structures is responsible for forming hydrogen bonds with minerals, proteins and carbohydrate components. Since they are electron donors, they act as free radical terminators, reacting with free radicals to form more stable components (Shahidi et al., 1995). Some of the most abundant flavanone present in grapefruit is cyanidin-glycoside, an anthocyanin also common in berry fruits (raspberry, black currant, blackberry, etc.), among flavonoids is naringenin and quercetin, a common flavanol in tea and several fruits, the main flavonol in onion, broccoli, and apple is catechin and the main isoflavones in soybean is daidzein, genistein and glycitein (D'Archivio, 2007).



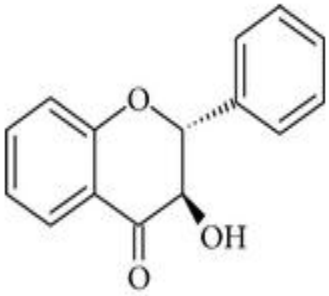
(Flavones)



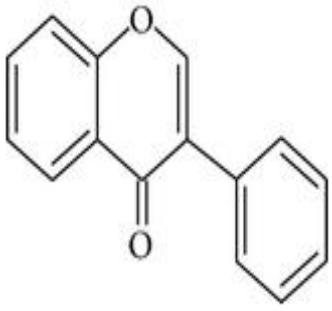
(Flavonols)



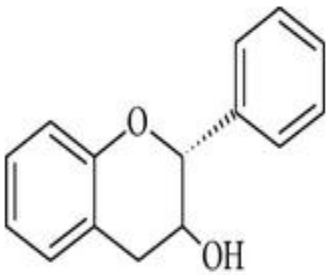
(Flavanones)



(Flavanonol)



(Isoflavones)



(Flavan-3-ols)

Figure 2.2: Chemical structure of some representative flavonoids (Kumar et al., 2013).

Antioxidants are a group of chemicals that that can protect cells from the damage caused by unstable molecules known as free radicals. The U.S Food and Drug Administration defines antioxidants as "preservatives that specifically retard deterioration, rancidity, or discoloration due to oxidation" (Specchio, 1992). Antioxidants may exist naturally or can be added during food manufacturing in order to maintain food quality and extend shelf-life. It is desirable that antioxidants should be cheap, inert, effective, with long term stability and harmless on color, flavor, and odor properties of food products (Reische et al., 1998; Rajalakshmi et al., 1996). On the basis of source, antioxidants are classified into two classes: natural and synthetic antioxidants. Various natural antioxidants such as thiols, ascorbic acid (AA) or polyphenols are used as reducing agents (Sies, 1997). Whereas, synthetic antioxidants are mainly lipophilic compounds that are extensively used in oil-in-water emulsions (Rajalakshmi et al., 1996). Some of the synthetic antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxyrotoluene (BHT) and propyl gallate (PG) are most commonly used (Hurrell, 2003). Antioxidants protect cells through different mechanism of action such as free radical scavenging, inactivation of peroxides and other reactive oxygen species, chelation of metals, and quenching of secondary lipid oxidation products (Decker, 1998). According to their mechanism of action antioxidants are classified as primary antioxidants and secondary antioxidants (Rajalakshmi et al., 1996). Primary antioxidants are the natural antioxidants that performs the function of chain breaking by reacting with the lipid radicals and converting them into more stable compounds (Hurrell, 2003). They include antioxidant mineral, antioxidant vitamin and phytochemicals (Hurrell, 2003). Various synthetic antioxidants such as BHA, BHT, PG, etc act as secondary antioxidants, which helps in capturing free radicals and stopping the chain reactions (Hurrell, 2003).

Natural antioxidants in humans diet can be used to prevent different health problems related to oxidative stress (Halliwell et al., 1990). Plants are considered as the first choice for the extraction of natural antioxidants, primarily the secondary metabolites consisting of various classes of phenolic compounds. Phenolics are commonly found in both edible and inedible plants. They are present in almost all plant organs and hence are an integral part of human diet such as cereals, oilseeds, fruits, vegetables, spices, beverages and pulses. For many years, phenolics were considered as antinutrients and little attention was paid to them. Polyphenols have been reported to combat various human diseases including atherosclerosis, arthritis, ischemia and reperfusion

injury of many tissues, central nervous system injury, gastritis, cancer, AIDS and many oxidative stress associated diseases (Kumpulainen et al., 1999). In addition to having antioxidant properties, phenolics control the organoleptic characteristics of plant-derived foods and beverages, affecting the color, flavour and texture of various foods (Tapas et al., 2008). They contribute to the bitterness, astringency and browning of fruit and fruit juices. In some instances, certain characteristics are both essential but undesirable during food processing. The astringency that is imparted by the presence of phenolics is attractive to impart the flavour of red wines, coffees, teas and dark chocolates but unattractive in food such as a smoky flavour in chocolate (Shahidi et al., 1995). Moreover, they are also known to obstruct the oxidative degradation of lipids, which is responsible for increasing the nutritional value of food (Srivastava et al., 2013).

Pulses, like other grains, represent a rich source of food phenolics. Among phenolics, polyphenols have relatively high concentrations in pulses, up to 2 % of the total content of beans and peas. Field peas has been reported to contain approximately 1050 tannic acid equivalents of polyphenols, with the majority of polyphenols located in the testa (cotyledon) (Shahidi et al., 1995). In general, pulses with light cotyledon have less polyphenols as compared to pulses with dark cotyledon. Furthermore, immature pulses have higher amounts of polyphenols than do mature pulses (Shahidi et al., 1995).

Pea seeds are a valuable source of nutrient compounds comprising of proteins, starch, oil, fibers, vitamins and minerals as well as non-nutrient phenolic compounds represented by simple phenolics, flavonoids and condensed tannins (Stanisavljevic et al., 2014). Peas are reported to contain a wide variety of phenolic substances, especially in varieties with dark colored seed coats (Agboola et al., 2010; Duenas et al., 2006; Troszynska et al., 2002a). Various legumes such as mung bean, fava beans, navy beans, lima beans, field peas, lentils, pigeon peas, lupines, chickpeas and cowpeas contained total phenolic acids in the range from 1.8 to 16.3 mg / 100 g (Sosulski et al., 1984). Phenolic distribution among peas has been found similar to lentils (Duenas, et al 2002). Remiszewski et al. (2006) found that total phenol content in field pea is 0.86 mg GAE / g (GAE, gallic acid equivalents) and contained 85 mg/g kaempferol, a type of flavonoid. In field pea, phenol content varied among cultivars and ranged from 162 to 325 mg CE /kg (CE, catechin equivalents) (Wang et al., 1998a). Some varieties of pea has been shown to have total phenolics content as high as 30.56 mg GAE / g in MBK 168 (catalog number of pea variety) and 113 mg / g

in fraction II (Stanisavljevic et al., 2014; Amarowicz et al., 2003). The content of phenolics in extract varies depending on the legume as well as solvent extraction technique used to extract these compounds. Xu et al. (2007) reported that yellow pea, green pea and chickpea showed the highest phenolic content when extracted with 50 % acetone but for lentils the total phenolic content (TPC) was highest when extracted with acidic 70 % acetone (+ 0.5 % acetic acid). Even soaking and germination of peas, beans and lentils had been found to change their phenolic composition (Lopez-Amoros et al., 2006; Oboh et al., 2006). Sosulski et al. (1984) found that field pea and pigeon pea flours contained only soluble esters, and isolated trans-ferulic acid, trans-p-coumaric and syringic acids upon hydrolysis. In another study, basic hydrolysis revealed the presence of vanillic, caffeic, p-coumaric, ferulic and sinapic acids in pea crude extract (Amarowicz et al., 2003). The HPLC analysis of seed coat of pea extract indicated the presence of some phenolic acids such as benzoic acid, cinnamic acids and cinnamic acid derivatives, flavone and flavonol glycoside (Troszynska et al., 2002a).

The main flavonoids characterized in the pea plant are as kaempferol-3-triglucoside, its *p*-coumaric acid ester, quercetin-3-triglucoside and its *p*-coumaric acid ester (Furuya et al., 2001). Amarowicz et al., 2003 isolated quercetin and kaempherol, procyanidin B2 and B3 in pea crude extract by HPLC analysis. The content of various flavonoids in seeds of green peas contained between: daidzein 1.746 - 2.688 mg / kg, genistein 0.412 – 0.706 mg / kg, kaempherol 0.621 - 1.484 mg / kg, apigenin 0.261 – 0.479 mg / kg and in yellow varieties of pea the content varied from 0.375 – 0.779 mg / kg daidzein, 0.115 – 0.158 mg / kg genistein, kaempherol 0.742 1.314 mg / kg, apigenin 0.462 – 0.698 mg / kg (Timoracka et al., 2010). Changes in flavonoids content in field pea has been reported to vary between varieties and time of storage.

Stanisavljevic et al. (2014) demonstrated the presence of bioactive phenolic constituents in the seed coat of colored pea varieties, using UHPLC-LTQ OrbiTrap MS and identified 41 phenolic compounds. The examined pea seed coats contained 12 phenolic acids (gallic, protocatechuic, chlorogenic, p-hydroxyphenylacetic, gentisic, caffeic, ferulic and p-coumaric acids, sinapic acid, syringic acid, rosmarinic acid and p-hydroxybenzoic), 5 flavanols (catechin, epicatechin, catechin gallate, gallocatechin, and epigallocatechin), 5 flavonols (quercetin, rutin, morin, kaempferol and galangin), 2 flavones (luteolin and apigenin), 3 flavanones (naringin, hesperetin and pinocembrin) as well as 10 flavonol glycosides. In addition, it has been shown that dark colored genotypes

exhibited more total phenolic contents and antioxidant activities in comparison with bright colored genotypes that have the highest metal-chelating capacities (Stanisavljevic et al., 2014).

Legumes have been investigated as a source of phenolic compounds showing antioxidant activity. In recent years, many studies were done on the phenolic contents and antioxidant activities of raw and processed pea seeds (Stanisavljevic et al., 2013; Xu et al., 2009; Han et al., 2008; Troszynska et al., 2002b). Hydrophilic phenolics in the extract of pea showed strong antioxidant activity (Tsuda et al., 1993). Many by-products have been developed from pea seed coat (Dueñas et al., 2006; Oomah et al., 2011). With the advent of pneumatic separation technology, seed coats can be separated from cracked legume seeds (Innocentini et al., 2009), the isolation of various components from the seed such as dietary fibers, phenolics and other bioactive compounds can be very useful for the food industry (Stanisavljevic et al., 2014). In peas, α-, ɣ- and ɗ-tocopherols were detected, with ɣ-tocopherol the most abundant ranging from 1.60 mg/100 g to 2.09 mg/100 g. Various studies on plants indicated that the available antioxidant activity of phenolic compounds can be enhanced by improving agricultural practices, post-harvest treatments and food formulation and processing conditions. In peas and beans, germination has shown to increase the significant amount of antioxidant activity (Lopez-Amoros et al., 2006). The various post germination factors affecting changes in phenolic compounds are presence of light, germination time and the type of seeds. In peas, for instance, value of p-hydroxybenz aldehyde, cis p-coumaric acid and trans-ferulic acid was enhanced (Lopez-Amoros et al., 2006) in the early stage of germination, an increase was observed after four days in the presence of light. An increase in phenolic content resulted in an increase in the antioxidant activity which was measured by free radical scavenging capability. In legumes such as chickpea, peas and lentils, the thermal processing was found to decrease antioxidant activity compared to raw legumes (Xu et al., 2009). However these changes were dependent on the type of legume and processing conditions. Steaming proved to be the best method to preserve phenolic and antioxidant components of peas.

Pownall et al. (2010) have isolated five antioxidative peptides form protein hydrolysates of pea protein showing strong radical scavenging and metal chelating activities. Ndiaye et al. (2012) reported antioxidant activity of pea protein hydrolysate against nitric oxide. Furthermore, hydrophobicity and net charge on amino acids were reported as important contributing factors to peptide antioxidant properties of yellow field pea seed protein hydrolysate. The antioxidant

potential of five extracts of pea seed coat in liposomal systems has been demonstrated by Troszynska et al. (2002b). Antioxidant activity of any given phenolic is dependent on three structural components of the molecule: an o-hydroxy structure in the B ring, 2,3 double bond with a 4-oxo function in the C ring and hydroxyl groups (-OH) on the 3- and 5- positions in the A ring with 4-oxo functions in the C ring. Methods for the assessment of antioxidant activity of any sample are broadly divided into two groups: the electron transfer assays (ET) and the hydrogen atom transfer assays (HAT). The principle behind ET-based methods involves the use of an oxidant (also called as probe) that accepts an electron from the donor antioxidants, resulting in change in the colour of the probe, proportional to its antioxidant activity. They comprises of three methods namely: Trolox equivalent antioxidant capacity (TEAC), ferric reducing/antioxidant power (FRAP), and the 2, 2-diphenyl-l-picrylhydrazyl assay (DPPH) (Huang et al., 2005). Although ET assays are relatively simple but they are time dependent. Whereas, in HAT-based methods, probe accepts hydrogen molecule from a donor antioxidant, resulting in emittance of fluorescence from probe. HAT assays are solvent and pH independent, and can be completed very rapidly. They include oxygen radical absorbing capacity (ORAC) and total radical trapping antioxidant parameter (TRAP). HAT assays are very fast since they are not dependent on pH and solvent but they are costly (Prior et al., 2005).

2.6 Proximate Analysis

2.6.1 Carbohydrates

Leguminous seeds are recognized for the high quality of plant based protein and carbohydrates (Berrios et al., 2010). Due to high nutritional value, lentil, dry pea, chickpea and dry bean are increasingly grown and consumed worldwide (Berrios et al., 2010). Field pea represents a rich source of protein, carbohydrates, fiber and amino acids and contains about 86 – 87 % total digestible nutrients which make them excellent in the diet (McKay et al., 2003). The composition of dry pea seeds is given in Table 2.4. The carbohydrate portion is mainly made up of sugars (mono-, di- and oligo-saccharides) and starch. Similar to corn, peas has a high energy level due to its high carbohydrates content with starch (54 %) accounting for most of this fraction (Anderson et al., 2002).

Table 2.2: Proximate composition of dry pea seeds (Adsule et al., 1989)

Constituent	Content (%)
Moisture	16.0
Carbohydrate	56.5
Protein	19.7
Fat	1.1
Minerals	2.2
Crude fibre	4.5

The carbohydrate–oligosaccharide fraction basically includes starch, soluble sugars and dietary fiber in pulses (Berrios et al., 2010). The soluble sugars mainly comprises of monosaccharides such as glucose, ribose, galactose and fructose, and disaccharides such as sucrose and maltose (Berrios et al., 2010). Most of the oligosaccharides of pulses are derived from the α-galactosides group, where galactose is present in a α-D-1,6-linkage. Galactosides includes sucrose derivative (raffinose, stachyose and verbascose), glucose galactosides (melibiose and manninotriose) and inositol galactosides (galactinol, galactopinitol and ciceritol) (Berrios et al., 2010; Sanchez-Mata et al., 1998; Bernabe et al., 1993; Quemener et al., 1983). Peas contain a significant amount of galactons and about 5 % oligosaccharides, consisting of sucrose (2 %), verbasose (1.5 %), stachyose (1 %) and raffinose (0.5 %) (Han et al., 2006). Verbascose is the highest molecular weight galacto-oligosaccharide, which is present at high levels in peas (Han et al., 2006).The cell walls contributes a significant amount of fibre, predominately made up of cellulose and lignin levels (Hickling, 2003). The amounts of different carbohydrates in pea are given in Table 2.5.

Peas have high levels of soluble sugars ranging from 8.0 % to 9.4 % that vary with different pea genotypes (Rodrigues et al., 2012). Canadian pea varieties have approximately 12.5 percent of non-starch polysaccharide content, which is mainly made up of glucose, uronic acids, arabinose, xylose and galactose (Igbasan et al., 1997). Tosh et al. (2013) indicated that in peas the neutral sugar fractions has the highest ratio of insoluble to soluble fibre at 3.8:1 (Table 2.7). However, germination (for sprouts) has been reported to change the carbohydrate composition of the dietary fiber of peas (Table 2.8) (Martín-Cabrejas et al., 2003).

Table 2.3: Carbohydrate content of peas (Reddy et al., 1984).

Constituent	Content (%)
Total sugars	5.3 - 8.7
Sucrose	2.3 - 2.4
Raffinose	0.3 - 0.9
Stachyose	2.2 - 2.9
Verbascose	1.7 - 3.2
Starch	36.9 - 48.6
Cellulose	0.9 - 4.9
Hemicellulose	1.0 - 5.1
Lignin	0.5 - 0.9
Total carbohydrates	56.6

Table 2.4: Sugar and oligosaccharide composition of *Solanum tuberosum* (potato), *Cicer arietinum* (chickpea), *Lens culinaris* (lentil) and *P. sativum* (field pea) powders, expressed as a percentage of the dried powders (Tosh et al., 2013).

	S. tuberosum	***C. arietinum***	***L. culinaris***	***P. sativum***
Sugars (%)	1.7	3.08	1.81	3.21
Sucrose (%)	ND[2]	3.04 ± 0.57^{a}	1.80 ± 0.04^{b}	3.17 ± 0.37^{a}
Glucose (%)	1.69 ± 0.01^{a}	0.04 ± 0.03^{b}	0.01 ± 0.01^{c}	0.04 ± 0.01^{b}
Oligosaccharides (%)	ND	2.02	2.75	3.73
Raffinose (%)	ND	0.53 ± 0.03^{a}	0.32 ± 0.01^{a}	0.48 ± 0.07^{a}
Stachyose (%)	ND	1.49 ± 0.07^{c}	1.79 ± 0.06^{b}	2.36 ± 0.39^{a}
Verbascose(%)	ND	ND	0.64 ± 0.01^{b}	0.89 ± 1.17^{a}
Total (%)	**1.07**	**5.1**	**4.56**	**6.94**

Data are expressed as means ± SD (n = 6), means within a row followed by the same letter are not significantly different at $p < 0.05$; ND, not detected.

Table 2.5: Neutral sugar distribution in the soluble and insoluble fibre fractions calculated as a percentage of total carbohydrates (Tosh et al., 2013).

	C. arietinum	*L. culinaris*	*P. sativum*
Soluble			
Arabinose (%)	6.74 ± 0.18^{a}	6.31 ± 0.01^{a}	5.58 ± 0.11^{b}
Galactose (%)	4.32 ± 0.07^{a}	2.81 ± 0.07^{b}	2.55 ± 0.04^{b}
Glucose (%)	5.58 ± 2.62^{a}	6.32 ± 1.16^{a}	2.81 ± 0.37^{b}
Mannose (%)	2.18 ± 0.24^{a}	2.32 ± 0.36^{a}	2.12 ± 0.40^{a}
Rhamnose (%)	1.23 ± 0.09^{a}	0.56 ± 0.02^{a}	0.85 ± 0.09^{a}
Xylose (%)	0.52 ± 0.04^{a}	1.17 ± 0.09^{a}	1.03 ± 0.06^{a}
Total (%)	**20.6 ± 3.2**	**19.5 ± 1.8**	**14.9 ± 1.1**
Insoluble			
Arabinose (%)	9.02 ± 1.9^{a}	8.09 ± 0.23^{a}	9.08 ± 1.07^{a}
Galactose (%)	1.17 ± 0.15^{a}	1.54 ± 0.05^{a}	1.65 ± 0.08^{a}
Glucose (%)	33.05 ± 8.6^{c}	36.4 ± 0.6^{b}	40.7 ± 6.13^{a}
Mannose (%)	0.69 ± 0.06^{a}	0.57 ± 0.019^{a}	0.76 ± 0.06^{a}
Rhamnose (%)	0.45 ± 0.07^{a}	0.48 ± 0.06^{a}	0.57 ± 0.10^{a}
Xylose (%)	1.10 ± 0.27^{b}	4.56 ± 0.37^{a}	1.10 ± 0.15^{b}
Total (%)	**46.0 ± 11.1**	**51.6 ± 1.5**	**57.2 ± 8.7**

Data are expressed as means ± SD (n = 6 for soluble fibre, n = 18 for insoluble fibre), means within a row followed by the same letter are not significantly different at $p < 0.05$. (Tosh et al., 2013)

Table 2.6: Soluble sugar contents of dry pea flour samples (g/100 g) (Berrios et al., 2010)

	Ribose	Fructose	Glucose	Galactose	Sucrose	Maltose	Melibiose	Raffinose	Ciceritiol	Stachyose
Raw	0.52[b]	0.12[a]	0.04[a]	0.72[c]	0.65[a]	0.19[b]	0.16[b]	1.56[b]	ND	2.02[a]
Raw-formulated	0.70[b]	0.21[b]	0.04[a]	0.06[a]	0.69[a]	0.13[ab]	0.12[ab]	1.41[b]	ND	2.19[a]
Extruded	ND	ND	ND	ND	1.3[c]	ND	0.09[a]	0.816[a]	ND	1.53[a]
Formulated-extruded	0.21[a]	0.1[a]	0.06[b]	0.13[b]	0.86[b]	0.18[a]	0.21[b]	1.44[b]	ND	2.44[a]

Values represent means of three replicate analyses. ND: not detected. * Means within a column followed by different letters are significantly different ($p < 0.05$).

Many health benefits are attributed with the consumption of pea seeds. Compared to some other pulses such as lupins and beans, peas have low levels of gas producing oligosaccharides. (Hickling, 2003). Pea starch contributes to slow glucose release with a low glycemic index (Winham et al., 2007; Rizkalla et al., 2002), whereas dietary fiber is used for gastrointestinal health, prevention of constipation (Marlett et al., 2002).

2.6.2 Starch

Starch is an abundant and one of the major polysaccharides used by plants for energy storage. It is widespread in seeds, roots and tubers as well as in stems, leaves, fruits and pollen (Perez et al., 1996). In peas, starch is localised in the parenchymatous tissue of the seed cotyledons. Starch is accumulated in granules, which mainly constitute amylose and amylopectin and limited amounts of protein, lipid, mineral, and water (Tester et al., 2004a). Starch granules of legumes are densely packed and their size and shape varies among the genotypes, size ranging from 1 to 100 µm and their shape can be spherical, lenticular, oval, or irregular (Eliasson et al., 2006; Jane, 2004; Tester et al., 2004b; Oates, 1997). Starch granules of field pea are larger in size than those of some other legume starches (Gujska et al., 1994). The size of starch granules of field pea varies between 14 – 32 µm (width) and 15 – 37 µm (length) and the shape is predominately oval, but round, spherical, elliptical and irregular shapes can be found as well (Ratnayake et al., 2002; Gujska et al., 1994).

The empirical hydrated formula of starch is $(C_6H_{10}O_5 \cdot H_2O)_n$. Starch is a polymer of D–glucose molecules connected with glycosidic bonds and is made up of amylose and amylopectin. Amylose

has a linear molecular structure with relatively few branches, usually 9 to 20 per molecule with about 99 and 1 % of glycosidic bonds in the α–1,4 and α–1,6 form, respectively (Jane, 2004). The molecular weight of amylose ranges between 1×105 to 1×106 Dalton (Da), the length of amylose chains range from 200 to 700 glucose molecules and the average degree of polymerization (DP) is between 324 to 4,920 glucose molecules (Tester et al., 2004a,b; Buléon et al., 1998; Oates, 1997). This difference in size and structure depends on the origin of starch. In round pea the amylose fraction of starch ranges between 33.1 to 48.8% whereas in wrinkled pea, it ranges from 60.5 to 88 % with a DP range from 1000 to 1400 and about 2 to 3.2 branches per molecule (Ratnayake et al., 2002). Unlike amylose, amylopectin is highly branched with around 95 % of the glycosidic bonds being in the α–1,4 form and approximately 5 % in the form of α–1,6 bonds (Buléon et al., 1998; Oates, 1997). It has a much larger molecular weight than amylose with a range between 1×107 to 1×109 Da length of amylopectin chains from 12 to 120 glucose units and the average degree of polymerization (DP) is between 9,600 to 15,900 glucose molecules. (Tester et al., 2004a,b; Oates, 1997). Average amylopectin DP in starches from four pea cultivars (Carneval, Carrera, Grande and Keoma) ranged from 22.9 to 24.2 (Ratnayake et al., 2001).

Based on the botanical origin, the proportion of amylose and amylopectin varies among starch sources and according to the relative levels of these components, starches can be categorized. Therefore, the amylose to amylopectin ratio is an important factor in determining the starch characteristics. For most species the concentration of amylose and amylopectin is about 25 % and 75 % respectively. In waxy cultivars such as barley, maize, potato, rice, sorghum, and wheat, starch contains less than 15 % amylose and is made up only of amylopectin. On the other hand, sources having greater than 36 % amylose are called high–amylose variants (Jane, 2004; Tester et al., 2004a; Oates, 1997). In general, pulses are known to have a higher content of amylose as compared with other grains. Legume grains such as pea are characterized by a high content of amylose (Hoover et al., 1991). Starch in smooth pea and wrinkled pea usually differ in their amylose/amylopectin ratios and by the presence of an intermediate material of low molecular weight (Ratnayake et al., 2002). Round pea cultivars have 33.1 to 49.6 % of amylose content and wrinkled pea cultivars has 60 to 88 % amylose content (Eliasson et al., 2006; Ratnayake et al., 2002). Adsule et al. (1989) demonstrated that amylose content of wrinkled pea is higher than that of smooth pea starch. Gujska et al. (1994) reported that the high amylose content of the starch made the field pea more suitable for extrusion cooking compared to other legume starches.

The content of starch is highly influenced by pea genotypes and method of fractionation employed for its isolation. In peas, starch is the largest carbohydrate component accounting for 54% of the total weight of pea. However, the yield of field pea starch ranges from 30 to 63% and its purity (starch %) ranges from 78.7 to 99.5 %. Its purity is dependent on microscopic observation (absence of any adhering protein) as well as composition (low N/protein and low ash content) (Ratnayake et al., 2001). Tulbek et al. (2007) reported that starch concentration varies significantly among different pea genotypes and ranges from 43.9 to 40.9 %.

There are two main types of starch on the basis of degradation: resistant starch (RS) and non-resistant starch (NRS). Resistant starch is defined as that fraction of starch that is not digested and absorbed in the small intestine of humans, but is readily fermented completely or partially into short chain fatty acids by microorganisms in the colon (Osorio-Diaz et al., 2003; Bravo et al., 1998; Garcia-Alonso et al., 1998; Hoover et al., 1991). Resistant starch is further classified into four types on the basis of resistance against enzymatic hydrolytic degradation: physically inaccessible starch (RS1), native granular starch (RS2), retrograded starch (RS3) and chemically modified starch (RS4) (Mikulíková et al., 2008). Most of the studies reported on RS have been performed on wheat, amylomaize, and waxy maize (Hoover et al., 1991). Recently, legume starches have received attention since they are a good source of RSI and RS2. Wrinkled pea seeds is found to be a very rich source of resistant starch (Mikulíková et al. 2005). The content of resistant starch is proportional to the content of amylose and is found less in round field pea than in wrinkled pea. The studies of RS from grain-legumes such as lentil, chickpea (Costa et al., 2006; Mahadevamma et al., 2004, 2003; Garcia-Alonso, 1998), moth bean, horse gram, black gram (Mahadevamma et al., 2004; Bravo et al., 1998), different kinds of beans (Costa et al., 2006; Osorio-Diaz et al., 2003; Garcia-Alonso, 1998), mung bean, pigeon pea (Mahadevamma et al., 2004, 2003) and peas (Costa et al., 2006; Lehmann et al., 2003; Vasanthan et al., 1998) has been investigated. From the above studies, the authors asserted that RS content is higher in raw legumes (1.2 - 21.4 %) as compared to the processed legumes (0.27 - 8.0 %) (Costa et al., 2006; Lehmann et al., 2003; Bravo et al., 1998; Garcia-Alonso, 1998). The annealing process has been shown to enhance the RS content from 8.4 to 14.1 % and from 6.5 to 9.5 % for field pea and lentil starches, respectively (Hoover et al., 2003; Vasanthan et al., 1998). For this reason, the ingestion of legumes either raw or processed, has many beneficial implications such as reduced glycemic and insulinemic postprandial responses, the management of diabetes, preventing constipation and colon cancer (Cui, 2005;

Mahadevamma et al., 2004, 2003; Hoover & Zhou, 2003; Ratnayake et al., 2002; Bravo et al., 1998; Garcia-Alonso et al., 1998). Additionally, Cui. (2005) reported that RS has other greater diversity of health benefits equivalent to other similar fiber-enriched food ingredients such as decreasing dietary caloric values, preventing obesity, reducing blood cholesterol levels and controlling cardiovascular diseases. Beside health benefits, starch is a valuable ingredient in the food industry as it impacts gelatinization, pasting, solubility, swelling and digestibility properties (Singh et al., 2010b; Wiseman et al., 2006; Carré, 2004).

2.6.3 Protein

Production of plant protein has recently gained attention in food and non-food industry (Sanchez-Vioque et al., 1999). This can be attributed to a growing trend of consumers toward healthy food, replacing protein based food of animal origin to plant origin. Beans, grains and leaves are possible sources of plant proteins (Jongen et al., 2001). Due to the higher protein content in legume grains (17 – 40 %) and cereals (7 – 13 %), they are extensively used for the production of vegetable protein (Costa et al., 2006). Among legumes, soybean is mainly used for industrial production of food protein isolates, but pea is increasing in consumption (Tomoskozi et al., 2001). The protein content of field pea varies with respect to variety and environment and ranges from 21 to 25 % (GL-Pro, 2005; Hickling, 2003; McKay et al., 2003; Anderson et al., 2002). Pea proteins comprises of 21 % albumin, 66 % globulins and 12 % glutelins (Tomoskozi et al., 2001; Deshpande et al., 1990; Gueguen, 1983). The globulin protein is composed of legumin and vicilin, in the ratio of 1:4.2 (Gueguen, 1983). Pisumin, an antifungal protein is isolated from honey pea (*P. sativum* var. macrocarpon cv. sugar snap) (Ye et al., 2003). Pea proteins are highly digestible with a well-balanced profile of amino acids (Hickling, 2003). In comparison to cereal grain, pea protein has high levels of essential amino acid, lysine and threonine (7.3 %, and 3.7 % of total N, respectively). However, they have relatively low levels of sulfur containing amino acids such as methionine, cystine, and tryptophan (GL-Pro, 2005; Hickling, 2003; Oelke et al., 2000). Composition of amino acids in pea protein is listed in Figure 1.1 (Chapter 1). In addition, they have a low level of trypsin inhibitor usually less than 4 TlU / mg (TIU, trypsin inhibitor units), making it a crude protein source in most diets (Hickling, 2003; McKay et al., 2003; Anderson et al., 2002). Similar to soybean (Chung et al., 2003), protein content in field pea seeds is negatively correlated with lipid content, total yield and starch content (Al-Karaki et al, 1997; Abrahamsson et al., 1993).

Several studies have shown the feasibility of field pea protein in applications for both food and non-food industries. Since pea protein possess good nutritional and processing characteristics (an excellent amino acid balance), good functional properties (such as gelling, whippability, emulsifying and foaming properties), researchers have reported it as a promising protein source in food ingredients and an alternative to soybean protein (Nunes et al., 2006). In addition, due to its low level of anti-nutritional factors, non-allergenic properties, neutral taste and color and its GMO-free status, they find applications in processed foods and pharmaceuticals (Qi et al., 2004b). In pharmaceuticals, fermented (by lactic acid bacteria) pea protein is found to prevent the formation of Angiotensin-II (the potent vasoconstrictor) from Angiotensin-I and therefore it can be incorporated in prevention and treatment of hypertension (Vermeirssen, 2003). Pea proteins are used in the manufacture of packaging materials, as surfactants in coatings, paints, adhesives as well as a matrix material for the micro-encapsulation (De Graaf et al., 2001; Qi et al., 2004b).

The Kejaldahl method (AACC, 1986) is widely accepted method for protein determination in legumes. Though highly reliable, this method has many drawbacks such as it is labor-intensive and time consuming because it involves separate steps for protein digestion and quantification by titration which results in analysis of limited samples at a time. Another drawback of this method is that it leads to overestimation of proteins in the sample consisting of a large portion of non-protein nitrogen. However, there are number of newer methods available for rapid quantification of protein which include colorimetric methods like Lowry assay, Bradford assay and Bicinchoninic Acid (BCA). The International Union of Pure and Applied Chemistry (IUPAC) name for BCA is 2,2'-diquinonyl-4,4'-dicarboxylic acid. The BCA assay has many advantages over other methods, as it is highly sensitive for quantification of insoluble proteins, decreased sensitivity to interferences, exhibit color stability, needs just one reagent and is time efficient. The assay consists of two steps - first the reduction of Cu^{2+} to Cu^{+} by the protein, and second the complex formation between Cu^{+} and BCA to form a purple chromophore which is freely soluble in aqueous solution. The purple chromophore is formed by the chelation of one Cu^{+} and two BCA molecules (Figure 3.2).

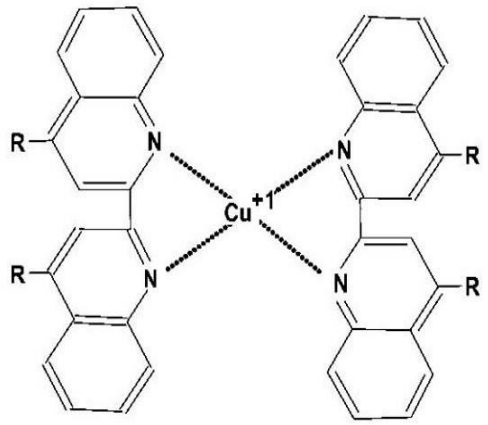


Figure 2.3: Complex formation between 2,2'-bicinchoninic acid and Cu^{+} (Owusu-Apenten, 2002)

2.6.4 Lipid

In legume seeds, stored lipids are the main source of dietary fat (Pattee et al., 1982). Research has investigated legume lipid composition, chemistry, flavor, off-flavor development, and their application in food industries (Pattee et al., 1982). Soybean and peanuts are major legume oilseeds with 18.0 - 22.0 % and 40 – 50 % oil respectively (Yoshida et al., 2004, 2003). Lipid from field pea have been investigated but there is limited published research on pea lipid content because of its low lipid fraction in the seed. The lipid content of pea ranges from 1.0 to 2.5 % of dry matter (Pryor, 2008; Ryan et al., 2007; Hickling, 2003; Anderson et al., 2002; El-Refai et al., 1987; Welch et al., 1984). Research by Sessa et al. (1977) reported 2.5 % of crude oil while identifying lipid-derived flavours of under-blanched pea seeds. In a study by the Canadian Grain Commission, 48 varieties were reported to have 1.0 to 1.7 % fat content (Canadian Grain Commission Grain Research Laboratory, 2004). However, some studies on field pea seeds reported total lipid of pea being as high as 4.7 % (Bastianelli et al., 1998) and 9.7 % (Letzelter et al., 1995). The lipid content of field pea seed varies with the environmental conditions, type of soil, variety and location (Srivastava et al., 2009; Welch et al., 1984). Even seed shape has a significant role with smooth peas possessing less lipid content than wrinkled varieties (Welch et al., 1984; Bastianelli et al., 1998). Nikolopoulou et al. (2007) found that the climatic conditions and soil characteristics of cultivated area and year significantly affected the amount of lipid (0.76 to 3.95 %) and its composition. The range of lipid content in peas obtained from different studies are summarized in Table 2.9. Peas grown in semi-arid locations had shown to have more fat production than those grown in arid areas (Al-Karaki et al., 1997). In addition, lipid content is found to reduce through

plant maturation (Daveby et al., 1993). The lipid or fat content of field peas is relatively low as compared to starch and proteins. The lipid content in the seed is independent of the protein content (Reichert et al, 1982). Whereas, data presented by Al-Karaki et al. (1997) confirms an inverse relation between yield, lipid, and starch content with protein and three types of sugar content (glucose, fructose and sucrose).

Table 2.7: Summary of earlier research on the total lipid content in *Pisum sativum.*

S.No.	**Study**	**Method of lipid extraction**	**% total lipids observed**
1.	*Khodapanahi et al., 2012*	Five extraction procedures were used: -Butanol -Hexane/isopropanol -Chloroform/methanol -Soxhlet with petroleum ether or with hexane,	0.9 - 5 %
2.	*Srivastava et al., 2009*	Association of Official Analytical Chemists (AOAC) method (1990)	1.1 - 1.5 %
3.	*Nikolopoulou et al., 2007*	Association of Official Analytical Chemists (AOAC) method (1998); solvent unknown	0.76 - 3.95 %
4.	*Ryan et al., 2007*	Hexane/Isopropanol (3:2 v/v)	1.5 %
5.	*Yoshida et al., 2007*	Chlorofom:methanol (2:1 v/v)	2.1 - 3.7 %
6.	*Daveby and et al., 2006*	Diethyl ether in a Tecator Soxtec System HT	1.9 - 2.6 %
7.	*Murcia and Rincon, 2006*	n-butanol (modified Morrison method)	1.2 - 3.5 %

8.	*Palander et al., 2006*	Diethyl ether (AOAC method 920.39), traditional Soxhlet extraction	2.7 %
9.	*Bastianelli et al., 1998*	Petroleum ether after acid hydrolysis - Coxon and Wright FAME by GC	1.9 - 4.7 %
10.	*Letzelter et al., 1995*	- Fourier-transform infrared spectroscopy Photoacoustic detection - Coxon and Wright method (quantitative FAME)	1.7 - 9.7 %
11.	*Hoover et al., 1988*	Hot n-propanol-water (3:1)	2.9 %
12.	*El-refai et al., 1987*	Fatty acid methyl esters (FAME) by GC-MS	0.41 %
13.	*Coxon and Wright., 1985*	- n-butanol (modified Morrison method) then FAME by GC (quantitative) -Gravimetric - chloroform:methanol (2:1) (modified Haydar and Hadziyev method) -Microgravimetric- chloroform:methanol (2:1)	1.4 - 4.0%
14.	*Welch and Grifiths., 1983*	FAME by GLC	1.37 - 2.80%

Methods for quantification of total lipids are broadly classified into two categories: extraction or non-extraction methods. Non-extraction methods are indirect methods which are based on the measurement of physical or chemical property of a sample (Akoh et al., 2002). Indirect methods include methods such as density measurement, dielectric, near-infrared spectroscopy, low-resolution nuclear magnetic resonance spectroscopy, ultrasonic, colorimetric and X-ray absorption (Akoh et al., 2002). For extraction methods, the lipids are separated from other compounds of the cell using the water insolubility property of the lipids (Rahman, 2008; Gunstone et al., 1994). Extraction methods are divided into two categories: solvents and non-solvent methods. Solvent methods are based on extraction of lipid content with one or more organic solvents. The method adopted for lipid extraction depends upon the nature of the sample (plant tissues, oilseeds, and marine samples) as well as the type of lipid composition. It is desirable to select a solvent which is highly soluble with the lipids and less or not soluble with other components of cells. Hexane, diethyl ether, petroleum ether, pentane, isopropanol, and methylene chloride are some of the solvents commonly used for extraction of lipids from oilseeds (Wrolstad, 2005; Akoh et al., 2002; Moreau et al., 2003). Beside single solvent extraction, methods involving the solvent combinations are used for the quantitative recovery of lipids. Such methods comprises of utilization of polar and non-polar solvent with different proportions. In non-solvent methods, lipid content is quantified by volumetric means, after digestion by chemical reagents. Dairy food analysis is performed using non-solvent methods (Wrolstad, 2005; Gunstone, 2004). Non-solvent methods include acid digestion methods, detergent method and physical method (Gunstone, 2004).

In plant, fatty acids are the major component of lipid content (Gunstone et al., 1994). In order to characterize the extracted oil, individual classes of fatty acids are first separated and then analysed. Lipids analysis methods comprises of bulk properties methods, chromatographic methods, spectrometric methods and enzymatic methods (Figure 2.4) (Akoh et al., 2002). Khodapanah et al., 2011 compared different extraction methods such as butanol extraction, hexane/isopropanol, chloroform/methanol, soxhlet extraction, Bligh & Dyer and microwave extraction and found that for field peas the most effective method was the Bligh & Dyer with 2.0 % of yield and Soxhlet being the least effective method with 0.8 % of yield.

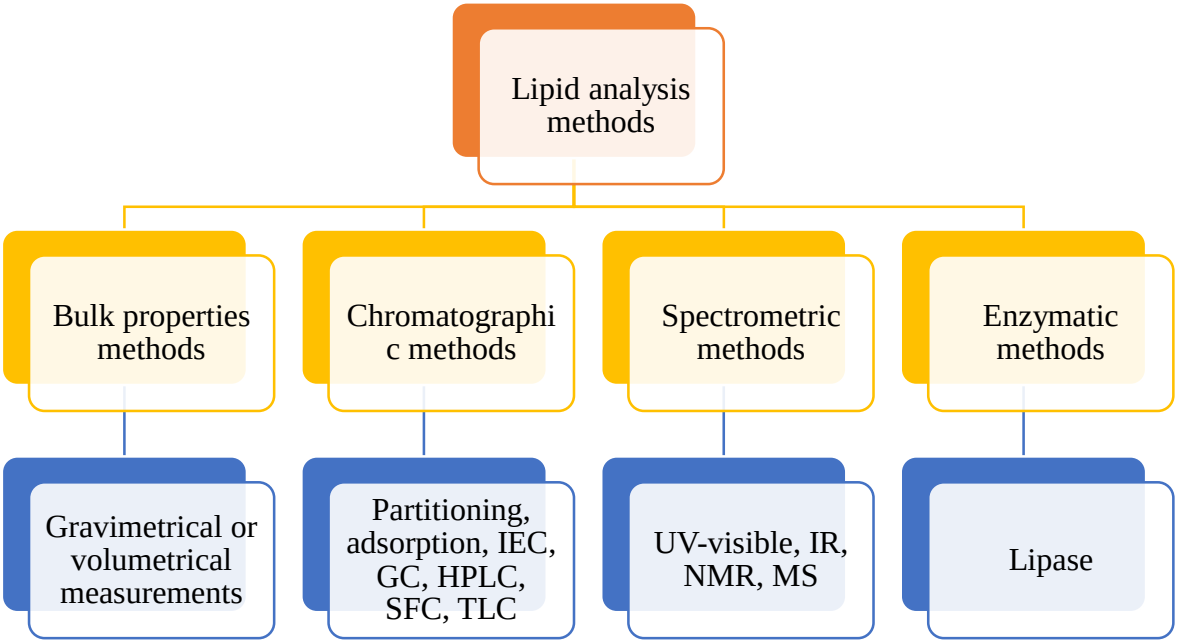


Figure 2.4: Various methods used for lipid analysis.

Field pea seed oil mainly consisted of 43.2 % neutral lipids, 3.2 % glycolipids and 53.6 % phospholipids, with the main components among them as 70% triacylglycerol in neutral lipid, 28% esterified sterol glycoside in glycolipid and 55 % phosphatidylcholine in phospholipid (Hoover et al., 1988). However, Yoshida et al. (2007), found slight difference in distribution of lipid content with phospholipids from 52.2 to 61.3 % and triacyglycerides from 31.2 to 40.3 %. Coxon et al. (1985) reported that 99 % of the total lipid content in field pea seeds was composed of palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18: 3) fatty acids. Summary of various fatty acids characterized in field pea are presented in Table 2.10. Like other grains, pea lipid is primarily composed of polyunsaturated fatty acid (Ryan et al., 2007). The fatty acid profile of different genotypes of field pea revealed that the amount of unsaturated fatty acids (79.2 - 86.2%) is higher than saturated fatty acids (15%) (Hickling, 2003; Kosson et al., 1994b). The unsaturated fatty acids basically comprises of linoleic (50 %), oleic (20 %) and linolenic (12 %) acids (Hickling, 2003; Kosson et al., 1994b). Among saturated fatty acids, pea seed lipid consists of 12.0 to 18.4 % palmitic acid and less than 4.2 % stearic acid. Welch et al. (1984) concluded that linoleic and oleic acids were the main unsaturated fatty acids of field pea, whereas palmitic was the main saturated fatty acid of field pea. Among the fatty acids in pea lipids (C16:0, C18:0, C18:1, C18:2, C18:3), only palmitic acid was significantly correlated with total lipid content (Welch et

al., 1984). Murcia et al. (1992) identified the fatty acid composition in field pea seeds of different sizes and indicated that in small (super fine from 4.7 to 7.5 mm; very fine from 7.6 to 8.2 mm) and medium fresh peas (fine from 8.3 to 8.8 mm) the most commonly found fatty acid is linoleic acid, whereas in larger seed accessions (middle from 8.9 to 10.2 mm) it is palmitic acid, and linolenic acid was the rarest fatty acid in all sizes. The authors further asserted that lipid accumulation halts when pea seeds are still quite small and when they grow in size, there is a change in lipid composition toward saturation of fatty acids. The fatty acid profile of pea differs with the cultivar, location, climate, season, and environmental conditions (Patte et al., 1982). Furthermore, distribution of lipid classes varies among the seed part. In smooth peas, oleic acid was present in higher quantity in germ lipid than in cotyledon lipid. Whereas, the hull lipids total saturated fatty acids are in higher concentration, usually palmitic acid (Kosson et al, 1994).

Table 2.8: Comparative table of the fatty acid composition of pea oil (%).

Fatty acids	Reported values %				
	Solis et al., 2013	Ryan *et al*., 2007	Wang & Daun, 2004	Murcia & Rincón, 1992	El-Saied *et al*., 1981
Palmitic (C16:0)	6.76	10.65	10.65	16.4	12.79
Stearic (C18:0)	2.86	3.29	3.29	15.2	2.41
Oleic (C18:1)	31.04	28.15	23.22	23.5	14.67
Linoleic (C18:2)	46.06	47.59	45.63	32.9	53.99
Linolenic (C18:3)	11.12	9.29	13.69	12	9.04
Arachidic (C20:0)	0.13	0.22	0.79	NR	NR
Gadoleic (C20:1)	0.25	0.21	0.62	NR	NR
Erucic (C22:1)	0.03	ND	0.24	NR	NR
Lignoceric (C24:0)	1.77	NR	0.33	NR	NR

NR- not reported, ND-not detectable

Pea seed lipids were found to contain four classes of sterols, namely: free sterol, sterol esters, sterylgylcosides and acylsterykglycosides (Miyazawa et al., 1974). Phytosterol, an unsaponifiable lipid fraction, has been reported in pea seeds with the concentration of 242 mg / 100 g on a dry-

mass basis (Ryan et al., 2007). Phytosterol has a broad range of biological effects, such as anti-inflammatory, anti-oxidative, anticarcinogenic activities and restraining the intestinal absorption of cholesterol.

2.7 Economic importance of field pea

2.7.1 Feed industry

Production of field pea is rapidly increasing throughout the world and a large portion of this pulse crop is now available for animal feed (Anderson et al., 2002). Different types of peas are used for different purpose such as fall-sown Austrian winter peas, which are primarily used as livestock feed. Austrian winter pea and maple pea are primarily used for forage or feed. In Europe and North America, whole seeds of field pea is ground and mixed with ground cereal grains for the manufacture of livestock feed (Anderson et al., 2002). Field pea has a high portion of digestible protein with well-balanced amino acid composition (Hickling, 2003) and is used as a source of protein concentrates for the formulation of livestock feed (Oelke, et al., 2000). Earlier research reported that field pea is used as a protein supplement for the formulation of livestock feed for swine, dairy cow, feeder calf, and poultry (GLPro, 2005; Hickling, 2003; McKay et al., 2003). The use of pea in terrestrial animal feeds include pig (Petersen et al, 2006; Stein et al., 2004; Brand et al., 2000), poultry (Nalle et al., 2011; Wiryawan et al., 1999) and pet animals like dogs and cats (Carciofi et al., 2008; DeOliveira et al., 2008; Bednar et al., 2001). Pea can be used with other grains to improve the feed protein quality (Hoang, 2012; Anderson et al., 2002). For example, the combination of field pea with canola results in an enriched diet for hogs, where canola will provide high levels of methionine and cystine to complement the lower levels in peas, and peas will provide high levels of lysine to complement the lower lysine levels in canola meal. Field pea is used extensively for aquaculture feed (Adamidou et al., 2009; Allan et al., 2004; Thiessen et al., 2003; Cruz-Suarez et al., 2001). Several reports have indicated field pea as a feed ingredient for rainbow trout (Burel et al., 1996; Gomes et al., 1995, 1993; Kaushik et al., 1993), silver perch (Allan et al., 1999, 1997; Booth et al., 1999), European sea bass (Gouveia et al., 2000, 1998; Santos et al., 1997), and blue shrimp (Davis et al., 2002; Cruz-Suarez et al., 2001). Several anti-nutritional factors such as tannins and anti-trypsin, have been completely or substantially eliminated in feed peas with the help of plant breeding (Castell et al., 1996). As a result, genotypes with a low trypsin inhibitor are

of greater interest for animal feed (Nalle et al., 2011; Morrison et al., 2007; Grosjean et al., 2000; Wang et al., 1998b).

2.7.2 Food industries

Legume seeds represents a good source of nutrition with a high proportion of protein, starch and oil. Apart from macronutrients, legumes contain appreciable amounts of dietary fiber, vitamins and minerals. Field peas are low in sodium and fat, high in protein, soluble and insoluble fibre, carbohydrates, B vitamins and minerals such as calcium, iron and potassium (Anonymous et al., 2011). As a result of the high nutritional factor, field peas show a high potential in human food applications (Rodrigues et al, 2012). About 10 % of field pea is consumed as whole or split seeds, which are either boiled or roasted and used in stews, soups, baking mixes, canned food, breakfast cereals, processed meats, health foods, pastas, and purees and the hull as bread fibre (Ratnayake et al., 2001; Saskatchewan Agriculture and Food, 2001). In field pea, three value-added components namely protein, starch and fiber have a huge potential in the food industry. Fiber fraction of pea is widely used in bakery products, including extruded snacks and special diets as it can be utilized as a texturing agent or enriching the fibre content of the food (Guillon et al., 2002). Field pea flour is known for its high quality protein, energy, vitamin B folate, gluten free trait and functional properties that are industrially important. There is growing interest in the use of pea protein as functional ingredients in the food industry. Tomoskozi et al. (2001) reported that the functional properties of pea protein isolate and its protein macro fractions (albumin and globulin) could potentially be used in several food applications. Due to its low level of anti-nutritional factors, non-allergenic properties, neutral taste and color and its GMO-free status, they find applications in the range of foods and pharmaceuticals (Qi et al., 2004b). There is growing interest among researchers in using pea proteins as analogues of meat and egg. Davis et al. (2010) investigated four meals with different amounts of soybeans or peas, namely: SOY pork chop (prepared from soybean), PEA pork chop, sausage partial PEA and PEA burger. The authors reported that utilization of pea meal provide equivalent energy. Various renowned companies such as Follow Your Heart and Hampton Creek Foods have launched an egg free food product that uses pea protein (Follow Your Heart, 2013; Hampton Creek Foods, 2013). However, chemically modified pea starch finds applications in deep-frozen dishes, dressings, extruded bakery products, instant soups and puddings. Unique physiochemical properties of field pea starch enable to be a

cheaper alternative for traditional starches with satisfactory results in noodle formation, canned foods, pates, cooked sausages and other foods (Czuchajowska et al., 1994).

2.7.3 Non-food applications

Pea protein products are used in the manufacture of films that can be utilized in packaging applications, surfactants in coatings, paints and adhesives (De Graaf et al., 2001). Advances in pea protein isolate indicated that it can be used as matrix material for the micro-encapsulation of 3-carotene (De Graaf et al., 2001). Additionally, pea starch finds many industrial applications since it is relatively cheap compared to traditional starches (Ratnayake et al., 2002). Field pea has a huge potential in detergent manufacture, carbonless paper production, waste water treatment, textiles, adhesives and plastics. It is used as an encapsulation agent, binding material for tablets, or disintegrating agent (Guillon et al., 2002; Agriculture and Agri-Food Canada, 1998).

Chapter 3

MATERIAL AND METHODOLOGY

3.1. Biological Samples

Out of 151 accessions of field pea (*P. sativum* L.*)* lipid content studied earlier in the laboratory, eight accessions catalogued as 29579, 43016, 45760, 29526, 29600, 42819, 36165 and 112351 were analyzed in this study. Seeds were procured from Agriculture and Agri-Food Canada (Saskatoon, Canada). These seeds were grown in a greenhouse and were harvested for two different years, with seeding on 7th April 2014 and 10th May 2015 at Macdonald Campus of McGill University (Ste-Anne-de-Bellevue: latitude 45.4039^{O} N. 73.9525^{O} W, QC, Canada). These seeds were surface sterilized by washing alternatively with distilled water and 70% ethanol prior to placing on moist tissue paper in petri plate and were allowed to germinate for 4 days, with regular sprinkling of water. The germinated seedlings were later on transferred to moistened rockwool cubes and were grown in a growth chamber (equipped with soft white light with light/dark period duration of 16/8, having 50 % relative humidity and the temperature was maintained at 20°C) for a week as shown in Figure 3.1. The plants in the rockwool were then planted in 1 L pots filled with a peat-vermiculite (2:1 volume ratio) mixture and were allowed to grow in the greenhouse (room was equipped with cool white fluorescent lamps (Model 830, Philips), an upward airflow distribution system providing ambient CO_2 conditions inside the room, having 70 % relative humidity and the temperature was maintained at 25 / 20°C during the light/dark period) until they were fully matured, dried and harvested (Figure 3.2). Harvested pods of the samples were dried in an oven at 40°C for 72 hr with no further treatment. Seeds were cleaned of dirt and other particles and stored in aerated sacs.

Figure 3.1: Germination of seeds of selected pea varieties in the growth chamber (equipped with soft white light with light/dark period duration of 16/8, having 50 % relative humidity and the temperature was maintained at 20°C).

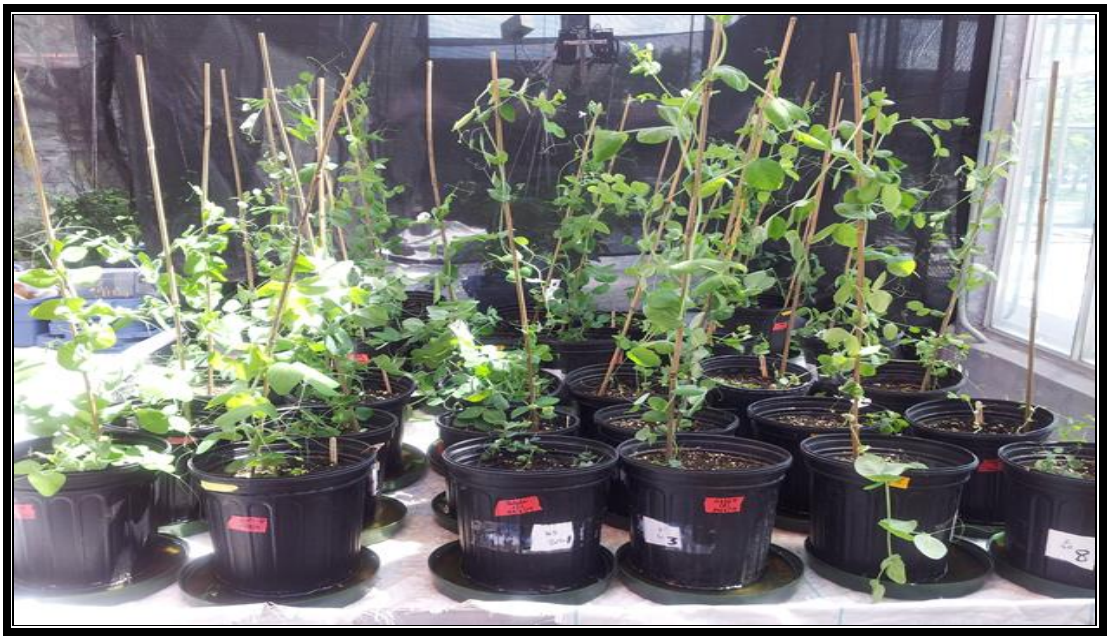
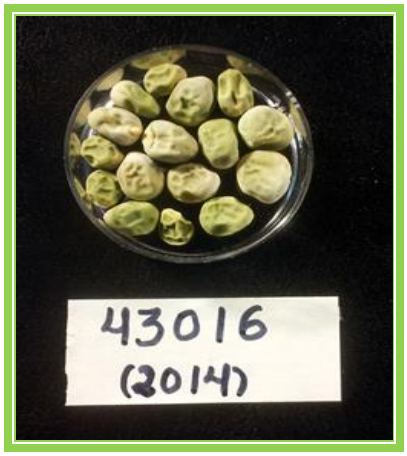


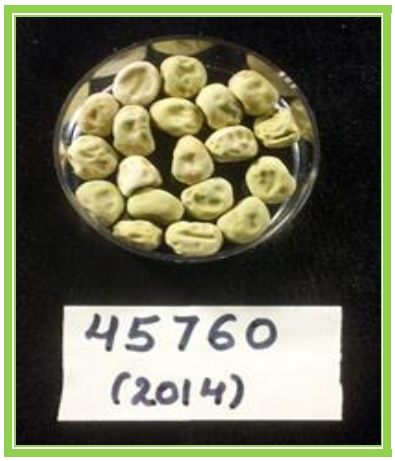
Figure 3.2: Varieties of *Pisum sativum* grown in a greenhouse (Macdonald Campus, Sainte-Anne-de-Bellevue, Canada).


29579
(2014)

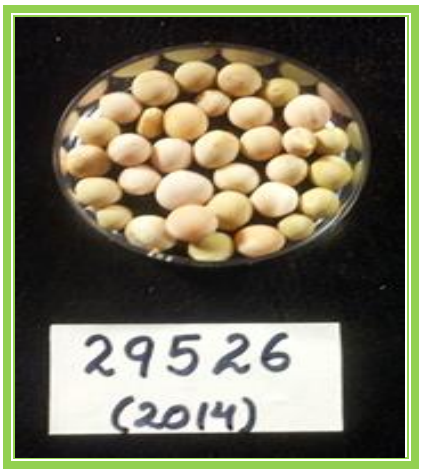
(a)


43016
(2014)

(b)


45760
(2014)

(c)


29526
(2014)

(d)


29600
(2014)

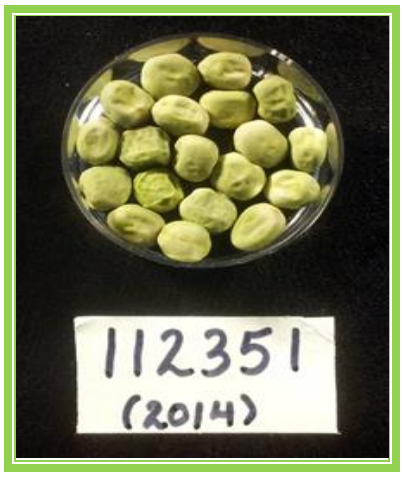
(e)


42819
(2014)

(f)


36165
(2014)

(g)


112351
(2014)

(h)


29579
(2015)

(i)

(j) (k) (l)

(m) (n) (o)

(p)

Figure 3.3: Harvested seeds of eight pea varieties grown for the year 2014 (a - h) and for year 2015 (i - p).

Seed characteristics such as seed colour and seed shape were visually compared and documented. Whereas, the width and height of healthy seeds were measured using vernier caliper and averaged among the seeds of same variety (Giami, 2002). Each seed was assumed as oblate spheroid with two segments (width and height) measuring maximum and minimum distance (Firatligil-Durmus et al., 2008).

The volume of the seed was therefore calculated using the Equation 3.1.

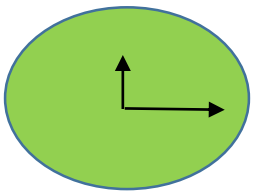


Figure 3.4: An oblate spheroid used to determine the volume of the seeds

$$V_{\text{oblate spheroid}} = \frac{4}{3}\pi a^2 b \qquad (3.1)$$

3.2 Materials

Pierce BCA Protein Assay Kit (Rockford, IL, USA) was used for determining the protein content and Megazyme Resistant Starch Kit (Megazyme, Ireland) was used for analyzing the starch content of the samples. All other chemicals used were of analytical grade (Fisher Scientific, Ottawa, Canada, and Sigma-Aldrich, Canada).

3.3 Plant Extraction

A measured amount of approximately 6 g of dried seeds were selected for each accession and ground in a grinder (Blackdecker, SmartGrind) until the field peas became a powder. One grams of each pea powder was extracted in 10 ml of methanol by maceration in a tube rotator (VWR, H005302, Mississauga, Ontario, Canada) with temperature control (37 °C) at a constant stirring rate of 200 rpm (Figure 3.5 a). After 24 h, infusions were filtered through Whatman No. 4 filter paper and filter cake was re-extracted twice with equal volume of solvents (Figure 3.5 b). Supernatants were combined and dried using a nitrogen evaporator (NEVAP-111, Berlin, MA, USA), at 40 °C until no observable liquid was visible. The obtained extracts were transferred into sterile sample tubes and methanol was added (ranging from 3-5 ml) in order to make a

concentration of 10 mg / ml (Figure 3.5 c). Plant extracts were stored in a refrigerator at 4 °C until further use.

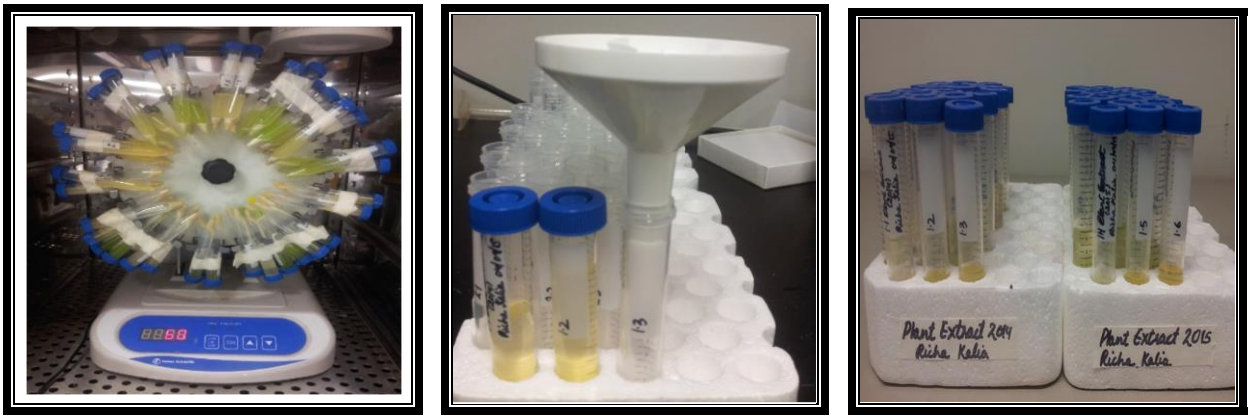


Figure 3.5: Overview of preparation of sample extracts in methanol (a-c). (a) Plant extraction in rotatory shaker, (b) Filtration of extract, (c) Methanolic sample extracts.

3.4 Proximate Analysis

3.4.1 Ash and Moisture Content

A measured amount of approximately 500 mg of ground field pea sample was weighed into a dry pre-weighed 30 ml porcelain crucible. The crucibles along with the samples were dried in an oven (Isotemp Oven, Fisher, 200 Series model 230F) for 16 hrs at 105 °C. Samples were removed from the oven and allowed to cool in drierite (AOAC, 2000). Samples were weighed and moisture content (wet basis) was calculated as below (Equation 3.3):

Dry matter (DM) was determined as follows:

% DM = (dry mass / wet mass) x 100 (3.2)

% Moisture = (100 - % DM) (3.3)

After determining the dry matter content, the crucible and the content were placed in a muffle furnace (Type 47900 & 48000 Furnaces, Barnstead International) for 240 minutes at 600 °C to determine the ash content. Thereafter, the crucibles were transferred to drierite and allowed to cool. Samples were weighed soon after cooling inorder to prevent moisture absorption (AOAC, 2000). The ash content was calculated as:

% Ash = (Ash mass / Wet mass) x 100 (3.4)

Figure 3.6: Ash content of selected seed varieties of *Pisum sativum* in crucible after 4 hr in furnace at 600 °C.

3.4.2 Protein Determinations

The total protein content of the field pea samples were determined by Bicinchoninic Acid Assay as describes by Chan and Wasserman (1993). Aqueous extract of the samples were prepared to a concentration of 1 mg/ml. 100 µl of each sample was taken in 2 ml microcentrifuge tubes and 20 µl of 2 % SDS was added to remove the interference of lipids with protein estimation (as specified in the kit) (Pierce Chemical Co., Rockford, IL). 2 ml of BCA reagent (Pierce Chemical Co., Rockford, IL) was added to the above mixture. The BCA Reagent was prepared by combining 50 ml of reagent A and 2 ml of reagent B (to eliminate the copper chelating agent) as specified in the kit (Pierce Chemical Co., Rockford, IL). The samples were then incubated at 37 °C for 30 minutes in a water bath (Isotemp 4100 R20 115VAC 60 Hz, Fisher Scientific) and were intermittently mixed on a vortex mixer (Standard 120V, Fisher) every 10 minutes. To stop the reactions, samples were cooled on ice for 5 minutes and centrifuged at 3000 rpm for 10 minutes. 0.2 ml of the supernatant was carefully transferred into another centrifuge tube and was diluted with 0.8 ml of BCA reaction buffer A. The solution was mixed using a vortex mixer (Standard 120V, Fisher). The absorbance of the sample was measured at 562 nm versus the blank using a spectrophotometer (LTQ XL, Thermo Scientific). Bovine serum albumin (Pierce Chemicals, stock concentration 2 mg/ml) was used as the protein standard. A standard curve of bovine serum albumin was prepared with concentrations of 0-100 µg / ml (7 data points evenly spaced) (Figure A4).

Figure 3.7: Protein test for selected seed varieties of *Pisum sativum* for the year 2014 and 2015.

3.4.3 Total Carbohydrate

Total carbohydrate in the field pea samples was determined using an anthrone method as explained by Hedge and Hofreiter, 1962. In this method, carbohydrates in hot acidic medium is first converted to hydroxymethyl furfural, which forms a green colored product with anthrone and has a maximum absorption at 630 nm. A measured amount of ground pea samples, of approximately 50 mg of the each sample was treated with 5 ml of 2.5 N HCl and kept in a boiling water bath (Isotemp 4100 R20 115VAC 60 Hz, Fisher Scientific) for three hours. The samples were cooled and sodium carbonate was added to neutralise it until the effervescence ceased. The solution was brought up to 50 ml with distilled water and centrifuged at 3000 rpm for 10 mins. 1 ml from the supernatant was further mixed with 4 ml of anthrone reagent and the reaction mixture was heated for 8 minutes in a boiling water bath and cooled at room temperature. The absorbance of the green coloured solution was determined at 630 nm versus the blank using the spectrophotometer (LTQ XL, Thermo Scientific). A standard curve of D-glucose was prepared with concentrations from 0 - 100 µg / ml (7 data points evenly spaced) (Figure A5). Total carbohydrate values were expressed as glucose equivalents (mg of glucose / volume of sample).

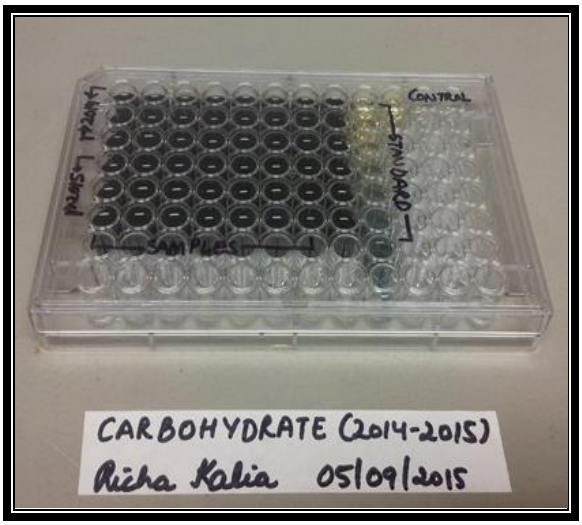


Figure 3.8: Total carbohydrate determination for selected seed varieties of *Pisum sativum* for the year 2014 and 2015 at 630 nm.

3.4.4 Total Starch

Resistant and non-resistant starch content of the samples was analyzed by using Megazyme Resistant Starch Assay Procedure which is based on AOAC Method 2000.02 and AACC Method 32-40.

3.4.4.1 Hydrolysis and Solubilisation of Non-resistant Starch

100 mg of each sample was weighed in a 15 ml centrifuge tube. 4 ml of freshly prepared pancreatic α-amylase containing dilute amyloglucosidase (AMG) (3 U / ml) (U denotes Units of activity of an enzyme) (as specified in the kit) was added to each tube, vortexed and incubated in a shaker at 37 °C for exactly 16 hrs. The tubes were removed from the incubator and the contents were treated with 4 ml of ethanol (99 % v/v) with vigorous stirring on a vortex mixer (Standard 120V, Fisher Scientific). The tubes were then centrifuged at 3300 rpm for 10 minutes. The supernatants were decanted carefully in separate 50 ml centrifuge tubes and the pellets were re-suspended in 2 ml of 50 % industrial methylated spirits (IMS) and mixed vigorously on a vortex mixer (Standard 120V, Fisher). Another 6 ml of 50 % IMS was added to the tubes, mixed and the tubes were centrifuged at 3300 rpm for 10 minutes. The supernatants were decanted and the suspension and centrifugation step was repeated once more.

3.4.4.2 Measurement of Resistant Starch:

Tubes were placed in an ice water bath and 2 ml of 2 M KOH was added to each tube to re-suspend the pellets. Tubes were then stirred for 20 minutes in a mechanical shaker and 8 ml of 1.2 M of sodium acetate buffer (pH 3.8) was added to each tube. This was followed by the addition of 0.1 ml of AMG (3300 U/ml). The contents were mixed well and the tubes were then placed in a water bath maintained at 50 °C. The tubes were incubated for 30 minutes with intermittent mixing on a vortex mixer (Standard 120V, Fisher) every 10 minutes. The tubes were centrifuged at 3300 rpm for 10 minutes. 0.1 ml of this supernatant was transferred to another tube and mixed with 3 ml of glucose determination reagent (GOPOD reagent) (provided in the kit). A blank was prepared by mixing 0.1 ml of 100 mM sodium acetate buffer (pH 4.5) and 3 ml of GOPOD reagent. D-glucose standard solution was prepared by mixing 0.1 ml of D-glucose (provided in the kit) with 3 ml of GOPOD reagent. All tubes containing samples, blank and D-glucose standard solution were incubated at 50 °C for 20 minutes. The tubes were brought to room temperature and absorbance was read at 510 nm against reagent blank using the spectrophotometer (LTQ XL, Thermo Scientific).

Figure 3.9: Test for resistant starch for selected seed varieties of *Pisum sativum* for the year 2014 and 2015.

The resistant starch content was determined as follows using Equation 3.6:

Resistant starch (RS) (g / 100 g sample), for samples containing less than 10% RS:

$RS = \Delta E \times F \times (10.3 / 0.1) \times (1 / 1000) \times (100 / W) \times (162 / 180)$ (3.5)

$RS = \Delta E \times (F / W) \times 9.27$ (3.6)

Where, ΔE = absorbance read against reagent blank

F = 100 (μg of D-glucose) / GOPOD absorbance for this 100 μg of D-glucose

3.4.4.3 Measurement of Non-resistant (Solubilised) Starch:

The supernatant solutions that were collected after the centrifugation of the initial washing with 99 % ethanol and after two subsequent washings with 50 % IMS (procedure continued after hydrolysis and solubilisation of non-resistant starch) were combined and the volume was made up to 100 ml in a volumetric flask using 100 mM sodium acetate buffer (pH 4.5). 0.1 ml of this solution was mixed with 10 μL of dilute AMG solution (300 U / ml) (as specified in the kit) and incubated for 20 minutes at 50 °C. Thereafter, 3 ml of GOPOD reagent was added to the samples. A blank was prepared by mixing 0.1 ml of 100 mM sodium acetate buffer (pH 4.5) and 3 ml of GOPOD reagent. D-glucose standard solution was prepared by mixing 0.1 ml of D-glucose (provided in the Megazyme Resistant Starch Kit, Megazyme, Ireland) with 3 ml of GOPOD reagent. All the samples, reagent blank and D-glucose standard solution were incubated at 50°C for another 20 minutes. The tubes were brought to room temperature and absorbance was measured at 510 nm.

Figure 3.10: Test for non-resistant starch for selected seed varieties of *Pisum sativum* for the year 2014 and 2015.

The non-resistant starch content was determined as follows using Equation 3.7:

Non-resistant (solubilised) starch (g/100g sample) = $\Delta E \times F$ X $(100 / 0.1) \times (1 / 1000) \times (100 / W) \times (162 / 180) = \Delta E \times (F / W) \times 90$ (3.7)

Where, ΔE = absorbance read against reagent blank

F = 100 (μg of D-glucose) / GOPOD absorbance for this 100 μg of D-glucose

The total starch content was calculated as the sum of resistant starch and non-resistant starch using Equation 3.8:

Total starch = Resistant starch + Non-resistant starch (3.8)

3.4.5 Lipid Determination

Lipid content of field pea was determined by extraction using a modified version of the method described by Ryan et al 2007. Two grams of ground field pea sample were placed in 50 ml Teflon-lined screw-capped glass centrifuge tubes (Fisher Scientific) of known mass. Two biological preplicates were completed for eight pea accession lines and one control for each sample. Lipids were extracted with 3 ml of hexane–isopropanol (3:2, v/v) at room temperature with constant stirring for 2 h. Thereafter, the samples were vortexed in vortex mixer (Standard 120V, Fisher) for 30 s followed by centrifugation at 6000 rpm for 10 min. Supernatant was carefully transferred into new 50 ml glass centrifuge tubes. The solid centrifuge pellets were washed twice with 2 ml of the hexane-isopropanol (3:2, v/v), each time vortexed for 30 s, centrifuged at 6000 rpm for 10 min and the hexane: isopropanol layers were recovered. The remaining centrifuge pellets was dried under nitrogen at 70 °C until the remaining solvent was completely evaporated. The sample tubes, along with the control were un-capped and placed in the oven for 24 h at 95 °C. The tubes were taken out of the oven and allowed to stand until they reached room temperature. Their final masses were measured and recorded. The difference between the initial and final mass of the control tube, represents the moisture loss during the drying period. The lipid percentage of the sample, was measured by subtracting the initial mass minus the final mass and minus the moisture loss based on the control sample for each sample tube. The recovered hexane–isopropanol extracts were centrifuged at 3000 rpm for 10 min and the supernatant transferred to a new known mass glass centrifuge tube. The recovered solvent was nitrogen evaporated in nitrogen evaporator (NEVAP-

111, Berlin, MA, USA) under 50 °C and lipids were dissolved in chloroform for storage and further sample preparation.

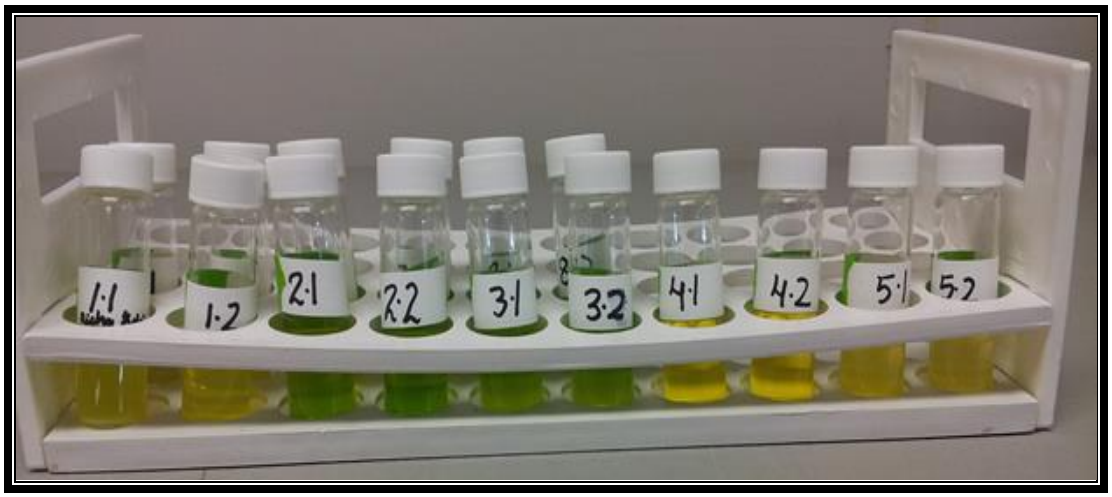


Figure 3.11: Lipid extracted from selected seed varieties of *Pisum sativum*.

3.5 Phytochemical Analysis

3.5.1 Flavonoids Determination

The content of total flavonoids in the examined plant extract was measured as reported by Zhishen et al. (1999) with little modification. In brief, 0.25 ml of each extract solution in the concentration of 10 mg/ml was mixed with 1.5ml of distilled water and subsequently with 0.15 mL of a $NaNO_2$ solution (5 %). After 5 mins of incubation, 0.15 mL of an $AlCl_3$ solution (10 %) dissolved in methanol (Quettier et al., 2000) and 1.0 mL of 1 M NaOH solution were sequentially added. The solution was vigorously shaken and the absorbance was measured at 510 nm versus the blank using spectrophotometer (LTQ XL, Thermo Scientific). Quercetin was used as a standard. A standard curve of quercetin was prepared with concentrations 0-100 µg / ml (7 data points evenly spaced) (Table A2). Total flavonoid values were expressed as quercetin equivalents (mg quercetin / g of dried extract).

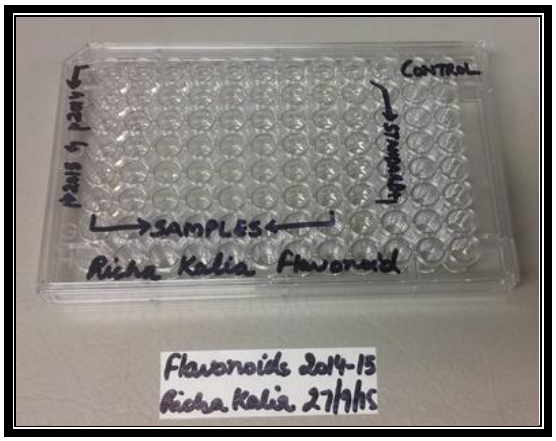


Figure 3.12: Flavonoid test for selected seed varieties of *Pisum sativum* for the years 2014 and 2015.

3.5.2 Antioxidant Activity Determination

Antioxidant activity in the peas samples was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical assay as reported by Stanojevic et al. (2009) with some modifications. In brief, 0.1 ml of methanolic extract solution (10 mg / ml) of each sample were mixed with 0.9 ml of freshly prepared 0.1 mM DPPH solution in methanol. The mixture was allowed to stand for 30 minutes in darkness at room temperature. The absorbance of the samples was measured at 517 nm versus the control using the spectrophotometer (LTQ XL, Thermo Scientific). The control sample contained 0.1ml methanol and 0.9 ml of DPPH. Ascorbic acid was used to generate a standard curve with concentrations between 0-100 µg / ml (data points evenly spaced) (Table A3). The capacity of scavenging free radicals was calculated as follows:

$$\text{Scavenging activity } \% = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) X\ 100 \qquad (3.9)$$

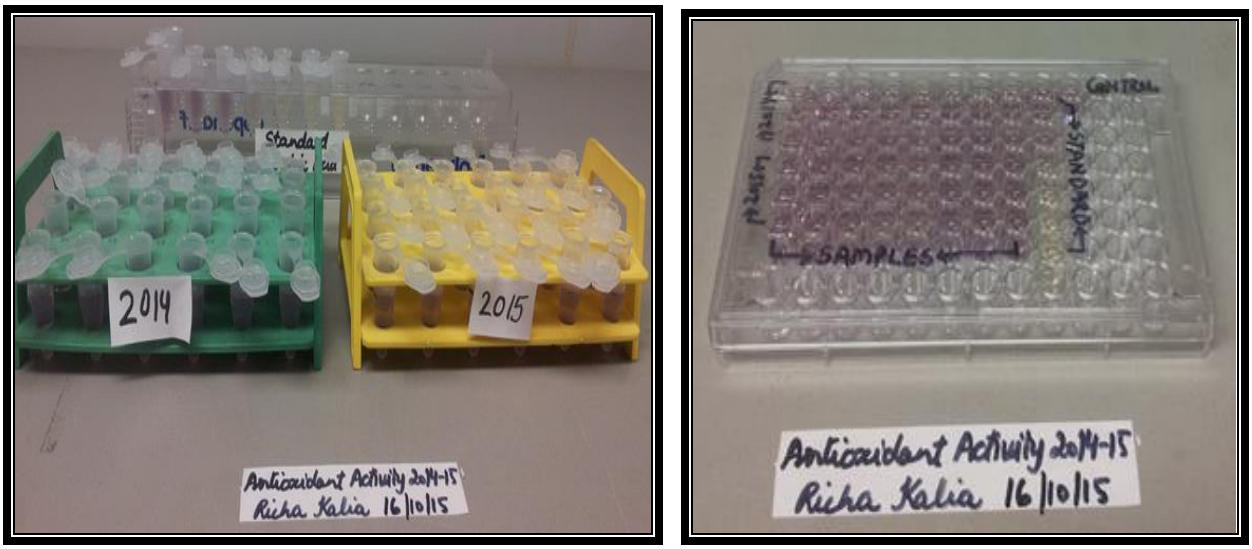


Figure 3.13: Antioxidant activity of selected seed varieties of *Pisum sativum* for the years 2014 and 2015.

3.5.3 Phenolic Determination

Phenolic content in the field pea sample were estimated using the spectrophotometric method by Singleton et al., 1999 with some modifications. A methanolic solution of the extracts in the concentration of 10 mg / ml was used in the analysis. The reaction mixture was prepared by mixing 0.25 ml of methanolic solution of extract, 0.25 ml of Folin-Ciocalteu's reagent and 2.25 ml of distilled water. After standing for 6 minutes, 2.5 ml 7 % $NaHCO_3$ was mixed. The blank contained 0.25 ml methanol, 0.25 ml Folin-Ciocalteu's reagent, 2.25 ml distilled water and 2.5 ml of 7 % of Na_2CO_3. After incubation for 60 min at room temperature, the absorbance of the samples was measured at 760 nm versus the blank using spectrophotometer (LTQ XL, Thermo Scientific). Gallic acid was used as standard to generate a standard curve with concentrations 0-100µg/ml (7 data points evenly spaced) (Table A1). The content of phenolics in extracts were expressed as gallic acid equivalents (mg of GA / g of dried extract).

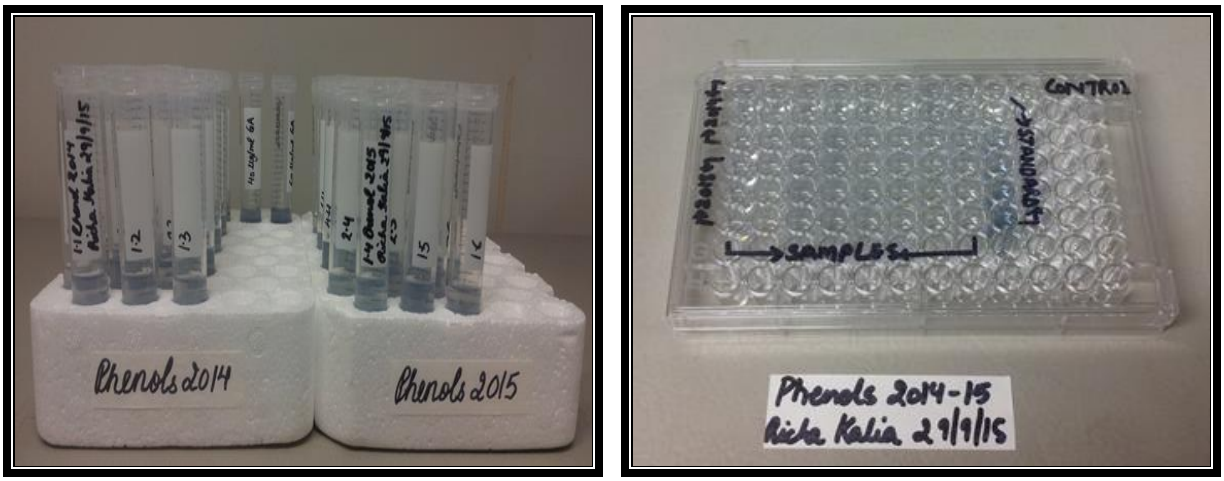


Figure 3.14: Total phenols test for selected seed varieties of *P. sativum* for the years 2014 and 2015.

3.5.4 Chlorophyll and Carotenoid Determination

Chlorophyll a, chlorophyll b and carotenoid in the plant extract were determined using the equation as reported by Holden (1976) and Smith Benitez (1955). 0.25 ml of methanolic extract of each pea samples was loaded in 96 well-microtitre plate. The absorbance of the plate was read at 666 nm, 653 nm and 470 nm for chlorophyll a, chlorophyll b and carotenoid respectively. The following equations are used for determination of chlorophyll and carotenoid in the sample (µg / ml of plant extract):

$$C_a = 15.65\, A_{666} - 7.34\, A_{653} \quad (3.10)$$

$$C_b = 27.05\, A_{653} - 11.21\, A_{666} \quad (3.11)$$

$$C_{x+c} = \frac{1000\, A_{470} - 2.86\, C_a - 129.2\, C_b}{245} \quad (3.12)$$

Where, C_a = Chlorophyll a

C_b = Chlorophyll b

C_{x+c} = Carotenoid

Figure 3.15: Pigment analysis for selected seed varieties of *Pisum sativum* for the years 2014 and 2015.

3.6 Statistical analysis

Statistical analyses of data were performed using SAS (SAS 9.4, SAS Institute, Inc, Cary, NC, USA). For most of the experimental variables the ANOVA residuals were not normally distributed, as a result non-normal distribution assumptions with generalized linear mixed ANOVA was appropriate (SAS PROC GLIMMIX) with the lognormal distribution specified (DIST=LOGN) and the year specified as a random component. The plausibility of the models were assessed with the Bayesian Information Criterion. For percentage variables the beta-binomial distribution was specified (DIST=BETA).). The lipid data was investigated more rigorously, and a generalized linear model ANCOVA was fit to the lipid data using SAS PROC GLIMMIX with scavenging activity as the covariate. The fit statistics indicated that the carotenoid variable was Gamma distributed (DIST=GAMMA).

For some of the distribution specifications, link functions were necessary. The link function transforms the Y side of the equation. For example the logit link function: Ln (pi/1-pi) = [model equation].

When a link function was necessary, the estimates of the model (which are used for inference) were of course not on the scale of measurement. To express the estimates of the least squares-means on the scale of measurement, the estimates were back-transformed by inverting the link function. Comparisons between least squares means were Scheffé-adjusted.

Chapter 4

RESULTS AND DISCUSSION

4.1 Phenotypic traits

Phenotypic traits were observed for the eight selected accessions which were grown for two separate years to maturity and produced seeds for the examination (Table 4.1).

Table 4.1: Phenotypic characters of selected seed varieties of *P. sativum*.

Year	Variety	Seed Colour	Seed surface	Seed Volume(mm^3)
	29579	brown-green	smooth	94.01
	43016	green	wrinkled	259.96
	45760	green	wrinkled	215.89
	29526	yellow	smooth	232.84
	29600	yellow-green	wrinkled	133.00
	42819	yellow	smooth	244.53
	36165	yellow	smooth	169.49
2014	112351	green	mix	219.86
	29579	brown-green	smooth	97.63
	43016	green	wrinkled	217.56
	45760	green	wrinkled	218.60
	29526	yellow	smooth	214.24
	29600	yellow-green	wrinkled	157.17
	42819	yellow	smooth	289.58
	36165	yellow	smooth	210.91
2015	112351	green	smooth	261.73

Different seed color, such as yellow, green and brown were observed among the cultivars, but the majority of seeds fall in the spectrum from yellow to green. The average length and width of pea seeds were 7.60 and 6.48 mm respectively, with different cultivars varying in their seed volume (Figure 4.1). Similar seed volume results were reported by Yalcin et al. (2007). Pea seeds were found to have smooth or wrinkled seed surface, which were equally distributed among the cultivars. Binary variables based on the smooth and wrinkled surface characteristics were highly negatively correlated. While smooth seed coat was strongly correlated with starch content ($r = 0.708$, $p = <.0001$) and negatively correlated with lipids ($r = -0.342$, $p = 0.0174$), but wrinkled seeds were negatively correlated with starch content ($r = -0.577$, $p = <.0001$) (Table A1). A study by Stickland et al. (1993) indicated that in wrinkled seeds one of the major isoforms of the enzyme that encodes starch-branching is missing, as a result, there is an overall lower starch synthesis in the pea.

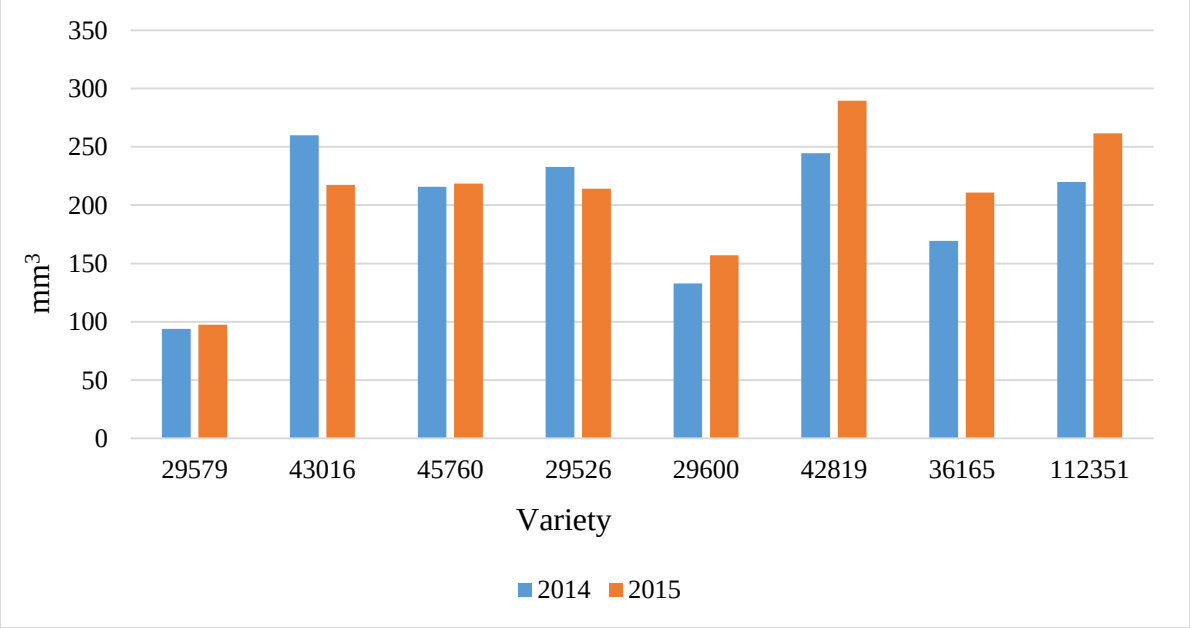


Figure 4.1: Graphical representation of seed volume arithmetic mean value for each varieties for the year 2014 and 2015.

4.2 Chemical composition

Relationships between field pea seed lipid, protein, starch, carbohydrate, ash, moisture, phytochemical constituents and pigments concentrations of eight pea cultivars were examined over two experimental years. Most of the variables were significantly influenced by cultivar and year (Table A1). The years differed markedly and was highly correlated with the moisture content (r = 0.865, p = < .0001), carbohydrate (r = -0.552, p = < .0001) and carotenoid (r = 0.866, p = < .0001). Significant differences were observed among pea varieties, differing in terms of chemical and phytochemical constituents (Table 4.1).

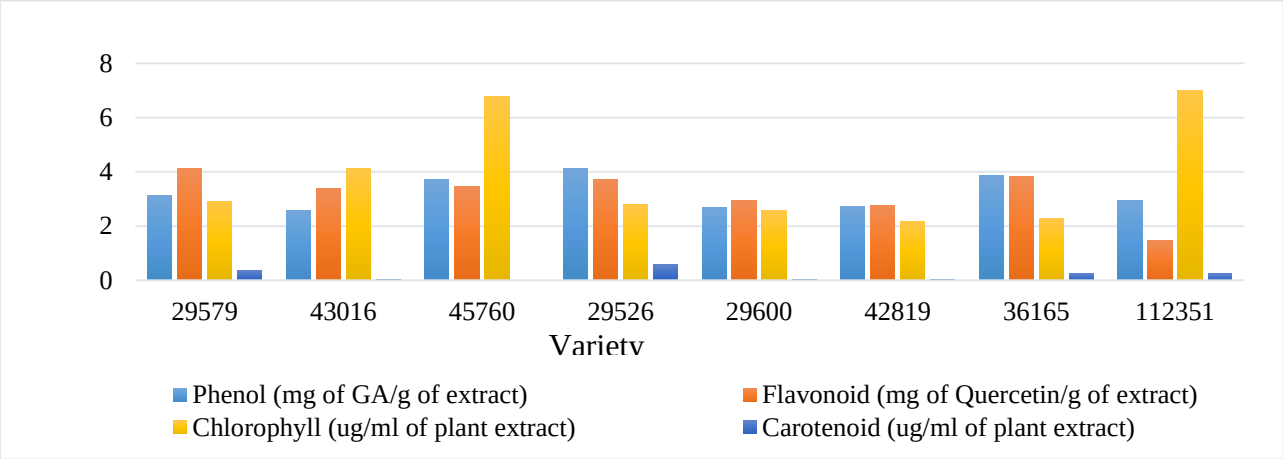


Figure 4.2: Graphical representation of arithmetic mean values of pigments and phytochemical analysis of Canadian field pea cultivars for the year 2014.

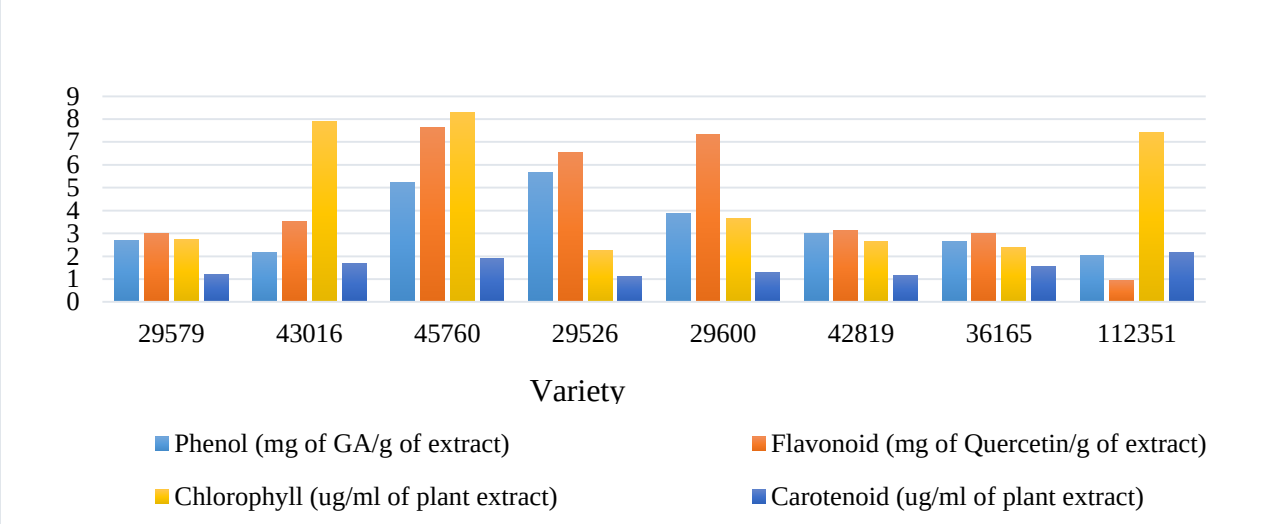


Figure 4.3: Graphical representation of arithmetic mean values of pigments and phytochemical analysis of Canadian field pea cultivars for the year 2015.

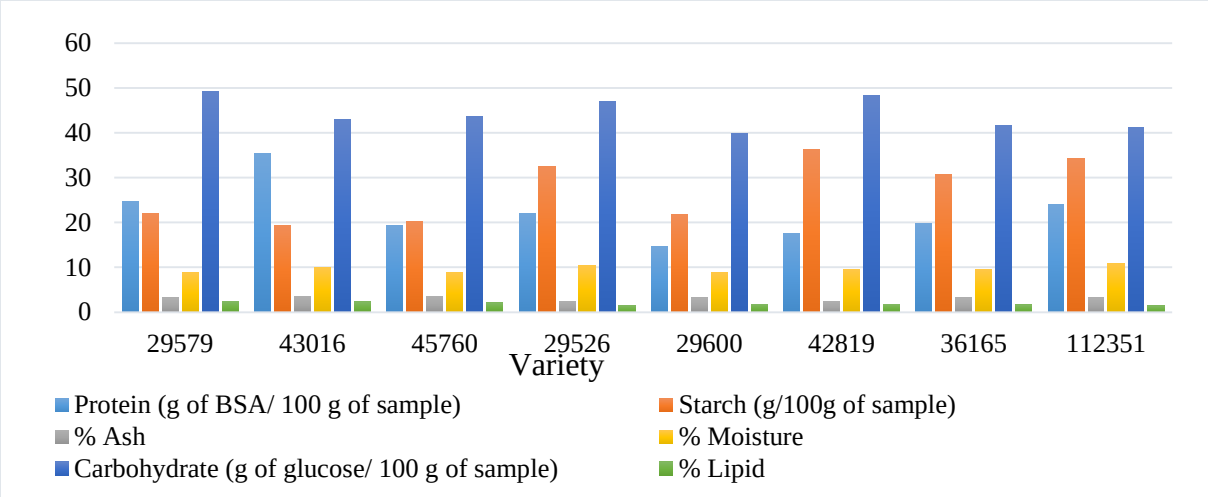


Figure 4.4: Graphical representation of arithmetic mean values of proximate analysis of Canadian field pea cultivars for the year 2014.

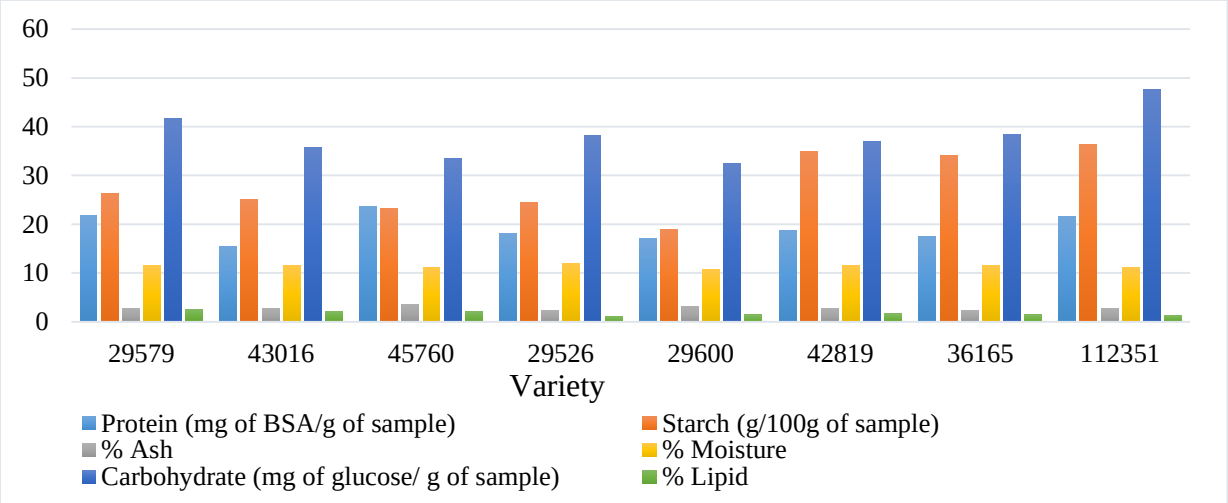


Figure 4.5: Graphical representation of arithmetic mean values of proximate analysis of Canadian field pea cultivars for the year 2015.

4.2.1 Proximate analysis

The results for proximate composition are presented in Table 4.2. Carbohydrate was the most abundant component in the field pea seeds, ranging from 357 to 453 mg of glucose / g of sample (Table 4.2, Figure 4.6). However, a higher amount of carbohydrate (51.0 - 56.5 %) was reported in the previous studies (Hickling, 2003, Anderson et al., 2002, Adsule et al., 1989).

Table 4.2: Least Squares Means of some chemical constituent for each variety.

	Protein	Starch	Carbohydrate
Variety	Back-transformed Mean(mg of BSA/g of sample)	Back-transformed Mean(g/100g of sample)	Back-transformed Mean(mg / g of sample)
29526	199	27.85	423
29579	228	24.01	453
29600	155	20.25	357
36165	184	32.11	400
42819	181	35.07	423
43016	232	21.93	390
45760	209	21.50	382
112351	225	34.75	443

*Back-transformed mean values are of estimates which are made on the natural log scale.

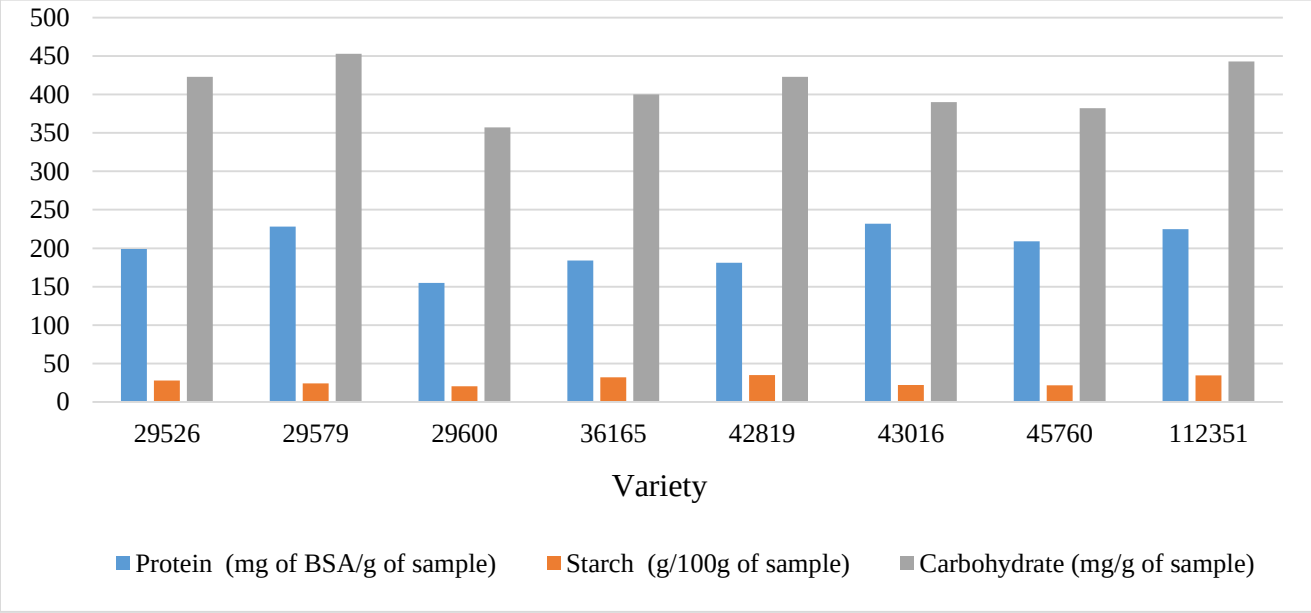


Figure 4.6: Graphical representation of LS-Mean of protein, starch and carbohydrate for each variety.

The concentration of starch measured had a wide range of variability from 20.25 to 35.07 g / 100 g of sample (Table 4.2). The starch content observed in the studies was less than that indicated by Reddy et al. (1984) and Dahl et al. (2012), with the value ranging from 36.9 to 49.0%. The resistant starch in seeds of field pea is comparatively less than non-resistant starch and ranges from 1.42 - 3.26 g / 100 g of sample (Table 4.3). Dahl et al. (2012) reported 2.1 to 6.3 % resistant starch which is similar to the results obtained for resistant starch content with the average of 2.51 g / 100 g of sample.

Table 4.3: Average values of resistant and non-resistant starch for the two experimental years for each pea cultivars

Variety	Resistant Starch(g / 100 g of sample)	Non-Resistant Starch (g / 100 g of sample)
29579	2.20	21.98
43016	3.01	19.24
45760	2.89	18.75
29526	2.31	26.14
29600	2.63	17.75
42819	1.42	34.15
36165	2.41	29.94
112351	3.26	32.02

The total protein contents of peas were 155 to 232 mg of BSA / g of sample (Table 4.2), within the range of 224 and 260 g / kg reported by Kotlarz et al. (2011). Accession 43016 has the highest protein concentration amongst accessions (Table 4.2). Earlier research has indicated that the protein content of field pea vary with respect to variety and environment and ranges from 15 to 39% (GL-Pro, 2005; Hickling, 2003; McKay et al., 2003; Anderson et al., 2002; Bressani et al., 1988; Davies et al., 1985).

The lipid results (Table 4.4) show an overall range of recovery from 1.3 % (variety 29526) to 2.6 % (variety 29579). Earlier research on peas lipid content also reported the lipid content that ranged from 1.55 to 2.5 % of dry matter (Khodapanahi et al., 2012; Pryor, 2008; Ryan et al., 2007; Murcia et al., 2006; Hickling, 2003; Anderson et al., 2002; El-Refai et al., 1987; Welch et al., 1984.

Furthermore, variation in lipid content was noted among varieties for the two years (Figure 4.10). Studies conducted by Khodapanahi et al. (2012) also confirms the influence of year on the lipid content.

Table 4.4: Lipid Least Square Means (%) produced by SAS PROC GLIMMIX.

Lipid Least Squares Means (%)					
Variety	Mean	Standard Error Mean	Scheffé-adjusted 95 % Confidence Limits		Scheffé Grouping*
			Lower Limit	Upper Limit	
29526	1.3	0.07	1.18	1.47	C
29579	2.6	0.11	2.37	2.80	A
29600	1.7	0.10	1.53	1.92	BC
36165	1.5	0.08	1.36	1.68	C
42819	1.7	0.08	1.55	1.88	BC
43016	2.2	0.10	2.03	2.43	AB
45760	2.1	0.09	1.89	2.27	AB
112351	1.4	0.07	1.26	1.57	C

* LS-means with the same letter are not significantly different.

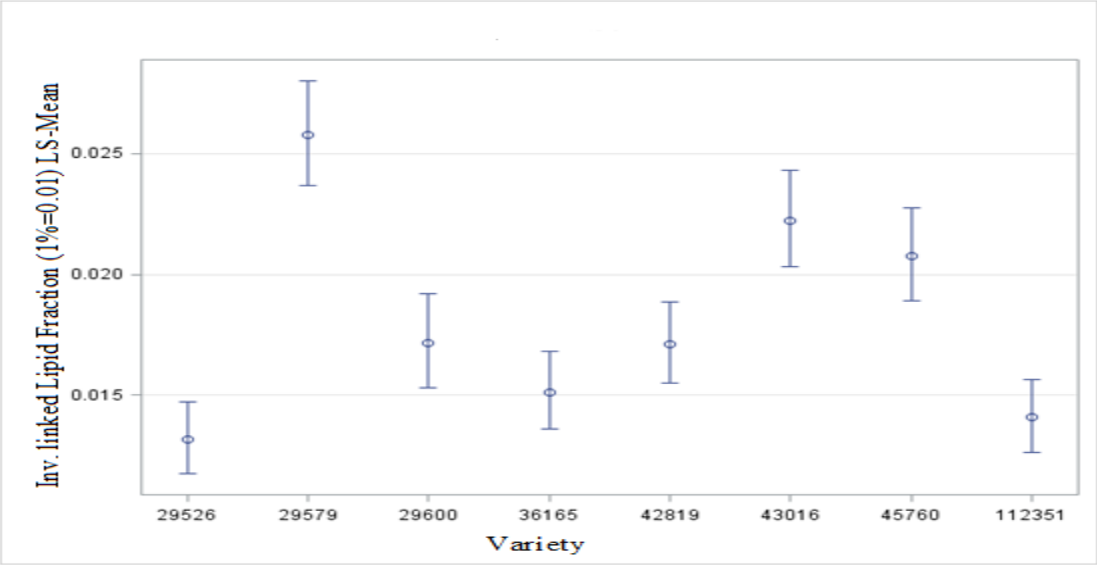


Figure 4.7: Least Square Means for variety with respect to lipid content.

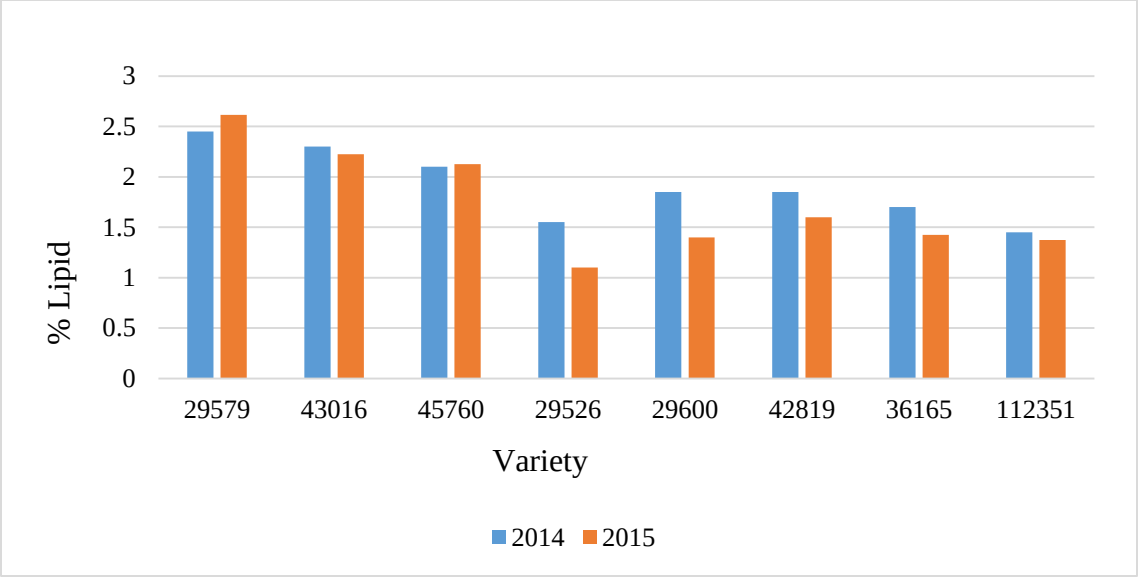


Figure 4.8: Graphical representation of total lipid content (arithmetic mean) throughout different varieties of *Pisum sativum* grown in 2014 and 2015.

Based on the Spearman correlation coefficients (Table A1), percent lipid content was positively correlated with variety 29579 (r = 0.569, p < .0001) and variety 43016 (r = 0.405, p = 0.0043). Lipid was negatively correlated with variety 29526 (r = -0.378, p = 0.0081) and variety 112351 (r =-0.400, p = 0.0048). While correlations are not additive, squared correlations may be added, and the sum of the squared correlations is 0.93. Therefore, Variety appears to contribute 11.67% of the variance in lipid content. Studies conducted by Welch et al. (1984) and by the Canadian Grain Commission (Canadian Grain Commission Grain Research Laboratory, 2004), confirmed that the lipid content of the field peas varies with the cultivars and ranges from 1.0 to 2.8 %.

Earlier researchers has reported a relationship between starch and lipid in the seed, where one is high the other is low, and the data confirms this well-known negative correlation (r =-0.419, p =0.0031). Starch content could therefore be considered to account for 17.54 % of the variance in lipid. Even the ash content was found to be positively correlated with lipid (r = 0.414, p = 0.0035). In a study by Ryszard et al. (1994), lipid content was found to be positively correlated with ash.

Furthermore, lipid content has been found to be influenced by seed color, seed shape and seed size. The brown colour was positively correlated to lipid content (r = 0.569, p < .0001), and the green colour was also positively correlated to lipid content (r = 0.457, p = 0.0011). The yellow colour

was negatively correlated to lipid content (r = -0.548, p < .0001). However, no previous research has been indicated on the relationship between seed color and lipid content. Surface smoothness was negatively correlated with lipid content (r = -0.342, p =0.0174). Seed volume has significant influence on lipid content (r = -0.279, p = 0.05). Studies conducted by Coxon et al. (1982) and Colonna et al. (1980) on the lipid content of different pea cultivars also confirmed that the lipid content is higher in wrinkled than in smooth peas. Similar results were obtained by Welch et al., (1984) and Bastianelli et al. (1998). Furthermore, Ryszard et al. (1994) reported that smooth pea cultivars contained less crude protein, free lipid, ash, glucose, and sucrose and more starch as compared to wrinkled pea cultivars. This was confirmed by the statistical analysis (Table A1) that smooth peas contained less ash, lipids and more starch, but in either case the effect of protein was insignificant. A study by Reichert et al. (1982) further supported that the lipid content in seed is independent of the protein content. However, the average ash and moisture content varies from 2.4 - 3.6 % and 9.8 - 11.2 % respectively (Table 4.5), which was supported by Dahl et al. (2012) and Hickling (2003), with the average of about 2.3 - 3.4%.

Table 4.5: Averages of moisture content and ash percentage for the selected field pea varieties for both the year.

Variety	**Moisture content %**	**Ash %**
29579	10.2	3.0
43016	10.8	3.2
45760	10.0	3.6
29526	11.2	2.4
29600	9.8	3.2
42819	10.6	2.6
36165	10.6	2.8
112351	11	3.0

4.2.2 Phytochemical analysis

The means of phenols, flavonoids, pigments and scavenging activity are presented in Table 4.6. The overall range of scavenging activity percent of pea seeds was from 17.23 to 27.70 %, which are supported by the results obtained by Bajacan et al. (2013) with the decrease in scavenging activity about 26.8 %. The phenol content of pea seeds varied from below 2.33 (43016) to 4.81 (29526) mg of GA / g of extract (Table 4.6). However, the concentration of flavonoid was 1.16 - 4.92 mg of quercetin/g of extract. Peas have been previously reported to contain a wide variety of phenolic substances and flavonoids (Agboola et al., 2010; Dueñas et al., 2006; Amarowicz et al., 2003; Troszynska et al., 2002a). In a study by Remiszewski et al. (2006) it has been reported that total phenol content in peas is 0.86 mg GAE / g. Wang et al. (1998a) reported that phenol content varied among pea cultivars and ranged from 162 to 325 mg CE /kg (CE, catechin equivalents) (Table 4.6). 3.04 to 5.35 mg/ kg of various flavonoids were reported by Timoracka et al. (2010). Phenol content was found to be highly correlated with flavonoids ($r = 0.752$, $p = < .0001$) and significant correlated with scavenging activity percent ($r = 0.313$, $p = 0.03$) (Table A1). Similar results were obtained for the correlation of scavenging activity percent and total phenol content ($r = 0.971$, $p < 0.05$) of peas seeds (Stanisavljevic et al., 2015) and for numerous legume seed extract (Amarowicz et al., 2003; Xu and Chang, 2007; Troszynska et al., 1997).

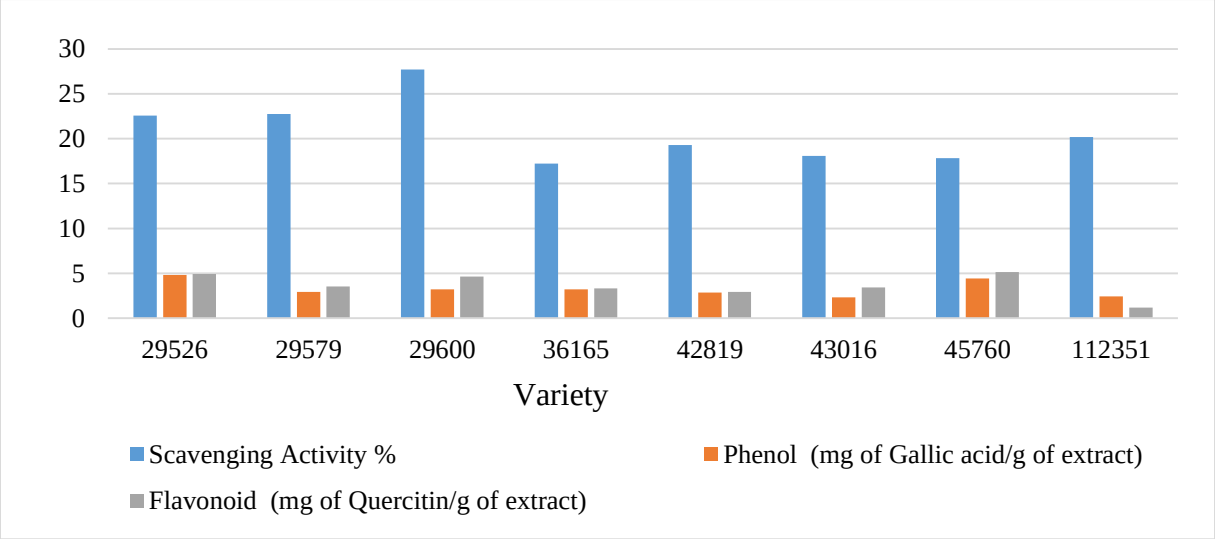


Figure 4.9: Graphical representation of LS-Mean of scavenging activity %, phenol and flavonoid for each variety.

Table 4.6: Least Squares Means of phytochemical constituent estimate for each *P. sativum* variety.

	Scavenging Activity %	Phenol	Flavonoid
Variety	Back-transformed Mean (%)	Back-transformed Mean (mg of Gallic acid/g of extract)	Back-transformed Mean (mg of Quercitin/g of extract)
29526	22.59	4.81	4.92
29579	22.74	2.91	3.52
29600	27.70	3.22	4.62
36165	17.23	3.19	3.30
42819	19.30	2.86	2.92
43016	18.09	2.33	3.41
45760	17.84	4.41	5.12
112351	20.17	2.43	1.16

*Back-transformed mean values are of estimates which are made on the natural log scale.

4.2.3 Pigment Analysis

The total chlorophyll content of pea seeds extract with a concentration of 10 µg/ml varied from 2.33 ug/ml of plant extract (yellow seeded variety 36165) to 7.50 ug/ml of plant extract (green seeded variety 112351) (Table 4.7). However, pea seeds were found to have much less carotenoid content ranging from 0.6-1.2 ug/ml in the seed (Table 4.7). Kaliyaperumal et al. (2013) also reported that the mean lutein (carotenoid) concentration ranging from 7.2 µg g^{-1} to 17.6 µg g^{-1}. Chlorophyll content was a more precise measure of seed greenness, and it was highly correlated to green colour and negatively correlated to yellow (Table A1). Earlier research indicated the presence of chlorophylls, carotenoids and xanthophylls are major chloroplast photosynthetic pigments which results in the green color of pea seeds the presence for these photosynthetic pigments (Edelenbos et al. 2001; Steet et al., 1996)**.** Furthermore, it was found that chlorophyll has strong positive correlation with wrinkled surface and negative correlation with smooth surface.

Table 4.7: Least Squares Means of pigments estimate for each *P. sativum* variety.

	Carotenoid	Chlorophyll
Variety	Back-transformed Mean (ug/ml of plant extract)	Back-transformed Mean(ug/ml of plant extract)
29526	0.9 ± 0.4[a]	2.51
29579	0.8 ± 0.4[a]	2.83
29600	0.8 ± 0.4[a]	3.06
36165	0.9 ± 0.5[a]	2.33
42819	0.6 ± 0.3[a]	2.40
43016	0.8 ± 0.4[a]	5.71
45760	1.1 ± 0.6[a]	7.44
112351	1.2 ± 0.6[a]	7.50

*Back-transformed mean values are of estimates which are made on the natural log scale.

Chapter 5

CONCLUSION

The main objectives of this study were to determine the content of nutrients and phytochemicals in *Pisum sativum* and to determine if there were significant correlations between the lipid content and other nutrients or pea seed characteristics. The secondary objective of this study was to determine if variety has any influence on the nutritional composition. Significant differences were observed among cultivars for each variable. Variety appears to contribute 11.67 % of the variance in lipid content. Most of the variables were significantly influenced by cultivar and year. The years differed markedly and were highly correlated with the moisture, carbohydrate and carotenoid content. Correlation between moisture content and carotenoid as well as between chlorophyll content and the green colour of the seeds were also noted. The seeds of the pea varieties differed in terms of chemical and phytochemical constituents. Data obtained support that peas are a high source of proteins, starch, and carbohydrate and antioxidant components. Carbohydrate are the major component of the pea seeds accounting for 453 mg of glucose / g of sample. The lipid extracted from the field pea was from 1.3 to 2.6 %, which were within the expectation of earlier research. No variety was found to exceed 2.6 % of lipid content in seeds in this study. Data from the study also revealed that the lipids are positively correlated to ash, brown color seeds and green color seeds, a negatively correlated to smooth surface, yellow colour, starch content and seed volume. On the basis of statistical analysis of phenotypic markers, significant modification in the field peas can be further performed to improve the nutritional quality. Comparison of data from the study and literature values of other oilseed crops (canola, soybean, etc.) indicated that pea has the potential to be developed into a bio-oil crop. This research supports the idea of developing a novel dual-purpose oilseed pea that allows for the production of protein and oil in pea seeds while being adapted to a colder climate.

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APPENDIX

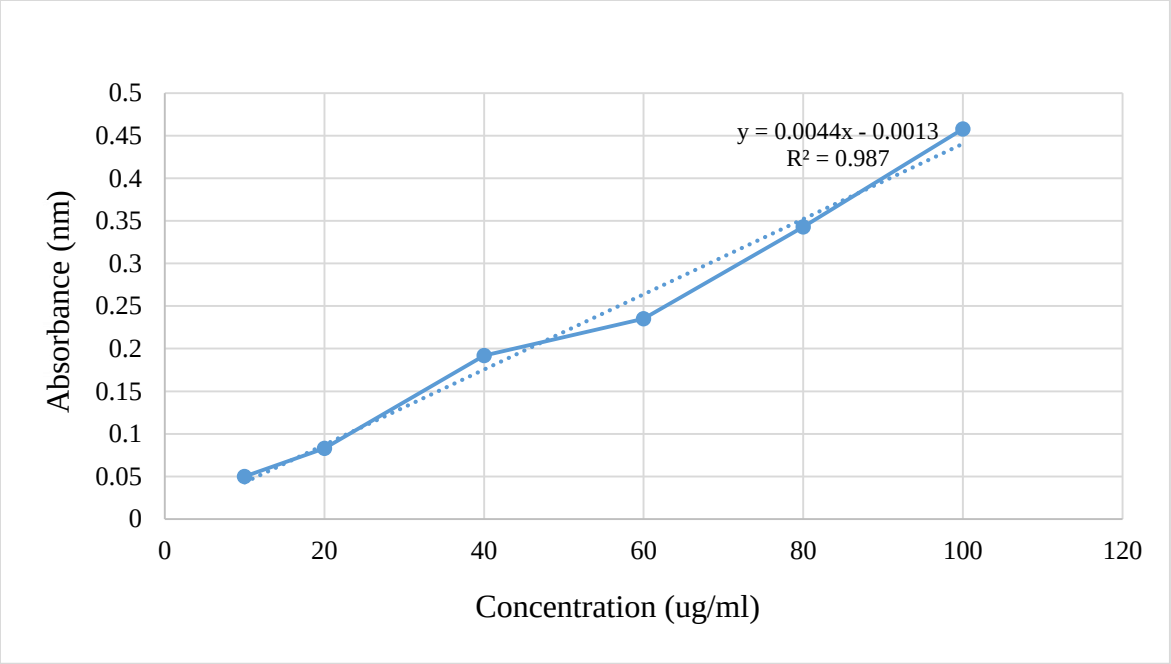

Figure A1: Standard calibration curve of gallic acid for the determination of total phenolic content.

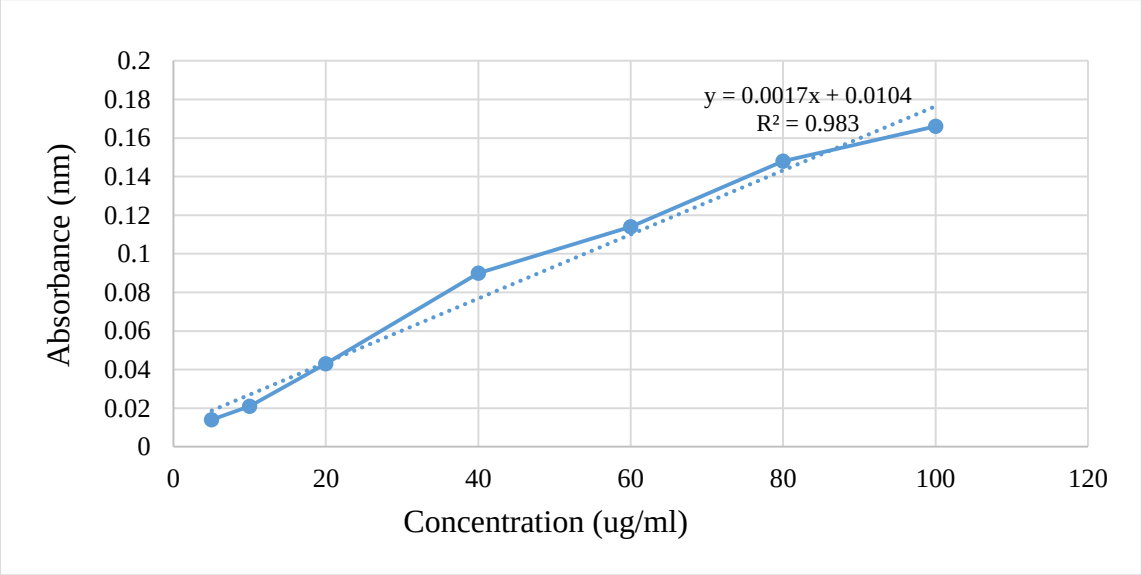

Figure A2: Standard calibration curve of quercetin for the determination of total flavonoid content.

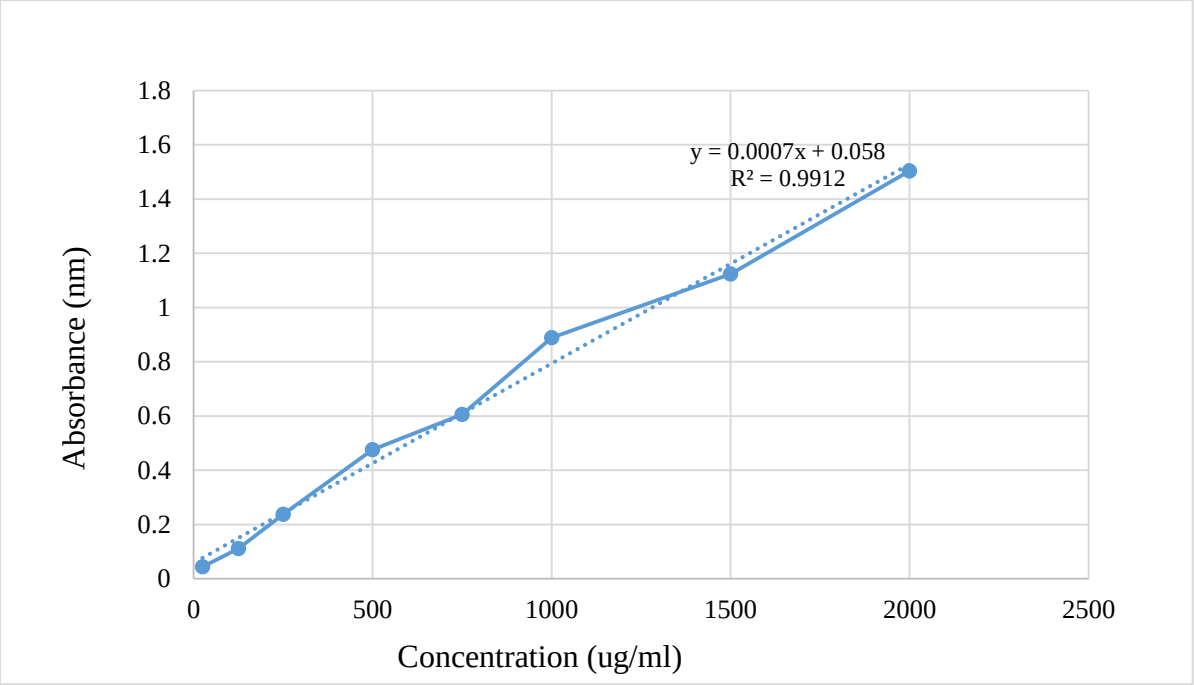

Figure A3: Standard calibration curve of BSA for the determination of total protein content.

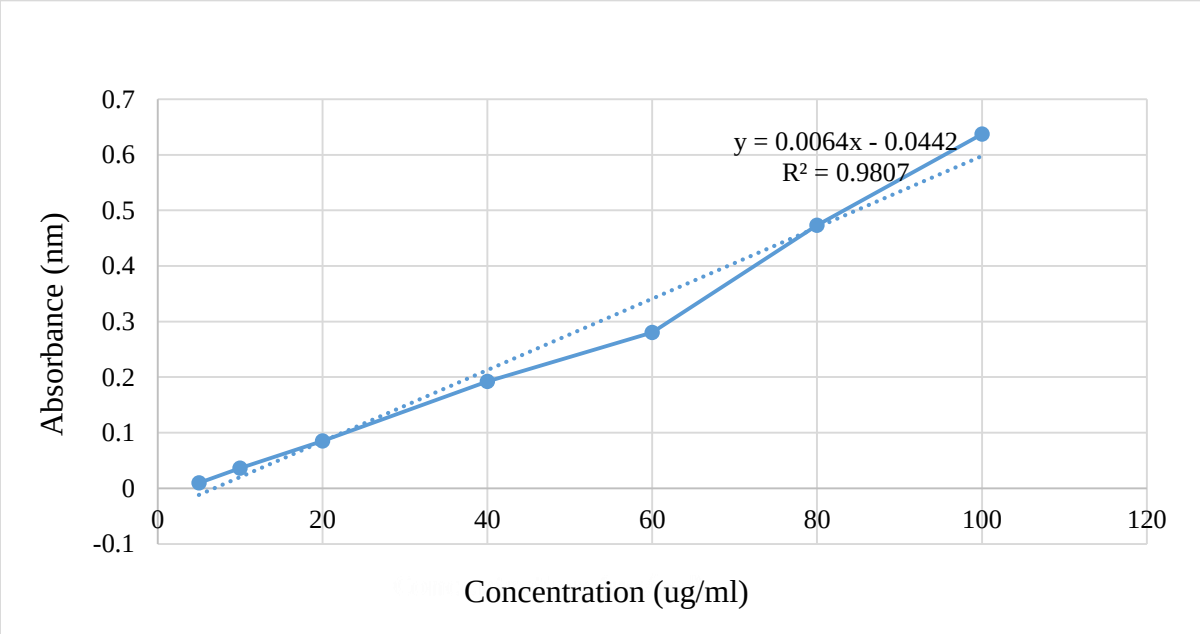

Figure A4: Standard calibration curve of glucose for the determination of total carbohydrate content.

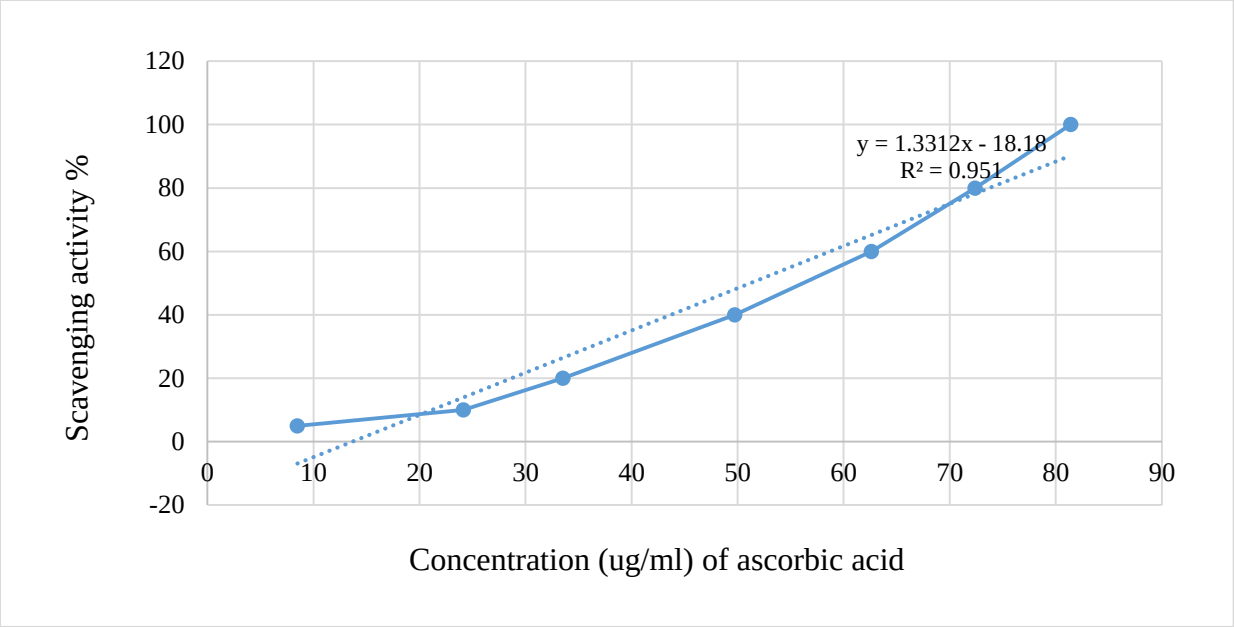

Figure A5: Graphical representation of percentage scavenging activity of DPPH free radicals by ascorbic acid at 517 nm.

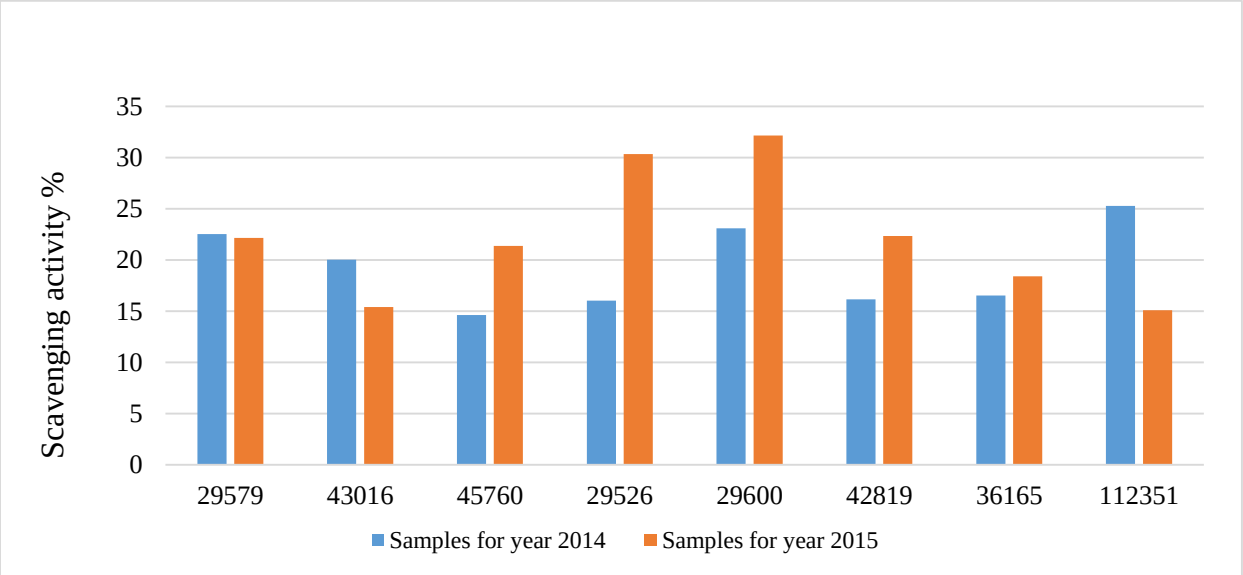

Figure A6: Graphical representation of scavenging activity % throughout different varieties of *Pisum sativum* grown in 2014 and 2015.

Table A1: SAS result presenting the Spearman Correlation Coefficient among different variables.

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Observations																								
	Lipid	**var29579**	**var43016**	**var45760**	**var29526**	**var29600**	**var42819**	**var36165**	**var112351**	**Year**	**Phenol**	**Flavonoid**	**Scavenging**	**Protein**	**Starch**	**Ash**	**Moisture**	**Carbohydrate**	**Chlorophyll**	**Carotenoid**	**Brown**	**Green**	**Yellow**	**Smooth**	**Wrinkled**	**Volume**
Lipid % Lipid	1.00000 48	0.56877 <.0001 48	0.40496 0.0043 48	0.25481 0.0805 48	-0.37766 0.0081 48	-0.15926 0.2796 48	-0.07280 0.6229 48	-0.21841 0.1359 48	-0.40041 0.0048 48	-0.20767 0.1567 48	-0.16117 0.2738 48	0.12852 0.3840 48	-0.12625 0.3925 48	0.18140 0.2172 48	-0.41880 0.0031 48	0.41375 0.0035 48	-0.28242 0.0518 48	0.10673 0.4703 48	0.19133 0.1927 48	-0.26424 0.0695 48	0.56877 <.0001 48	0.45693 0.0011 48	-0.54776 <.0001 48	-0.34192 0.0174 48	0.22296 0.1277 48	-0.27919 0.0574 47
var29579	0.56877 <.0001 48	1.00000 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	0.00000 1.0000 48	-0.09098 0.5386 48	0.04322 0.7705 48	0.17056 0.2464 48	0.22287 0.1279 48	-0.13642 0.3552 48	0.02120 0.8863 48	-0.06228 0.6741 48	0.30013 0.0382 48	-0.07731 0.6015 48	-0.01592 0.9145 48	1.00000 <.0001 48	0.29277 0.0434 48	-0.37796 0.0081 48	0.29277 0.0434 48	-0.33333 0.0206 48	-0.57813 <.0001 47
var43016	0.40496 0.0043 48	-0.14286 0.3327 48	1.00000 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	0.00000 1.0000 48	-0.40939 0.0039 48	0.00227 0.9878 48	-0.17738 0.2278 48	0.10461 0.4792 48	-0.30011 0.0382 48	0.19076 0.1940 48	0.10380 0.4826 48	-0.11596 0.4325 48	0.36380 0.0110 48	-0.01819 0.9023 48	-0.14286 0.3327 48	0.29277 0.0434 48	-0.37796 0.0081 48	-0.48795 0.0004 48	0.42857 0.0024 48	0.24441 0.0978 47
var45760	0.25481 0.0805 48	-0.14286 0.3327 48	-0.14286 0.3327 48	1.00000 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	0.00000 1.0000 48	0.40939 0.0039 48	0.29573 0.0413 48	-0.15237 0.3012 48	0.04548 0.7589 48	-0.33195 0.0212 48	0.55108 <.0001 48	-0.18683 0.2035 48	-0.18417 0.2102 48	0.45929 0.0010 48	-0.03865 0.7942 48	-0.14286 0.3327 48	0.29277 0.0434 48	-0.37796 0.0081 48	-0.48795 0.0004 48	0.42857 0.0024 48	0.07050 0.6377 47
var29526	-0.37766 0.0081 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	1.00000 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	0.00000 1.0000 48	0.48672 0.0005 48	0.29345 0.0429 48	0.10689 0.4696 48	0.03639 0.8060 48	0.07730 0.6015 48	-0.50868 0.0002 48	0.24911 0.0877 48	0.12733 0.3885 48	-0.30013 0.0382 48	0.04320 0.7706 48	-0.14286 0.3327 48	-0.48795 0.0004 48	0.37796 0.0081 48	0.29277 0.0434 48	-0.33333 0.0206 48	0.14571 0.3284 47
var29600	-0.15926 0.2796 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	1.00000 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	0.00000 1.0000 48	0.05004 0.7356 48	0.18199 0.2157 48	0.37524 0.0086 48	-0.34795 0.0154 48	-0.40470 0.0043 48	0.21195 0.1481 48	-0.26987 0.0636 48	-0.30013 0.0382 48	-0.05457 0.7126 48	-0.08185 0.5802 48	-0.14286 0.3327 48	0.29277 0.0434 48	0.37796 0.0081 48	-0.48795 0.0004 48	0.42857 0.0024 48	-0.38072 0.0083 47
var42819	-0.07280 0.6229 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	1.00000 48	-0.14286 0.3327 48	-0.14286 0.3327 48	0.00000 1.0000 48	-0.15693 0.2868 48	-0.22976 0.1162 48	-0.10006 0.4986 48	-0.16374 0.2661 48	0.39561 0.0054 48	-0.33912 0.0184 48	0.04152 0.7793 48	0.06594 0.6561 48	-0.38198 0.0074 48	-0.15234 0.3013 48	-0.14286 0.3327 48	-0.48795 0.0004 48	0.37796 0.0081 48	0.29277 0.0434 48	-0.33333 0.0206 48	0.38154 0.0081 47
var36165	-0.21841 0.1359 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	1.00000 48	-0.14286 0.3327 48	0.00000 1.0000 48	0.02957 0.8419 48	-0.01365 0.9266 48	-0.18648 0.2044 48	-0.12736 0.3884 48	0.31376 0.0299 48	-0.14837 0.3142 48	0.04152 0.7793 48	-0.10004 0.4987 48	-0.41382 0.0035 48	0.08185 0.5802 48	-0.14286 0.3327 48	-0.48795 0.0004 48	0.37796 0.0081 48	0.29277 0.0434 48	-0.33333 0.0206 48	-0.10341 0.4891 47
var112351	-0.40041 0.0048 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	-0.14286 0.3327 48	1.00000 48	0.00000 1.0000 48	-0.31842 0.0274 48	-0.57326 <.0001 48	-0.03639 0.8060 48	0.22970 0.1163 48	0.38651 0.0067 48	0.02120 0.8863 48	0.08304 0.5747 48	0.20691 0.1582 48	0.40472 0.0043 48	0.18190 0.2160 48	-0.14286 0.3327 48	0.29277 0.0434 48	-0.37796 0.0081 48	0.29277 0.0434 48	0.04762 0.7479 48	0.24911 0.0913 47
Year	-0.20767 0.1567 48	0.00000 1.0000 48	0.00000 1.0000 48	0.00000 1.0000 48	0.00000 1.0000 48	0.00000 1.0000 48	0.00000 1.0000 48	0.00000 1.0000 48	0.00000 1.0000 48	1.00000 48	-0.04964 0.7376 48	0.16852 0.2522 48	0.16998 0.2481 48	-0.24820 0.0889 48	0.09324 0.5285 48	-0.30842 0.0329 48	0.86505 <.0001 48	-0.55194 <.0001 48	0.14738 0.3175 48	0.86626 <.0001 48	0.00000 1.0000 48	0.00000 1.0000 48	0.00000 1.0000 48	0.00000 1.0000 48	-0.12599 0.3935 48	0.07217 0.6298 47
Phenol Phenol (mg of GA/g of extract)	-0.16117 0.2738	-0.09098 0.5386	-0.40939 0.0039	0.40939 0.0039	0.48672 0.0005	0.05004 0.7356	-0.15693 0.2868	0.02957 0.8419	-0.31842 0.0274	-0.04964 0.7376	1.00000	0.75168 <.0001	0.31350 0.0300	0.04038 0.7852	-0.25096 0.0853	0.15320 0.2985	-0.05999 0.6855	-0.17267 0.2406	-0.12489 0.3977	-0.04431 0.7649	-0.09098 0.5386	-0.24549 0.0926	0.27079 0.0627	-0.03418 0.8176	-0.00152 0.9918	-0.10122 0.4984

Spearman Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Lipid	var29579	var43016	var45760	var29526	var29600	var42819	var36165	var112351	Year	Phenol	Flavonoid	Scavenging	Protein	Starch	Ash	Moisture	Carbohydrate	Chlorophyll	Carotenoid	Brown	Green	Yellow	Smooth	Wrinkled	Volume
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Flavonoid	0.12852	0.04322	0.00227	0.29573	0.29345	0.18199	-0.22976	-0.01365	-0.57326	0.16852	0.75168	1.00000	0.30862	-0.00152	-0.51092	0.20622	0.03438	-0.30487	-0.00505	0.17105	0.04322	-0.03419	0.15348	-0.32790	0.14256	-0.22508
Flavonoid (mg of Quercitin/g of extract)	0.3840	0.7705	0.9878	0.0413	0.0429	0.2157	0.1162	0.9266	<.0001	0.2522	<.0001		0.0328	0.9918	0.0002	0.1597	0.8166	0.0351	0.9728	0.2451	0.7705	0.8176	0.2977	0.0229	0.3338	0.1282
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Scavenging	-0.12625	0.17056	-0.17738	-0.15237	0.10689	0.37524	-0.10006	-0.18648	-0.03639	0.16998	0.31350	0.30862	1.00000	-0.04282	-0.28052	0.11759	0.17391	-0.34522	-0.05207	0.04740	0.17056	0.12273	0.12936	-0.03107	0.14403	-0.37157
Scavenging Activity %	0.3925	0.2464	0.2278	0.3012	0.4696	0.0086	0.4986	0.2044	0.8060	0.2481	0.0300	0.0328		0.7726	0.0535	0.4261	0.2371	0.0163	0.7252	0.7490	0.2464	0.4060	0.3809	0.8339	0.3287	0.0101
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Protein	0.18140	0.22287	0.10461	0.04548	0.03639	-0.34795	-0.16374	-0.12736	0.22970	-0.24820	0.04038	-0.00152	-0.04282	1.00000	-0.01868	0.26639	-0.13979	0.25060	0.22348	-0.11348	0.22287	0.17400	-0.39863	0.13516	-0.04397	0.06541
Protein (mg of BSA/g of sample)	0.2172	0.1279	0.4792	0.7589	0.8060	0.0154	0.2661	0.3884	0.1163	0.0889	0.7852	0.9918	0.7726		0.8997	0.0672	0.3433	0.0858	0.1268	0.4425	0.1279	0.2369	0.0050	0.3597	0.7667	0.6622
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Starch	-0.41880	-0.13642	-0.30011	-0.33195	0.07730	-0.40470	0.39561	0.31376	0.38651	0.09324	-0.25096	-0.51092	-0.28052	-0.01868	1.00000	-0.60010	0.28958	0.23297	-0.26809	0.21044	-0.13642	-0.53739	0.25265	0.70824	-0.57749	0.38911
Starch (g/100g of sample)	0.0031	0.3552	0.0382	0.0212	0.6015	0.0043	0.0054	0.0299	0.0067	0.5285	0.0853	0.0002	0.0535	0.8997		<.0001	0.0459	0.1110	0.0654	0.1511	0.3552	<.0001	0.0832	<.0001	<.0001	0.0069
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Ash	0.41375	0.02120	0.19076	0.55108	-0.50868	0.21195	-0.33912	-0.14837	0.02120	-0.30842	0.15320	0.20622	0.11759	0.26639	-0.60010	1.00000	-0.50511	-0.15501	0.55358	-0.30724	0.02120	0.68052	-0.51871	-0.65156	0.70651	-0.16205
% Ash	0.0035	0.8863	0.1940	<.0001	0.0002	0.1481	0.0184	0.3142	0.8863	0.0329	0.2985	0.1597	0.4261	0.0672	<.0001		0.0003	0.2928	<.0001	0.0337	0.8863	<.0001	0.0002	<.0001	<.0001	0.2765
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Moisture	-0.28242	-0.06228	0.10380	-0.18683	0.24911	-0.26987	0.04152	0.04152	0.08304	0.86505	-0.05999	0.03438	0.17391	-0.13979	0.28958	-0.50511	1.00000	-0.47793	0.03073	0.73977	-0.06228	-0.22690	0.04119	0.24108	-0.23527	0.23046
% Moisture	0.0518	0.6741	0.4826	0.2035	0.0877	0.0636	0.7793	0.7793	0.5747	<.0001	0.6855	0.8166	0.2371	0.3433	0.0459	0.0003		0.0006	0.8358	<.0001	0.6741	0.1209	0.7810	0.0988	0.1075	0.1191
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Carbohydrate	0.10673	0.30013	-0.11596	-0.18417	0.12733	-0.30013	0.06594	-0.10004	0.20691	-0.55194	-0.17267	-0.30487	-0.34522	0.25060	0.23297	-0.15501	-0.47793	1.00000	-0.17525	-0.38307	0.30013	-0.06368	-0.13686	0.41006	-0.41079	0.04047
Carbohydrate (mg of glucose/ g of sample)	0.4703	0.0382	0.4325	0.2102	0.3885	0.0382	0.6561	0.4987	0.1582	<.0001	0.2406	0.0351	0.0163	0.0858	0.1110	0.2928	0.0006		0.2335	0.0072	0.0382	0.6672	0.3536	0.0038	0.0037	0.7871
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Chlorophyll	0.19133	-0.07731	0.36380	0.45929	-0.30013	-0.05457	-0.38198	-0.41382	0.40472	0.14738	-0.12489	-0.00505	-0.05207	0.22348	-0.26809	0.55358	0.03073	-0.17525	1.00000	0.27967	-0.07731	0.74866	-0.76099	-0.52500	0.65332	0.12975
Chlorophyll (ug/ml of plant extract)	0.1927	0.6015	0.0110	0.0010	0.0382	0.7126	0.0074	0.0035	0.0043	0.3175	0.3977	0.9728	0.7252	0.1268	0.0654	<.0001	0.8358	0.2335		0.0542	0.6015	<.0001	<.0001	0.0001	<.0001	0.3847
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Carotenoid	-0.26424	-0.01592	-0.01819	-0.03865	0.04320	-0.08185	-0.15234	0.08185	0.18190	0.86626	-0.04431	0.17105	0.04740	-0.11348	0.21044	-0.30724	0.73977	-0.38307	0.27967	1.00000	-0.01592	0.01864	-0.07219	0.09475	-0.16977	0.04949
Carotenoid (ug/ml of plant extract)	0.0695	0.9145	0.9023	0.7942	0.7706	0.5802	0.3013	0.5802	0.2160	<.0001	0.7649	0.2451	0.7490	0.4425	0.1511	0.0337	<.0001	0.0072	0.0542		0.9145	0.8999	0.6258	0.5218	0.2487	0.7411
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Brown	0.56877	1.00000	-0.14286	-0.14286	-0.14286	-0.14286	-0.14286	-0.14286	-0.14286	0.00000	-0.09098	0.04322	0.17056	0.22287	-0.13642	0.02120	-0.06228	0.30013	-0.07731	-0.01592	1.00000	0.29277	-0.37796	0.29277	-0.33333	-0.57813
	<.0001	<.0001	0.3327	0.3327	0.3327	0.3327	0.3327	0.3327	0.3327	1.0000	0.5386	0.7705	0.2464	0.1279	0.3552	0.8863	0.6741	0.0382	0.6015	0.9145		0.0434	0.0081	0.0434	0.0206	<.0001
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Green	0.45693	0.29277	0.29277	0.29277	-0.48795	0.29277	-0.48795	-0.48795	0.29277	0.00000	-0.24549	-0.03419	0.12273	0.17400	-0.53739	0.68052	-0.22690	-0.06368	0.74866	0.01864	0.29277	1.00000	-0.77460	-0.60000	0.68313	-0.27421
	0.0011	0.0434	0.0434	0.0434	0.0004	0.0434	0.0004	0.0004	0.0434	1.0000	0.0926	0.8176	0.4060	0.2369	<.0001	<.0001	0.1209	0.6672	<.0001	0.8999	0.0434		<.0001	<.0001	<.0001	0.0622
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47

Spearman Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Lipid	var29579	var43016	var45760	var29526	var29600	var42819	var36165	var112351	Year	Phenol	Flavonoid	Scavenging	Protein	Starch	Ash	Moisture	Carbohydrate	Chlorophyll	Carotenoid	Brown	Green	Yellow	Smooth	Wrinkled	Volume
Yellow	-0.54776	-0.37796	-0.37796	-0.37796	0.37796	0.37796	0.37796	0.37796	-0.37796	0.00000	0.27079	0.15348	0.12936	-0.39863	0.25265	-0.51871	0.04119	-0.13686	-0.76099	-0.07219	-0.37796	-0.77460	1.00000	0.25820	-0.37796	0.00941
	<.0001	0.0081	0.0081	0.0081	0.0081	0.0081	0.0081	0.0081	0.0081	1.0000	0.0627	0.2977	0.3809	0.0050	0.0832	0.0002	0.7810	0.3536	<.0001	0.6258	0.0081	<.0001		0.0764	0.0081	0.9499
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Smooth	-0.34192	0.29277	-0.48795	-0.48795	0.29277	-0.48795	0.29277	0.29277	0.29277	0.00000	-0.03418	-0.32790	-0.03107	0.13516	0.70824	-0.65156	0.24108	0.41006	-0.52500	0.09475	0.29277	-0.60000	0.25820	1.00000	-0.87831	0.04517
	0.0174	0.0434	0.0004	0.0004	0.0434	0.0004	0.0434	0.0434	0.0434	1.0000	0.8176	0.0229	0.8339	0.3597	<.0001	<.0001	0.0988	0.0038	0.0001	0.5218	0.0434	<.0001	0.0764		<.0001	0.7630
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Wrinkled	0.22296	-0.33333	0.42857	0.42857	-0.33333	0.42857	-0.33333	-0.33333	0.04762	-0.12599	-0.00152	0.14256	0.14403	-0.04397	-0.57749	0.70651	-0.23527	-0.41079	0.65332	-0.16977	-0.33333	0.68313	-0.37796	-0.87831	1.00000	-0.01577
	0.1277	0.0206	0.0024	0.0024	0.0206	0.0024	0.0206	0.0206	0.7479	0.3935	0.9918	0.3338	0.3287	0.7667	<.0001	<.0001	0.1075	0.0037	<.0001	0.2487	0.0206	<.0001	0.0081	<.0001		0.9162
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	47
Volume	-0.27919	-0.57813	0.24441	0.07050	0.14571	-0.38072	0.38154	-0.10341	0.24911	0.07217	-0.10122	-0.22508	-0.37157	0.06541	0.38911	-0.16205	0.23046	0.04047	0.12975	0.04949	-0.57813	-0.27421	0.00941	0.04517	-0.01577	1.00000
Seed Volume (mm3)	0.0574	<.0001	0.0978	0.6377	0.3284	0.0083	0.0081	0.4891	0.0913	0.6298	0.4984	0.1282	0.0101	0.6622	0.0069	0.2765	0.1191	0.7871	0.3847	0.7411	<.0001	0.0622	0.9499	0.7630	0.9162	
	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47