The Influence of Genotype and Environment on the Nutritional Composition of Field Peas Grown in Canada

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Food Science

University of Manitoba

Winnipeg, MB

"Though its root groweth old,
Though its stalk seen to die,
Though it rest within the earth
Ignoring wind and sun and sky,
Yet at the scent of water
Will it sprout again and branch,
A crown of buds weigh its boughs
A robe of leaves its frame enhance"

- Written by my aunt, Gail Sitarz

ACKNOWLEDGMENTS

I am grateful to have had the support and assistance of numerous individuals and organizations for my thesis project. Thank you to Dr. David Hatcher, my advisor, and to my examining committee, Dr. Susan Arntfield and Dr. Ning Wang, for all of your help, support and encouragement. Thank you to Debbie Salazar and Bruno Klassen, Analytical Services, Grain Research Laboratory, Canadian Grain Commission, for keeping me sane during the Total Dietary Fibre analysis phase. Thank you to Wendy Barker (aka Work Mom) and Dr. Pham Hung (visiting research scientist), Wheat Enzymes, Grain Research Laboratory, Canadian Grain Commission, for your assistance and patience during the phenolic analysis phase. Thank you to my SAS coach, Dr. Margaret Anderson, for enabling me to see that SAS software and statistics are not entirely evil entities. Thank you to Jonathon Muller, my friend and personal editor; I think I got the better deal. I owe a huge thank you to my friends and colleagues at the Grain Research Laboratory, I felt like I was part of the team from the moment I started my research.

I gratefully acknowledge the financial support of Pulse Canada for my thesis project. Thank you to Peter Watts, Dr. Julianne Curran and Heather Maskus from Pulse Canada for your advice and support. Thank you to Dr. Tom Warkentin, Crop Development Centre, University of Saskatchewan, for providing the field pea samples and providing input and advice on the project's progression. The financial support of the Grain Research Laboratory, Canadian Grain Commission, for the purchase of supplies and use of equipment is gratefully acknowledged.

Last, but not least, I thank my friends and family for their unwavering support. Thank you to my husband, Neil, for being so supportive of this endeavor throughout the good and stressful times. To my family, I know that you did not understand fully what I was doing, but you understood completely why I had to do it. To my friends, Nadine, Erin, Niki, Darren, Julia and Sergey, I thank you for your unfaltering support, even when I've been less than attentive. I am truly blessed to have you in my life.

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LIST OF ABBREVIATIONS

ADF	Acid Detergent Fibre
AOA	Antioxidant Activity
CE	Catechin Equivalents
CF	Crude Fibre
CVD	Cardiovascular Disease
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic Acid
ET	Electron Transfer
FRAP	Ferric Reducing/Antioxidant Power
GC	Gas Chromatography
G x E	Genotype by Environment Interaction
HAT	Hydrogen Atom Transfer
IDF	Insoluble Dietary Fibre
LDL	Low Density Lipoprotein
LSD	Least Significant Difference
LC	Liquid Chromatography
NDF	Neutral Detergent Fibre
ORAC	Oxygen Radical Absorbing Capacity
PE	Peroxidase-Catalyzed Enzymatic
PPO	Polyphenol Oxidase
ROS	Reactive Oxygen Species
SDF	Soluble Dietary Fibre
TDF	Total Dietary Fibre
TE	Trolox Equivalents
TEAC	Trolox Equivalent Antioxidant Capacity
TFA	Trifluoroacetic Acid
TLC	Thin Layer Chromatography
TPC	Total Phenolic Content
TRAP	Total Radical trapping Antioxidant Capacity
UPLC	Ultra-Performance Liquid Chromatography

ABSTRACT

Six field pea (*Pisum sativum*) varieties from five different growing locations in Saskatchewan in the 2006 and 2007 growing years were analyzed to determine the effect of genotype, environment and year on the total dietary fibre, insoluble dietary fibre, soluble dietary fibre, total phenolic content, simple phenolic content and antioxidant activities. Samples were analyzed for dietary fibre using the enzymatic-gravimetric method of fibre analysis in accordance to the AACC method 32-05. Growing location had a very significant effect ($p<0.0001$) on the IDF, SDF and TDF content. Genotype had a strong effect ($p<0.0001$) on both IDF and TDF while having no significant effect ($p=0.4556$) on SDF content. Crop year also displayed a significant effect on SDF and TDF ($p<0.0001$) while having a smaller effect on IDF content ($p=0.0139$). Green varieties yielded significantly higher IDF ($p=0.0041$) and TDF ($p=0.0028$) than yellow varieties. Significant genotype x location (0.0155) and location x year ($p=0.0002$) interaction terms were also observed for TDF. The total phenolic contents were assessed using the Folin-Ciocalteu method of total phenolic content (TPC) analysis, while the contents of 10 individual simple phenolic acids were assessed using reversed-phase UPLC. A significant genotype, environment, and genotype by environment (G x E) interaction effect on the TPC was observed. The seed coat colour and growing season did not show a significant effect on the TPC. The UPLC analysis showed that ferulic acid comprised the majority of the phenolic content of the field pea samples. There was also a genotype, seed coat colour, location, growing season and G x E effect on the total simple phenolic acid content. As well, a modified microplate method for antioxidant activity using the free radical DPPH was assessed against the

conventional cuvette method based system. Both methods showed that genotype ($p<0.05$) and location ($p<0.05$) had a significant effect on antioxidant activity. A larger, significant effect was seen in the genotype by environment (G x E) interaction ($p<0.0001$) in the 2007 and 2008 growing years. Growing year did not have a significant on antioxidant activity. Although there was some variation in the resulting AOA values between the two methods, these differences were found not to be statistically significant by means of a folded F-Test ($p < 0.05$), and the AOA between the two methods was highly correlated ($R^2 = 0.8866$). This indicates that a microplate may be used in place of cuvettes to determine AOA using the DPPH free radical to increase testing speed while reducing the amount of sample and reagent used in testing. The research performed on the influence of genotype and environment could potentially allow plant breeders, food scientists and nutraceutical manufacturers to manipulate field pea genotypes and growing conditions to attain an ideal nutritional profile for use in functional foods and nutraceuticals.

1.0 INTRODUCTION

Field pea (*Pisum sativum* L.) is one of the most important pulse crops produced in the world, second only to common bean (*Phaseolus vulgaris* L.) (Tar'an *et al,* 2004). As the leading exporter of field pea in the world (Agriculture & Agrifood Canada [AAFC], 2008), the significance of this pulse crop to the Canadian agriculture sector is profound. Estimates indicate that the demand for field pea and its fractions will only increase in the future, as its potential as a functional food ingredient and importance as a high quality livestock feed become more apparent. Good market prices for field pea, combined with their nitrogen fixing capabilities which offset the high costs of nitrogen fertilizer are expected to increase field pea production in Canada by approximately 10% (AAFC, 2008). The increasing awareness by consumers over health issues coupled with the extensive promotion of the health benefits of pulse consumption has supported the pulse industry's growth. Food manufacturers are striving to develop products that include elements such as whole pulses and pulse ingredients that have proven health advantages. Like other pulses, field peas are an excellent source of protein, dietary fibre, complex carbohydrates, potassium, phosphorus, and are low in fat and sodium (AAFC, 2008).

Despite the importance of this crop, there is very little research available on the nutritional composition of Canadian-grown field pea, and negligible information on the effect of genotype and environment on the nutritional composition of this commodity. The majority of genotype by environment (G x E) research on field pea to date pertains to protein content and anti-nutritional factors. Ali-Khan & Youngs (1973) found that there was a significant effect of genotype, location, growing season, as well as a G x E interaction effect on the protein content of Canadian-grown field pea. Similarly, Santalla,

Amurrio & De Ron (2001) found a significant environmental and G x E interaction effect on the protein content of Spanish-grown field pea. Not only did Nikolopoulou, Stasini, Alexis & Iliadis (2007) find a significant location and year interaction effect on the protein content of field pea grown in Greece, but they also discovered that there was a significant location by year interaction effect on the ash, fat, and non-starch polysaccharide content. The same study also concluded that location has a significant effect on the starch, tannin and phytic acid content, while growing year had a significant effect on the raffinose series oligiosaccharides, tannin and phytic acid content. Wang *et al* (1998) determined that there was a significant G x E and genotype effect on the total phenolic content of Canadian field pea using an acidified methanol extraction process. Black, Brouwer, Mears & Iyer (1999) proved a significant G x E effect on the total dietary fibre, carbohydrate, protein and fat content of Australian field pea. Wang & Daun (2004) demonstrated a significant genotype and environment (as indicated by crude protein content) interaction effect on the starch, acid detergent fibre, neutral detergent fibre and fat content, and a genotype effect on the ash and phytic acid content of Canadian field pea. Wang, Hatcher & Gawalko (2008) elucidated the significant effect that genotype has on the protein, starch, ash, total dietary fibre (TDF), insoluble dietary fibre, trypsin inhibitor activity, phytic acid and resistant starch content of Canadian field pea.

The existing research on the G x E effect on the protein and anti-nutritional factors in field pea implicates the potential for a G x E effect on other nutritional components. The fact that there is an evident G x E effect on the protein content of field pea and that the protein content of field pea affects the content of other nutritional factors

(Wang & Daun, 2004) makes this phenomenon especially probable. As cited above, there have been studies examining the G x E effect on the TDF in Australian field peas and studies of the effect of genotype on the TDF in Canadian field peas. However, there has been no research published on the G x E effect on the TDF content of Canadian field peas. Although the phenolic content of field pea has been investigated, very few have analyzed the effect of G x E on phenolic content. A study that explored the effect G x E on the phenolic content of Canadian field pea was undertaken by Wang *et al* (1998). However, the method of phenolic extraction used in this study involved a simple acidified methanol hydrolysis, which resulted in the release of the smaller amounts of free phenolic components, not the plentiful bound phenolic components (Shahidi & Naczk, 1995; Nardini *et al*, 2002; Ross, Beta & Arntfield, 2009). Additionally, there remains no published research on peroxidase, an enzyme which causes polyphenol-catalyzed browning in food, and any potential G x E effect on its activity in field pea.

The objective of this thesis was to analyze the effect of G x E on some of the lesser known, but nutritionally important, components of Canadian field pea. These components include the total dietary fibre, insoluble fibre and soluble fibre, as well as the total phenolic content, individual phenolic components, antioxidant activity and peroxidase activity. Such information is valuable to the functional food industry and to manufacturers of functional food and nutraceutical products. Plant breeders and agronomists could also use this information to assess the quality of field peas produced in Canada and to make improvements and recommendations based on these findings.

2.0 LITERATURE REVIEW

2.1 Background

Field pea (*Pisum sativum* L.) is an annual, herbaceous, climbing plant belonging to the leguminosae family. When the seeds are harvested when they are immature they are referred to as garden peas. Garden peas are typically eaten as a vegetable and may be canned, frozen or eaten fresh. 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. Mature field pea is often used for animal and livestock feed but can be dehulled, split, ground into flour or fractionated into its component parts and used in food products for human consumption (Kay, 1979). Whole or parts of the pea crop, such as seeds and other plant remnants, may be used for silage (Davies, Berry, Heath & Dawkins, 1985). For the purpose of this thesis, field pea will refer to the dried mature seeds of *Pisum sativum* L.

2.1.1 Production and Consumption of Field Peas

Field pea has been cultivated and used as a diet staple and forage food since prehistoric times. It is believed that the domestication of peas originated approximately 9000 years ago in south-west Asia and spread into Europe in the Neolithic era (Davies *et al*, 1985; Saskatchewan Pulse Growers, 2000). A cool weather crop, peas thrive in the subtropics and in high altitude tropical areas as a winter crop, and it thrives as a summer crop in cooler climates, such as Canada and Russia (Kay, 1979). Field peas are used as a primary food source in many cultures due to their high carbohydrate and lysine-rich protein content. Field peas are also used extensively for livestock forage, particularly in Russia, North America and Europe (Saskatchewan Pulse Growers, 2000).

Field pea is a highly important commodity for Canada's agriculture industry. Field pea has become an important crop for the Canadian agriculture industry. Canada is the largest field pea producer in the world, providing 25% the world production and accounting for 50 % of world exports, with the majority of exports going to Europe, South America and Asia (AAFC, 2008). In 2008, Western Canada produced 2.3 million tonnes of dry peas (Wang, 2008). Canadian field pea exports are important to the Canadian agricultural economy – 75% of Canadian field peas were exported at a value of $500 million in the 2007-2008 growing seasons (AAFC, 2008). Domestically, approximately 25% of Canadian field pea production is used locally, primarily as livestock forage. Like other pulses, field peas are increasingly in demand as ingredients in food products as whole, split or pea flour, or as fractionated protein or fibre isolates (AAFC, 2008).

Field peas are produced primarily in Western Canada with Saskatchewan being the largest field pea producer in Canada, followed by Alberta and Manitoba. The production of field pea is well suited to the Canadian prairies, as this crop prefers cooler growing temperatures (daytime highs between 13 - 23°C) in moist, sub-humid regions, and is not tolerant to heat or drought (Saskatchewan Pulse Growers, 2000). Field peas are best suited to black soil zones with well drained, clay loam soils (AAFC, 2008).

Yellow and green field peas are the primary types of pea grown in Canada, along with smaller amounts of Austrian winter, maple and marrowfat peas. Round field pea seeds are preferred in Canadian field pea production as they are of higher market value. Field peas with yellow cotyledons account for 80% of Canadian field pea production, while field peas with green cotyledons account for 18% of production. Austrian winter,

marrowfat and maple peas comprise the remaining 2% (AAFC, 2008). Yellow field pea production is preferred over green field pea production because yellow peas provide a 10-15% higher yield than green peas, and green field peas are susceptible to bleaching, which can downgrade their quality and value (Saskatchewan Pulse Growers, 2000).

2.2 Nutritional Composition of Field Peas

Pulses are a renowned source of nutrients and are recognized as a food source with numerous health-promoting advantages. Once considered a "poor man's meat", health organizations world-wide are advocating the consumption of pulses to help reduce the risk of certain cancers, diabetes, heart disease and obesity (Leterme, 2002). An increasing number of health conscious and nutrition savvy consumers are turning to pulse crops as a diet staple, as they are a nutrient-dense protein source high in carbohydrate, dietary fibre, vitamins, minerals and non-nutritive bioactive components.

Table 1 depicts the nutritional content of field peas. Like other pulses, field peas are an excellent source of protein. The protein found in pulses like field peas is considered to be highly nutritious, as it is high in the amino acid, lysine, an amino acid that is deficient in cereal crops (Saskatchewan Pulse Growers, 2000; Leterme & Muños, 2002; AAFC, 2008). However, pulses are deficient in the sulphur-containing amino acids such as methionine (Kay, 1979). For those consuming pulses as a primary source of protein, this deficiency can be easily overcome by supplementing their intake with cysteine and methionine rich cereals such as wheat, rice, barley and rye.

Table 1. The Nutritional Composition of Field Pea

Component	Amount (g/ 100 g, unless otherwise stated)
Protein (N x 6.25)*	23 - 31
Crude Fat*	2.0 - 4.0
Carbohydrate*	
Starch	20.0 - 50.0
Total α-Galactosides	5.1 - 8.7
Raffinose	0.3 - 1.6
Stachyose	0.7 - 1.5
Verbascose	1.6 - 4.2
Dietary Fibre	15 - 21
Sucrose	0.7 - 5.7
Minerals (mg/100 g) †	
Fe	7.36
Zn	3.01
Ca	96
Mg	132
Bioactive Components‡	
Phytate-phosphorus †	0.06 - 0.33
Phytates	0.2 - 1.3
Trypsin Inhibitor Activity (mg/g)	4.4-12.5
Total Polyphenols [§]	0.25
Phenolic Acid[§]	0.001 - 0.003
Tannins[§]	0.0 - 1.3
Saponins [§]	0.1 - 0.3

*Guillon & Champ, 2002
†Sandberg, 2002
‡Champ, 2002
[§] % as dry matter

Additionally, field peas are also an excellent source of carbohydrates. Amylose, a component of starch made up of linear strands of glucose (Jenkins & Donald, 1995) comprises the majority of the starch fraction in pulses (20-35% of starch is comprised of amylose) and is beneficial for use in frozen foods, extruded bakery products, instant puddings and soups and dressings. As well, foods that have a high amylose:amylopectin ratio, when exposed to heat processing, tend to contain higher amounts of resistant starches as the heat causes the amylose to retrograde into its component resistant starches (Åkerberg, Liljeberg & Björck, 1998). The α-galactosides, such as raffinose, stachyose and verbascose, are resistant starches that are not digested in the upper part of the digestive tract, and are therefore fermented by bacteria in the colon. α-galactosides can be considered undesirable components of pulses, as their fermentation in the colon results in the production of gases and results in flatulence. However, recent research has found that these components have nutraceutical benefits and are now identified as prebiotic agents. Besides gases, the fermentation of α-galactosides in the colon produces short chain fatty acids (SCFA) which are linked to reduced incidence of carcinomas and adenomas in the colon and the prevention of cancers. These SCFA are also believed to contribute to reduced triacylglycerol and cholesterol levels, which can result in a potential reduction in the risk of heart disease (Guillon & Champ, 2002).

The dietary fibre fraction of field peas is used extensively in the production and supplementation of commercial fibre preparations. Fibre is present in the testa or hull (outer) and the cotyledon (inner) of the seed. The testa consists of about 89% TDF components, while the cotyledon consists of only 55% TDF (Guillon & Champ, 2002). Its light colour and bland flavour makes pea fibre ideal for food processing (Guillon &

Champ, 2002). Inner pea fibre, comprised primarily of cellulose as well as some pectin and hemicellulose (has a water binding and fat binding capacity, making it ideal for use as a fat replacement and texturing agent. Outer pea fibre can be added to foods without modifying the technical structure, and is incorporated into bakery products, snack foods, cereals and extruded products (Guillon & Champ, 2002).

Pulses contain a number of non-nutritive bioactive components that are believed to have nutraceutical properties. Phytate, also known as inositol hexakisphosphate**,** is used by plants as a major form of phosphorus storage. Although they readily chelate with minerals, limiting their bioavailability, phytates are believed to possess anticarcinogenic and cardiovascular disease reducing properties (Champ, 2002). Polyphenols, including simple phenols, tannins and flavonoids, serve as a first line of defense against pathogens in plants, and are required for plant growth and development. Polyphenols are responsible for the astringency and colour of many plants foods, including pulses. Like phytates, polyphenols have the ability to bind with minerals and reduce their bioavailability. However, the consumption of plant-based polyphenols, including those in pulses, correlates to a reduced risk of several health conditions, including cancer and cardiovascular disease (Champ, 2002).

2.3 Dietary Fibre

As public interest in nutrition and health increases, so does the demand for foods that provide a high nutritional impact. Fibre is becoming an increasingly popular supplement with health conscious consumers. This is not surprising, as increased fibre intake is linked to a decreased risk of certain cancers, heart disease and diabetes (Schafer

et al, 2003). Fibre is an important component in the human diet as it helps to prevent constipation, increase fecal bulk and lower plasma cholesterol (Schneeman, 1987). Foods that are rich in dietary fibre can contribute to the prevention of type 2 diabetes by slowing the digestion and absorption of carbohydrates and improving glycemic control (Venn & Mann, 2004). High fibre diets may likewise be able to lower the risk of colorectal cancers in various ways. Dietary fibre binds to carcinogens and prevents the interaction of carcinogens and bile acids with colonic mucosal cells (Harris & Ferguson, 1993). Dietary fibre also increases fecal bulk, expediting transit time through the colon, thereby reducing colonic exposure to these carcinogens (Harris & Ferguson, 1993). The degradation of fibre in the large intestine results in the production of short chain fatty acids, which have been shown to reduce the risk of colorectal cancers (Harris & Ferguson, 1993). In addition, an increased intake of foods that are high in dietary fibre, particularly those that are high in protein such as legumes, can help prevent hypertension (Lee *et al*, 2008) Food manufacturers are responding to consumers' desire for increased dietary fibre content in food by incorporating high-fibre ingredients into breads, baked goods and other bakery products (Vetter, 1984).

Field peas are not only an excellent source of protein, vitamins and minerals, but they are high in dietary fibre, especially insoluble fibre (Wang *et al*, 2008). The dietary fibre fraction of field peas has potential for use not only as a nutritional supplement but as a functional food ingredient in developing novel foods. Pea fibre can be used in baking applications and can replace up to 10% of wheat flour content without sacrificing bread loaf quality (Sosulski & Wu, 1988). When incorporated into baked goods, pea fibre develops into a light cream colour and has only a very mild pea flavour and aroma,

producing consumer-acceptable products (Vetter, 1984). Incorporating inner pea fibre into lean ground beef (10-14% fat) patties has been shown to improve tenderness and cooking yield without compromising the desired juiciness and flavour of the product (Anderson & Berry, 2000). Supplementing enteral formulas with pea fibre along with fructo-oligiosaccharides has been shown to increase the feeling of satiety in patients requiring enteral formulas as their sole source of nutrition (Whelan *et al,* 2006).

2.3.1. Dietary Fibre Analysis

The American Association of Cereal Chemists (AACC) defines dietary fibre as "…the edible parts of plants or analogous carbohydrates that are resistant of digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine" and includes polysaccharides, oligosaccharides, lignin and associated plants substances (M^{c}Cleary, 2008). The method chosen for this study reflects this definition, as the enzymatic-gravitational method of TDF analysis (32-07) outlined by the AACC is able to include soluble dietary fibre fractions such as pectins, gums and certain hemicelluloses, (Asp *et al,* 1983) as opposed to other detergent-based methods. This unique quality, along with ease of use and cost effectiveness, is why the enzymatic-gravitational method is a preferred method of TDF, IDF and SDF analysis (Gordon, 2007).

Despite the relevance and utility of this method, few studies have evaluated the total dietary fibre (TDF), insoluble fibre (IDF) or the soluble fibre (SDF) content of field pea. Most field pea fibre studies analyze acid detergent fibre (ADF), neutral detergent fibre (NDF) or crude fibre (CF) content, which are more applicable for feed applications than dietary fibre analysis for human consumption. The NDF of a sample is determined

by the sum of the insoluble hemicellulose, cellulose and lignin present (Reichert & Mackenzie, 1982), while the ADF consists primarily of cellulose and lignin (Sandberg *et al*, 1981). Although NDF, ADF and TDF are not the same entity, the enzymatic-gravitational TDF assay measures many of the same fibre components. Enzymatic-gravitational TDF assays measure not only the cellulose, insoluble hemicellulose and lignins, but also water soluble components such as soluble hemicelluloses, gums and pectins (Asp *et al*, 1983). There are even fewer studies reporting the effect of genotype or environment, and their interactions on the dietary fibre content of field peas. To this date, there are no studies that analyze the effect of genotype x environment on the TDF, IDF and SDF content of field peas grown in Canada. Black *et al* (1998) studied the TDF content of 61 field pea cultivars grown in Australia over 2 years and found that genotype had a very significant effect ($p<0.001$) on TDF content in field peas, while growing year had no influence. Wang and Daun (2004) found a strong variety x environmental effect on the acid detergent fibre ($p<0.01$) and neutral detergent fibre (NDF) ($p<0.05$) on Canadian field peas. Warkentin, Sloan & Ali-Khan (1997) found significant ($p<0.05$) differences in ADF content between cultivars and growing locations. Al-Karaki & Ereifej (1999) found a significant ($p<0.05$) genotype by environment effect on the protein content in field peas grown in Jordan, indicating a possible genotype x environment effect on fibre, echoing the conclusions of Reichert & MacKenzie (1982) that NDF content varies inversely with protein content in Canadian-grown field peas. Such information is essential in order to market and utilize the TDF component of Canadian field pea.

2.4 Peroxidase Content

Peroxidase is a common, heat stable enzyme that is found in plants and plant products, including fruits, vegetables and grains. There is a direct relationship between the peroxidase activity and the development of off-flavours, off-odours and off-colours in food (Burnette, 1977). Peroxidases have the ability to perform single-electron oxidation on phenolics and aromatic components in plant matter in the presence of hydrogen peroxide (Burnette, 1977, Tomás-Barberán & Espín, 2001). This oxidation results in the production of melamines, causing enzymatic browning (Tomás-Barberán & Espín, 2001).

Peroxidases are widely distributed in plant tissues in both the intra- and extracellular environments, and are a primary response to physiological and pathogenic stressors (Luhová, Lebeda, Hedererová & Peč, 2003). Plant tissues contain reactive oxygen species (ROS) such as hydrogen peroxide, which are thought to be formed spontaneously or catalyzed by superoxide dismutase found in the cytosol, chloroplasts and mitochondria of plant cells (Wojtaszek, 1997). High amounts of ROS are generated in response to external stimuli (i.e. cutting). Peroxidase begins to react with ROS to produce melamine, a brown coloured pigment. This pigmentation results in off-colours, and may also result in changes in odour and flavour. Green peas have been found to have 3 peroxidase isozymes: one neutral and two cationic isozymes (Halpin, Pressy, Jen & Mondy, 1989).

Although peroxidases are desirable in some food processing methods, such as in the production of black tea, they are, more often than not, a detriment to grain and vegetable crops. Peroxidase in immature wheat kernels can restrict seed elongation during development and may cause enzymatic browning in noodle products (Hatcher &

Barker, 2005). The presence of peroxidase is especially of concern with frozen vegetables, such as green beans and peas, as peroxidase is a relatively heat stable enzyme and blanching procedures are typically not sufficient to inactivate the enzyme (Güneş & Bayindirli, 1993). Peroxidase activity has also been shown to have a detrimental effect on the colour of processed pea puree (Icier, Yildiz, & Baysal, 2006). The production of end-product field peas with reduced peroxidase activity would be preferential from a consumer and processing standpoint as opposed to inactivating the enzyme using thermal or chemical means.

2.5. Polyphenols in Field Peas

Phenolics can be defined as "substances containing an aromatic ring bearing one or more hydroxyl substituents, including their functional derivatives" (Shahidi & Naczk, 1995). They are secondary metabolites in plants and defend against pathogens and ultraviolet radiation damage (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). Many phenolics are also responsible for the brightly coloured hues of plants (Shahidi & Naczk, 1995). Humans consume phenolics via plant foods, such as cereals, oilseeds, fruits, vegetables, spices, beverages and pulses.

Phenolics play a complex role in the quality and nutritional impact of food. For years, researchers considered phenolics to be an antinutrient, meaning that their presence has a negative impact on the digestibility and absorption of nutrients. However, recent research has discovered that food phenolics have antioxidant capabilities and may have anticancer properties (Shahidi & Naczk, 1995). The presence and nature of different phenolics also has an important influence on the flavour of various foods. For example,

the astringency that is imparted by the presence of phenolics is essential to the flavour of red wines, coffees, teas and dark chocolates. Such flavours, however, are not always desirable and may impart undesirable flavours in food such as a smoky flavour in chocolate that has not been processed properly being one common example (Shahidi & Naczk, 1995). Phenolics are also important to the aesthetics of food. Some phenolics are a substrate for enzymes, such as polyphenol oxidase, that are responsible for browning in fruits such as apples. This same reaction is essential for imparting the proper flavour and appearance of cocoa, dates and tea (Shahidi & Naczk, 1995).

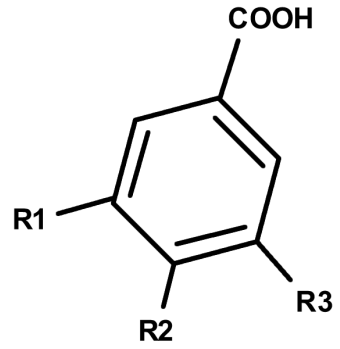
Pulses, like other grains, are a high source of food phenolics. Pulses are especially high in a group of phenolics known as polyphenols, which can reach up to 2% of the total content of beans and peas. Field peas can contain upwards of 1050 tannic acid equivalents of polyphenols, with the majority of polyphenols located in the testa (cotyledon) (Shahidi & Naczk, 1995). In general, pulses that have dark-coloured testae contain higher amounts of polyphenols than pulses that have light or white-coloured testae. In addition, immature pulses tend to have higher amounts of polyphenols than do mature pulses. This may be due to the polymerization of these components to larger, more insoluble polymers (Shahidi & Naczk, 1995).

2.5.1. Structure of Polyphenols

In general, pulses contain two types of phenolic components: phenolic acids and flavonoids. The phenolic acids can be further divided into two classes: the benzoic acids and the cinnamic acids (Manach *et al*, 2004). Phenolic acids can occur naturally bound as

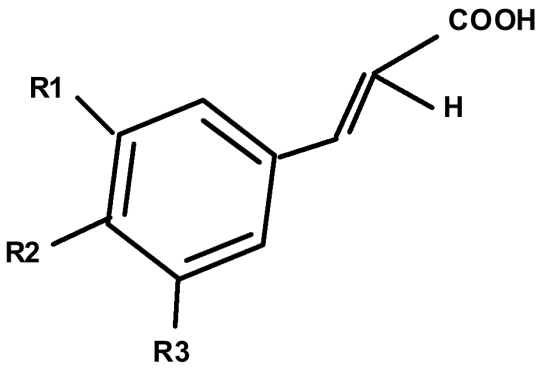
Figure 1. Structures of the Phenolic Acids and Flavonoids Found in Field Pea

Benzoic Acid Derivatives



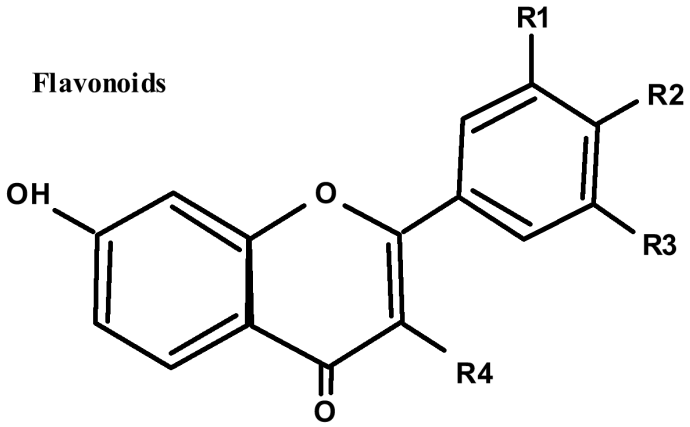
Gallic Acid R1 = R2 = R3 = OH
Protocatechuic Acid R1= R2 = OH, R3 = H
4-Hydroxybenzoic Acid R1 = R3 = H, R2 =OH
Vanillic Acid R1 = OMe, R2 = OH, R3 = H
Syringic Acid R1 = R3 = OMe, R2 = OH

Cinnamic Acid Derivatives



Caffeic Acid, R1 = R2 = OH, R3 = H
***p*-Coumaric Acid, R1 = R3 = H, R2 = OH**
Ferulic Acid, R1 = OMe, R2 = OH, R3 = H
Sinapic Acid, R1 = R2 = OMe, R3 = OH
Cinnamic Acid, R1 = R2 = R3 = H

Flavonoids



Quercetin, R1 = H, R2 = R3 = OH, R4 = rhamnoside
Rutin, R1 = H, R2 = R3 = OH, R4 = rhamnoglucoside

(Hayes, Smyth & M^{c}Murrough, 1987; Spáčil, Nováková & Solich, 2008)

esters and glycosides or conjugated with organic acids. Figure 1 shows the various phenolic structures. Benzoic acids consist of a C_6-C_1 backbone and are comprised of an aromatic ring and carboxylic acid. Cinnamic acids consist of a C_6-C_3 backbone and are also comprised of an aromatic ring and carboxylic acid. The phenolic acids have R groups located at the 3-, 4- and 5- positions of the ring structure. The flavonoids are C_{15}, three-ringed structures (C_6-C_3-C_6) that are often bound to various sugars in the aglycone or glycoside form. Flavonoids can be further broken down into smaller groups, including the flavones, flavonols, flavanones, flavonols, anthocyanins, chalcone, isoflavanones and isoflavones (Lee, 2000). Each of the ring structures may contain hydrogen, hydroxyl, methoxyl or rhamnoglucoside R groups. The hydroxyl groups on the ring structures readily form hydrogen bonds with minerals, proteins and carbohydrate components. They also are electron donors and act as free radical terminators, reacting with free radicals to form more stable components (Shahidi & Naczk, 1995).

2.5.2 Antioxidant Activity of Polyphenols.

In order to understand why the polyphenolic content plays such an important role in the nutritional, sensory and nutraceutical functions of pulses, the oxidative activity of polyphenols must be examined. Oxidation occurs when a chemical component undergoes a loss of electrons or hydrogen atoms by a free radical. In food systems, this can result in the loss of quality, colour, flavour, texture and nutritive value. In human health, oxidation plays a key role in carcinogenesis and cardiovascular disease. Autoxidation, or an oxidation chain reaction, involves three steps: initiation involves the initial production of free radicals, propagation involves the continued production of free radicals and finally

termination involves the binding of free radicals to form non-radical products (Shahidi & Naczk, 1995). The polyphenols in pulses act as primary antioxidants, which react directly with high energy free radicals to form more stable products, in contrast to secondary antioxidants, which slow the initial chain reaction by breaking down hydroperoxides. There are three proposed methods by which chemicals act as antioxidants: they may act as free radical terminators by donating a hydrogen atom; they may chelate with metal ions; or they may act as oxygen scavengers. Polyphenols act as antioxidants by participating in all three activities (Shahidi & Naczk, 1995). The positioning of the hydroxyl groups seems to have an effect, as having a second hydroxyl group at the ortho or para position of the phenol tends to increase the antioxidant activity. Once hydrogen has been donated to quench a free radical, a phenoxy radical is formed. The phenoxy radical remains fairly stable due to resonance delocalization around the aromatic ring of the phenol. However, at high concentrations, polyphenols may lose their antioxidant activities and become pro-oxidants, thereby acting as reaction initiators (Shahidi & Naczk, 1995).

2.5.3. Polyphenols and Nutrient Bioavailability.

Pulses are an essential part of African, Asian, Central and South American diets as they are high in dietary fibre, complex carbohydrates and are an excellent source of protein. However, the oxidative activity of polyphenols tends to reduce the bioavailability of many nutrients. Most early literature classified polyphenols as antinutrients and discussed ways to reduce either the amount, or the effects, of naturally occurring polyphenols in pulses. Reducing the polyphenol content of pulses may be especially

important to individuals who rely on this food source as a primary source of carbohydrates and protein.

2.5.3.1 Polyphenols and Proteins. There are two ways in which polyphenols interfere with ingested proteins and their nutritive value. Polyphenols in pulses prevent food proteins from being digested by binding to them and forming insoluble complexes. Polyphenols also bind to proteolytic enzymes, inhibiting the digestion of proteins in food. Polyphenols form complexes with proteins by forming hydrogen bonds with the reactive groups of protein molecules. Oxidized phenolics are reactive towards the methylthiol group in methionine and ε-amino group in lysine and form complexes that make proteins indigestible to monogastric animals such as humans (Shahidi & Naczk, 1995).

There are many factors influencing the affinity polyphenols have for proteins. Polyphenols can form soluble and insoluble complexes with proteins depending on the size, conformation and charge of the given protein. For example, proteins that tend to conform to globular structures, such as lysosyme and ribonuclease, have less affinity towards polyphenols than do proteins with open structures, such as gelatin. Proteins having the highest affinity for polyphenols tend to have high molecular weights and are largely composed of hydrophobic amino acids, such as proline (Shahidi & Naczk, 1995). The structure of the polyphenol also plays an integral role in its ability to bind with proteins. Polyphenols with at least three flavonol subunits, as well as polyphenols that have three orthohydroxy groups, as opposed to two on the B ring, create a tighter bond with proteins. At lower protein concentrations, the precipitation of polyphenol-protein structures is due to the formation of a hydrophobic layer of polyphenols on the surface of the protein. At higher protein concentrations, the precipitation is a result of both the

cross-linking of polyphenols with different protein molecules as well as the formation of a hydrophobic polyphenol layer on the protein surface. Such interactions may also occur with enzymes, and could inhibit the activity of amylases, proteases and β-glucosidases (Shahidi & Naczk, 1995).

2.5.3.2 Polyphenols and Carbohydrates. As with proteins, polyphenols form indigestible complexes with carbohydrates. Polyphenols that have larger molecular weights and have open conformational structures are more likely to form complexes with carbohydrate molecules. For example, polyphenols tend to have an affinity towards cyclodextrins and bind within the centre of the cyclodextrin molecule (Shahidi & Naczk, 1995). Carbohydrates that have formed complexes with polyphenols are less susceptible to enzymatic degradation, making the carbohydrate unavailable to humans as a source of energy and nutrition. However, this lack of digestibility may have some benefits, as studies have shown that certain phenolic acids may have flatulence-inhibiting properties, particularly in soybean (Shahidi & Naczk, 1995).

2.5.3.3 Polyphenols and Vitamins & Minerals. Another reason polyphenols are classified as an antinutrient is their ability to form insoluble complexes with micronutrients. Divalent metallic ions, such as iron, are particularly susceptible to bonding and precipitation by polyphenols, thereby inhibiting their nutritional bioavailability (Shahidi & Naczk, 1995). This phenomenon is especially apparent in chronic tea drinkers, who are especially susceptible to iron-deficient anemia. The polyphenols in the tea bind to iron, preventing its absorption and lowering the retention of iron in their bloodstream (Shahidi & Naczk, 1995). Phenolic acids that contain galloyl or catechol moieties are the most successful at chelating iron. Certain polyphenols also

have a tendency to chelate with vitamins. For example, caffeic acid has an oxidative effect on thiamine, while tannic acid causes the precipitation of vitamin B_{12} (Shahidi & Naczk, 1995).

2.5.4. Sensory Characteristics of Polyphenols in Pulses.

Polyphenols are important to the sensory evaluation of pulses, as they contribute to the flavour, colour and texture of pulses. In some instances, certain characteristics are both essential and undesirable in food processing. For example, astringency in wine and enzymatic browning effect in cocoa are beneficial. However, the presence of polyphenols in pulses is usually undesirable from a sensory perspective. The incorporation of pulse fractions into food is limited by their characteristic colours and flavours, and enzymatic browning reactions are problematic to processing applications. Polyphenols also tend to impart medicinal flavours in high quantities. For example, feedstuffs for livestock that are high in polyphenols may carry over to the animal and give the animal muscle a phenolic taste (Shahidi & Naczk, 1995).

Polyphenols also play a role in the enzymatic browning reaction of plant foods. One such enzyme, polyphenol oxidase (PPO), is a common enzyme found in plants. In living plants, PPO acts as an antipathenogenic, producing insoluble polymers that prevent the spread of viruses, bacteria and fungi to healthy parts of the plant (Shahidi & Naczk, 1995). PPO causes browning to occur when it catalyzes the oxidation of phenolics to ortho-quinones, which in turn react with the amino and sulfhydryl groups of proteins and enzymes, forming high-molecular weight complexes that are brown in colour (Shahidi & Naczk, 1995; Hutchings, 1994). This reaction is beneficial when processing coffee, tea,

cocoa and dates, but undesirable when processing plant-based foods, including pulses. In these cases, the reaction can be slowed or eliminated through various methods. Blanching, or applying high temperature water or steam for short periods of time, can be enough to denature the enzyme. However, steam and hot water blanching may result in the loss of water soluble vitamins and nutrients (Shahidi & Naczk, 1995). Reducing the pH below 4.0 may also denature PPO, preventing the onset of enzymatic browning. Although many phenolics are substrates for the PPO reaction, some may have a preventative effect. Studies have found that protocatechuic acid inhibits the enzymatic browning of broad beans (*Vicia faba L.*) (Hutchings, 1994).

The polyphenol content in pulses may also be responsible for the "hard-to-cook" phenomenon. Pulses are notoriously hard to prepare when dried, as they need to be soaked and cooked for long periods of time. Pulses that have elevated polyphenol content tend to take longer to cook than pulses with lower polyphenol content (Shahidi & Naczk, 1995). Extended storage of pulses, particularly in hot and humid conditions, results in phenol metabolism and encourages the formation of polyphenol-protein complexes (Shahidi & Naczk, 1995). Higher PPO levels tend to enhance the hardness of the cotyledon (Shahidi & Naczk, 1995). This is due to the ability of PPO to oxidize polyphenols and allow them to form strong protein-polyphenol complexes. Soaking pulses in a saline solution reduces this effect (Shahidi & Naczk, 1995). Dehulling the seeds is one of the most effective ways of reducing the hardening of the cotyledon, as the hull of the seed contains the highest amount of polyphenol. Soaking also removes a large amount of polyphenol, and dehulling the cotyledon after soaking further reduces the

polyphenol content. Treatment with alkali solution of sodium and potassium hydroxide can also result in significant reductions of polyphenols in pulses.

2.5.5 Nutraceutical Effect of Polyphenols

Like many other phytochemicals, some of the same chemical properties of polyphenols that result in undesirable sensory or processing effects are beneficial from a pharmacological or nutraceutical perspective. For example, the chelating properties of polyphenols that bind to minerals and prevent their absorption may help to prevent cancer. The antioxidant properties of polyphenols show promise for use in the food industry as natural preservatives, and in the functional foods and nutraceutical industry as nutritional components with antiviral, anti-inflammatory and anticarcinogenic effects (Shahidi & Naczk, 1995).

2.5.5.1 Polyphenols and Cardiovascular Disease (CVD)

Not only are polyphenols powerful antioxidants in plant and food systems, they are powerful antioxidants *in vivo.* This is especially true in the case of CVD and the ability of polyphenols to inhibit the oxidation of low density lipoprotein (LDL) cholesterol. Animal trials have found that supplementing food and water sources with polyphenols, such as catechin or quercetin, or polyphenol-rich beverages such as dealcoholized wine or pomegranate juice, reduced the oxidation of LDL, decreased the susceptibility of aggregation of LDL, and reduced the development of atheromatous lesions in apoE- deficient mice. In humans, high consumption of polyphenols has been shown to improve endothelial dysfunction, a condition in which the function of the cells that line the inner surface of blood vessels and arteries is compromised. Often,

endothelial dysfunction is a precursor and prognostic tool for CVD risk. The consumption of foods high in polyphenols may also have an antithrombotic effect by preventing platelet aggregation (Scalbert, Manach, Morand, Rémésy & Jiménez, 2005). Studies have demonstrated an inverse relationship between flavonoid consumption and the risk of CVD. One study suggested that higher intakes of flavonoids (> 30 mg/ day) resulted in a 50% decrease in coronary heart disease mortality compared to those who consumed low amounts (<19 mg/day) of dietary flavonoids (Flight & Clifton, 2006).

2.5.5.2 Polyphenols and Cancer Prevention

In recent years, the chemopreventative effect of polyphenols has been the focus of rigorous research. It is thought that the antioxidant effect of polyphenols may prevent mutagenesis and tumour development. There are many mechanisms that are believed to give polyphenols their cancer preventing effect. Flavonoids seem to modulate cytochrome p-450 isoenzymes that subsequently inhibit the metabolic activation of carcinogens (Scalbert *et al*, 2005). Flavonoids deactivate radical oxygen species and scavenge active oxygen species. Flavonoids also deactivate ultimate carcinogens and reduce their bioavailability. Additionally, flavonoids inhibit the metabolism of arachidonic acid, which has been identified as a biomarker for various cancers, and they inhibit the activity of protein kinase C and other kinases that act as signal pathway regulators to cancerous cells. Quercetin, a common flavonoid in pulses, has been found to have excellent antimutagenic properties (Shahidi & Naczk, 1995, Scalbert *et al*, 2005). Flavonoids are proving to have anticarcinogenic effects and are demonstrating potential

in the prevention of many cancers, including lymphocytic leukemia and lung cancer (Wagner, 1979).

2.5.5.3 Antiviral and Antibacterial Properties

Not only do polyphenols possess antioxidant properties, they possess antibacterial and antiviral properties, as well. Several studies have researched the antimicrobial properties of polyphenols with hopes to develop a natural antimicrobial. Esters of gallic acid and p-hydroxybenzoic acid have shown the ability to inhibit the growth and toxin production of *Clostridium botulinum* types A and B. Ferulic acid and *p*-coumaric acid has been found to inhibit *Saccharomyces cerevisiae* (Shahidi & Naczk, 1995). There is a synergistic quality between different types of polyphenols when examining the microbial inhibitory effect. A study analyzing the antimicrobial properties of proanthocyanidins, flavonols and benzoic acids on *Sacchromyces bayanus* and *Pseudomonas fluorescens* found that there is a synergistic relationship between benzoic acids and proanthocyanidins or flavonols that is not seen in isolation (Shahidi & Naczk, 1995). The antimicrobial effect of proanthocyanidins and flavonols together is additive, however.

There are also some studies that indicate that polyphenols could be used as antivirals (Bakay, Musci, Beladi & Gabor, 1968; Jassim & Naji, 2003). Polyphenols from strawberries have been shown to deactivate the polio, enteric and herpes viruses (Konowalchuk & Speirs, 1976), while quercetin, a common polyphenol in pulses, has been proven to deactivate herpes simplex virus type 1, para influenza virus type 3 and polio type 1 virus. Quercetin may also act synergistically with interferon to increase the antiviral effect (Shahidi & Naczk, 1995).

2.5.5.4 Polyphenols and their Antiglycemic Effect

Recent research has shown that diets containing high polyphenol content may help reduce the risk of metabolic disorders such as type 2 diabetes mellitus. It appears that elevated levels of dietary polyphenols result in a lower than expected glycemic index due to inhibited metabolism and the absorption of glucose. Foods that are high in polyphenols slow the absorption of glucose into the bloodstream and prevent a post-meal spike in plasma glucose levels. Diets rich in polyphenols have also displayed an ability to lower plasma insulin and glucose-dependent insulinotropic polypeptide as well as increase glucagons-like polypeptide-1 levels after eating (Cifford, 2004). There are many theories as to how the ingestion of polyphenols results in a lower glycemic index. One such theory points to the ability of polyphenols to form strong bonds with and inhibit enzymes, including α-amylase, α-glucosidase or maltase (Cifford, 2004). Other theories suggest that polyphenols interfere with active glucose transporters such as SGLT1 (sodium-glucose cotransporter enzyme) in the duodenum (Clifford, 2004) as well as GLUT1 (glucose transporter enzyme) and GLUT2 (Scalbert *et al,* 2005). Several polyphenols, including quercetin glycosides, have been shown to interact with SGLT1, thereby interfering in glucose transport (Clifford, 2004).

2.6. Methods of Determining the Phenolic Content of Pulses

Currently there is no standardized method of measuring the phenolic content of pulses, the relevant literature containing a variety of alternatives for its analysis and assessment. Most methods of phenolic content analysis can be broken down into three groups: total phenolic content analysis, chromatographic analysis of individual phenolic

components, and antioxidant activity analysis. Most literature relies on a combination of two or more of these methods for reporting phenolic content. All three types of analyses were used in this thesis and the different types of assays were assessed before proceeding with the phenolic analysis.

2.6.1 Total Phenolic Content (TPC) Assessment

There are several different methods that can be used to assess the TPC of field pea. The most commonly used is the redox-based Folin-Ciocalteu method developed by Singleton & Rossi (1965). The use of the Folin-Ciocalteu reagent in the TPC assay was developed to replace the use of the traditional Folin–Denis phenol reagent, which had produced erratic colour development and a possible underestimation of the phenolic content of the sample being analyzed (Singleton & Rossi, 1965). Another redox-based method, the Prussian blue test, has been cited in literature for use in the estimation of TPC (Budini, Tonelli & Girotti, 1980; Deshpande & Cheryan, 1987). Other methods, such as the vanillin assay, have been used, but were not considered for this thesis. The primary reason for their exclusion was that the vanillin and other assays detect only tannic acid content, and do not detect the smaller, yet nutritionally important, phenolic acids (Deshpande & Cheryan, 1987). More recently, a peroxidase-catalyzed method of TPC determination was developed by Stevanato, Fabris & Momo (2004), with modifications by Ma & Cheung (2007).

2.6.1.1 Peroxidase-Catalyzed Enzymatic Method of TPC Analysis

There are many advantages to using the peroxidase-catalyzed enzymatic (PE) method for TPC analysis as opposed to more conventional redox reactions. The PE method works by forming phenoxyl radicals from existing sample polyphenols that in turn bind to 4-aminophenazone and form a quinone-imine dye. In brief, peroxidase enzyme (from horseradish) in the presence of hydrogen peroxide is oxidized and reacts with the phenolic components of the sample to form phenolic radicals. These radicals then react with aromatic substrates, including the aromatic amine group of 4-aminophenazone to form a quinone-imine coloured product. The estimated TPC is based on the ability of these individual phenoxy radicals to react with aromatic components and form components with a high molar absorbance that can be read spectophotometrically at 500 nm. The results were then compared to the absorbances of known amounts of catechin, which provided a linear response with a correlation coefficient of 0.993, to determine the TPC (Stevanato *et al*, 2004).

2.6.1.2 Redox-Based Methods of TPC Analysis

The redox-based methods are the most commonly used methods of TPC analysis due to their ease of use, and the fact they require little in the way of equipment and reagent. The method relies on the reducing power of the phenolic components to quench certain oxidizers, such as phosphotungstic and phosphomolybdic acid in the Folin-Denis and Folin-Ciocalteu methods (Deshpande & Cheryan, 1987). The Prussian blue test is another redox method that is based on the reduction of ferric ions to ferrous ions by phenolic components and the subsequent formation of a ferricyanide-ferrous ion complex

with a high molar absorbency (Price & Butler, 1977). A major drawback of the redox-based methods is that other reducing substances, such as sulphites and ascorbic acid, may interfere with the results and give artificially high phenolic content estimates (Stevenato *et al*, 2004).

2.6.2 Chromatographic Analysis of Polyphenols

Chromatography can be defined as a physical separation method in which components are separated and distributed between a stationary phase and a mobile phase that percolates through the stationary phase (Siouffi, 2000). Many methods of chromatography have evolved over the years, utilizing various mobile and stationary phases. Thin-layer chromatography (TLC) is a method of separation in which the stationary phase is a flat surface containing a thin layer of adsorbent. In more common use, column chromatography involves adsorbent packed into a narrow tube, or column. The mobile phase can also consist of different states of matter. In gas chromatography (GC), the mobile phase is a gaseous state, while in liquid chromatography (LC), the mobile phase is a liquid (Yost, Ettre & Conlon, 1980). The work for this thesis will focus on liquid chromatography using a column, with special attention being placed on ultra-performance liquid chromatography (UPLC) and its utilization in separating phenolic components in pulses. There are several different ways in which LC can separate chemical components:

2.6.2.1 Normal Phase Chromatography

In normal phase chromatography, the stationary phase of the column is comprised of a strongly polar packing material, while the mobile phase is comprised of a non-polar liquid. As the sample travels through the column, the least polar components elute faster, while the more polar components are retained in the column longer than their less polar counterparts (Yost *et al*, 1980). Because this method is based on the use of a polar stationary phase and a non-polar mobile phase, normal-phase chromatography is used to separate components that have some solubility in non-polar organic solvents. This is especially the case with fat-soluble vitamins, such as vitamins A and D, tocopherols, tocotrienols, diglycerides and many arachidonic acid metabolites (Larson, Tingstad & Swadesh, 2001).

2.6.2.2 Reversed-Phase Chromatography

In reversed-phase chromatography, the stationary phase of the column is comprised of a non-polar packing material, while the mobile phase is comprised of a polar liquid. As the sample travels through the column, the polar components are strongly attracted to the mobile phase and are the first to be eluted out of the column. The more non-polar components are retained in the column, the lower the affinity of the component to the mobile phase dictating a longer period of retention. (Yost *et al,* 1980).

Reversed-phase chromatography is extremely versatile and can be used for many applications. This method tends to work well with slightly hydrophobic components, as the principle of separation is based on hydrophobic affinity. The non-polar components interact readily with the non-polar stationary phase via hydrophobic interactions. This

gives the component more of an affinity to the stationary phase than to the more polar mobile phase, until the polarity of the mobile phase decreases to the point where the component has more affinity for the mobile phase than the stationary phase. In reverse-phase chromatography, the more polar components elute first, while the least polar components elute last. Because of this principle, reverse-phase separation can be used for aromatic hydrocarbons, carboxylic acids, some hydrophobic amines, peptides and carbohydrates, lipids and other hydrophobic components such as pigments and dyes (Shah & Maryanoff, 2001).

2.6.2.3 Ion-Exchange Chromatography

In ion-exchange chromatography, the stationary phase has an ionic charge that is opposite to the analyte(s) to be separated. The stronger the electrostatic interaction between the analyte and the stationary phase, the longer it will take for the analyte to elute from the column. The mobile phase varies in its pH and polarity to control the elution time from the column (Yost *et al*, 1980). There are two different types of ion-exchange (active) groups that are bound to the basic structure of the stationary phase. Cationic exchange groups are acidic groups, while anion-exchange groups are basic groups. Strongly acidic and strongly basic groups retain their charges regardless of the pH of the mobile phase within normal column operating pH. However, weak acids are charged only when the pH is greater than or equal to their pKa + 1, and weak bases are charged only when the pH is less than or equal to their pKa – 1. This makes using weakly acidic and basic groups in the matrix desirable, as the pH of the mobile phase buffer can be manipulated to change the charge of the matrix, thus allowing for greater control of

elution of analytes that would otherwise be strongly adsorbed. For this reason, weak exchange materials are used to elute strong acidic and basic analytes while strong exchange materials are used to elute weakly acidic and basic analytes (Rizzi, 1998). Ion-exchange chromatography lends itself to the detection of many ionic components such as inorganic ions, the separation of sugars and carbohydrates, amines and amino acids, peptides, proteins and in some cases, organic and phenolic acids (Swadesh, 2001).

2.6.2.4 Size-Exclusion Chromatography

In size-exclusion chromatography, the stationary phase consists of a material that has pore sizes within the desired range of the molecular size of the sample being separated (Yost *et al*, 1980). The principle behind this method is size exclusion; larger molecules that are not small enough to fit into these pores are eluted first out of the column while smaller molecules penetrate into the pores. The shape of the pore can also be manipulated, such that only molecules of a certain shape and size can penetrate the pores. Stationary phases from silica and cross-linked polymer gels can be used. This method of exclusion is used primarily with macromolecules such as proteins (Rizzi, 1998).

2.6.2.5 Chromatography for Phenolic Separation in Pulses

The mechanism of separation strongly depends on the chemical structure of the component to be analyzed. Food phenolics can be described as “a wide range of compounds that possess an aromatic ring bearing a hydroxyl substituent and include their functional derivatives, such as esters, methyl ethers and glycosides” (Lee, 2000).

Phenolic glycosides are water soluble, while aglycones are more hydrophobic. The polarity of the phenolics is dependent on the availability of the hydroxyl group, with hydroxyl groups at the 4-position giving the highest polarity, followed by hydroxyl groups at the 3- and 2- positions. The increase of methoxy groups or the presence of an ethylenic side chain in a cinnamic acid can also reduce polarity. Their aromatic ring structures give phenolics the ability to absorb ultraviolet light (Lee, 2000).

In most cases, reversed-phased chromatography is the preferred method of phenolic analysis. This is due to the varying polarity of the different phenolics, from slightly polar to extremely hydrophobic. This variance in polarity allows for the more polar phenolics to be eluted first, followed by less polar phenolics, with the most hydrophobic components eluting last. Typically, C_{18} silica columns are used as the stationary phase, but various publications have reported using polystyrene columns and silica particles with smaller ligands (C_6-C_8) (Lee, 2000).

In reversed-phase chromatography, water is typically used with some form of organic solvent (usually methanol or acetonitrile) in a gradient for the mobile phase. There are many reasons why methanol would be preferred over acetonitrile. Methanol is less toxic than acetonitrile, is significantly less expensive, and can be used in a higher concentration than acetonitrile without fears of column deterioration. However, acetonitrile gives better and sharper peaks than methanol, and it gives a better resolution in a shorter period of time than methanol (Lee, 2000). Provided that budgets allow for it, acetonitrile is a preferred organic solvent for phenolic separation. Because the pH range preferred for reversed-phase separation of phenolics is low (between pH 2 – 4) there is a chance that the phenolic groups may ionize and increase retention in an undesirable

manner. Therefore, acetic or trifluoroacetic acid is often added to the mobile phase. However, the addition of acetic acid may cause a noisy baseline; the addition of 0.1% trifluoroacetic acid results in a transparent mobile phase, permitting the phenolics to elute in a symmetrical band (Lee, 2000).

There have been many recent advances in column chemistry and technology that have changed the way stationary phases are designed. Traditionally, larger, irregularly shaped particles were used in the stationary phase. More recently, spherical particles have been used in columns. The spherical shaped particles allow for uniform packing without random gaping or voids, providing the best bed stability (Waters Corporation, 2007). Silica is the preferred stationary phase in reversed-phase chromatography; polymer based phases have lower performance levels, especially with smaller molecules, and carbon based phases are less efficient. Metal oxide phases such as alumina and zirconia have greater stability than silica, but they are not as efficient, and they are prone to ionization (Doyle & Dorsey, 1998). Silica phases use a variety of ligands, from C_4 to C_{18}, some with phenyl groups and some with embedded polar groups. Unbonded particles are useful for very polar compounds while the C_{18} ligands allow for retention of hydrophobic groups and particles. (Waters Corporation, 2007). Because the separation of the phenolics is based on hydrophobic interactions with the stationary phase, a silica C_{18} column is typically preferred.

The size of a column is important when developing a separation method. Smaller diameter columns increase the sensitivity and are ideal for analytical and diagnostic work, while larger diameter columns increase column capacity. Increasing the column length increases the resolving power, while shorter columns reduce the elution time.

Smaller particle sizes (< 2.0 μm) are preferred, as they result in faster analysis and an increase in sensitivity and resolution. To get these results, however, high amounts of pressure are produced for an equivalent flow rate in UPLC systems (Waters Corporation, 2007).

2.6.3. Methods of Analyzing Antioxidant Activity in Field Pea

Long regarded as an antinutrient that needed to be eliminated or reduced from pulse crops, polyphenols are now considered an integral part of nutrition and are believed to reduce the incidence of many diseases. Polyphenols in pulses possess antioxidant activity, which is key to the nutraceutical or pharmacological effect that a component has on human health. Because it is the antioxidant activity of the phenolic components and not the amount of phenolics present in the product, per se, that provides the nutraceutical benefit of the product, knowing the antioxidant activity to be more important than knowing the actual phenolic content (Rice-Evans *et al*, 1996). Different phenolics have different antioxidant activities. The radical scavenging ability of any given phenolic is based on three structural components of the molecule:

1. An *o*-hydroxy structure in the B ring, which allows for electron delocalization and higher radical stability;
2. A 2,3 double bond with a 4-oxo function in the C ring. This allows for delocalization of electrons around the B ring and allows for stability of phenoxy radicals due to resonance effect of the aromatic nucleus;
3. Hydroxyl groups (-OH) on the 3- and 5- positions in the A ring with 4-oxo functions in the C ring, allowing for increased radical scavenging ability.

For example, although quercetin and catechin have similar structures, quercetin has a significantly higher antioxidant activity. This is because quercetin meets all three structural components for ideal antioxidant activity while catechin only meets two – it is missing the 4-oxo function in the C ring. This slight difference in chemical structure decreases the antioxidant activity of catechin to almost half that of quercetin (Rice-Evans *et al*, 1996). Therefore, the antioxidant activity of a given food is largely dependent on its phenolic composition. Potentially, different genotypes or different growing locations could affect the phenolic composition and therefore, the antioxidant activity (AOA) of field pea.

There are several published methods for assessing the antioxidant activity of foods. The most common can be divided into two groups: the electron transfer assays (ET) and the hydrogen atom transfer assays (HAT). ET-based methods include the Trolox equivalent antioxidant capacity (TEAC), ferric reducing/antioxidant power (FRAP), and the 2, 2-diphenyl-1-picrylhydrazyl assay (DPPH). ET assays are based on the addition of an oxidant (probe) that in turn extracts an electron from an antioxidant source, which causes a colour change in the probe. The change in colour of the probe is proportional to the antioxidant activity present (Huang, Ou & Prior, 2005). HAT-based methods include oxygen radical absorbing capacity (ORAC) and total radical trapping antioxidant parameter (TRAP). HAT methods are based on the ability of a probe to extract a hydrogen molecule from a donor antioxidant. In the ORAC and TRAP methods, the probe emits fluorescence, and the antioxidant activity is measured as loss of fluorescence over time (Prior, Wu & Schaich, 2005*)*. There are many advantages and disadvantages to each method, as outlined below.

2.6.3.1 Use of HAT Methods to Assess AOA

One of the primary advantages of using HAT methods is the biological relevance of the assay. It is believed that hydrogen atom transfer methods most closely mimic the way antioxidants react within biological systems. In addition, HAT assays are solvent and pH independent, and can be completed very rapidly. HAT assays may report erroneously high AOA due to the presence of reducing agents other than phenolics (Prior *et al,* 2005*).* The biggest drawback for using these methods for this thesis work is the financial cost associated with such methods.

2.6.3.2 Use of TEAC to Assess AOA

The TEAC method assesses the ability of phenolic antioxidants to donate an electron to the intensely coloured, radical form of 2, 2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and form a colourless, stable form of ABTS that can be read spectrophotometrically. The TEAC method is advantageous as it is simple to use, can be used over a wide pH range, and ABTS is soluble in aqueous and organic solvents. However, the generation of the radical ABTS solution can take a long time (up to 16 hours) and the reaction itself may take a long period of time to occur (Prior *et al*, 2005). Moreover, the ABTS radical working solution is time dependant, and if the solution used is not always the same age, it could cause differences in the reported AOA (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos & Byrne, 2006).

2.6.3.3 Use of FRAP to Assess AOA

The FRAP assay was originally developed to measure reducing power in plasma, but has been adapted to measure the reducing power of plant antioxidants. This method measures the ability of antioxidants to reduce colourless ferric 2, 4, 6-tripyidyl-*s*-triazine to the intensely blue ferrous 2, 4, 6-tripyidyl-*s*-triazine. Although this method is easy to use and can be easily automated, the reduction of ferric iron differs between the various polyphenols, and may take several hours to react (Prior *et al,* 2005).

2.6.3.4 Use of DPPH to Assess AOA

The DPPH assay is based on the ability of antioxidants to reduce the purple coloured DPPH radical to its colourless DPPH stable form. The decrease in absorbance is inversely proportionate to the AOA of the sample. The main drawback of the DPPH method is that other reducing agents may react with the DPPH radical, resulting in the overestimation of the phenolic AOA. Despite this limitation, the DPPH method is preferred as it is easy to use, rapid, and, unlike the TEAC method, the reagent does not need to be generated and used within a specific time span for each performance of the test. (Prior *et al*, 2005).

3.0 MATERIALS AND METHODS

3.1. Materials

Three yellow pea genotypes – Cutlass, Eclipse and SW Marquee – and three green pea genotypes – CDC Striker, Cooper and SW Sergeant – were obtained from the Crop Development Centre at the University of Saskatchewan. Seed samples were derived from three standard replicate provincial variety evaluation trials conducted at several locations in Saskatchewan. In this study, samples were derived from the 2006 and 2007 growing seasons from five of these locations: Saskatoon, Swift Current, Indian Head, Melfort and Rosthern. Samples were taken from two separate plots at each location from each growing year. Samples were cleaned of dockage, ground using an Udy Cyclone Sample Mill (Fort Collins, CO), passed through a 0.5 mm screen and stored at -22ºC in sealed plastic bags until analyzed.

Table 2 lists all the chemicals used for the analyses of the nutritional components of the pea samples. A Foss Fibertech 1023 Tecator (Eden Prairie, MN) was used to separate the TDF components. A LECO Dumas Combustible Nitrogen Analyzer Model FP-528 (St. Joseph, MI) (Williams, Sobering & Antoniszyn, 1998) was used to analyze the protein content of the fibre fractions. A Beckman Coulter Avanti® J-E Centrifuge (Palo Alto, CA) was used when needed. A Molecular Devices SpectraMax M5 Microplate Reader with Cuvette Port (Sunnyvale, CA) was used to analyze cuvettes and microplates for the TPC and AOA testing. The 4.5 mL Plastibrand® disposable cuvettes as well as the Costar® EIA/RIA 96 well polystyrene microplates were supplied by Fisher Scientific (Oakville, ON). Syringe filters were supplied by Pall Life Sciences

Table 2. Chemicals Used for Chemical Analyses of Field Pea Samples

	Chemicals
Sigma Aldrich Co. (Oakville, ON)	Acid Washed Celite for TDF Analysis Tris[hydroxymethyl]aminomethane (TRIS) 2-[N-morpholino]ethanesulfonic acid (MES) L-ascorbic acid Ethylenediaminetetraacetic acid (EDTA) Acetonitrile, Chromasolv® for HPLC Ethyl acetate, ≥ 99.5% ACS Diethyl ether, Chromasolv® for HPLC Folin-Ciocalteu Reagent, 2N 2,2-Diphenyl-1-picyl-hydrazyl (DPPH) (+)-Catechin hydrate (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox™) Hydrogen peroxide (30% w/w) 4-dimethylaminoantipyrine (4-aminophenazone) Potassium phosphate Potassium hydroxide
Fluka Scientific (Oakville, ON)	Trifluoroacetic acid (TFA)
Fisher Scientific Co. (Ottawa, ON)	95 % Histoprep Ethyl Alcohol Acetone, Certified ACS, HPLC grade Methanol, HPLC grade Sodium carbonate Sodium hydroxide (NaOH - for phenolic analysis) Sodium Acetate Trihydrate
Megazyme International Ltd. (Bray, Ireland)	Total Dietary Fibre Assay Kit - contained α-Amylase, Protease and Amyloglucosidase enzyme reagents
BDH Chemicals (Mississauga, ON)	Sodium hydroxide (NaOH - for TDF)
EMD Biosciences Inc. (Darmstadt, Germany)	Hydrochloric acid (HCl - for TDF) Acetic acid, glacial Sulphuric acid
Pierce Chemical Co. (Rockford, IL)	1-Step ABTS (2,2'-azino-bis[3-ethylbenzthiazoline-6-sulphonic acid] Peroxidase Conjugate Stabilizer/Diluent
ACROS Organics (Geel, Belgium)	Hydrochloric acid (HCl - for phenolic analysis)

(East Hills, NY) and syringes were supplied by Fisher Scientific (Oakville, ON). A Waters Acquity™ UPLC with a Photodiode Array Detector (Milford, MA) was used to separate the phenolic samples from the field pea extract. An Agilent Zorbax® Eclipse Plus 1.8 μm Reverse Phase C18 30 mm column was used to separate the phenolic components from the UPLC. As well, a Buchler Instruments Rotary Flash Evaporator (Fort Lee, NJ) was used to condense the phenolic samples after extraction.

3.2 Methods

3.2.1. Total Dietary Fibre

Total dietary fibre content was determined in accordance to AACC Approved Method 32-07 (American Association of Cereal Chemists [AACC], 2000). The data in this document are expressed on a dry basis. Duplicate 1.0 g samples of ground pea were suspended in pH 8.2 (24ºC) MES/TRIS buffer. MES/TRIS was used instead of phosphate buffer to eliminate the need to adjust the pH before adding protease, thereby reducing total volume for filtration as per Lee, Prosky & DeVries (1992). Samples were digested with heat-stable α-amylase for 35 minutes (95-100ºC) and protease for 30 minutes (60ºC). A 0.561 M HCl solution was added to the suspension to reduce the pH to between 4.2-4.8 prior to digesting with amyloglucosidase for 30 min (60ºC). The enzyme digest was filtered through a Foss 30 mL - P2 40-60 μm fritted glass crucible on a Foss Fibertech 1023 Tecator (Eden Prairie, MN). The filtrate was collected in a flask and diluted with 4 volumes of 95% ethanol. The mixture was heated in a water bath (60ºC), then allowed to sit overnight to permit water-soluble materials to precipitate. The following day the water soluble fraction was filtered using the P2 crucibles and the

remaining solvent fraction was discarded. Both insoluble and soluble fibre crucibles were rinsed with ethanol, followed by acetone, and allowed to dry overnight in a 103ºC oven. One set of duplicate residues was ashed in a muffle furnace (525ºC) overnight. The other set of insoluble and soluble residues were analyzed for protein content (N x 6.25) using the LECO Dumas Combustible Nitrogen Analyzer. Insoluble dietary fibre and soluble dietary fibre content were calculated as follows:

$$\text{DF \%} = \frac{(\text{mean residue wt} - \text{residue protein wt} - \text{residue ash wt} - \text{blank wt})}{\text{mean sample weight}} \times 100$$

Total Dietary Fibre % = Insoluble Dietary Fibre % + Soluble Dietary Fibre %

The results for the dietary fibre content are reported in dry basis.

3.2.2. Peroxidase Analysis

The peroxidase content of the field pea samples was analyzed using a method developed by Hatcher & Barker (2005). A 0.5 g sample of ground field pea was added to 5 mL of cold (4°C) sodium acetate buffer (0.1M, pH4.2), vortexed for 10 seconds and centrifuged in the cold (4°C) at 10,000 x G for 10 minutes. The supernatant was decanted and filtered through a 25 mm, 1.0 µm glass fibre membrane syringe filter. The supernatant and reagents were kept on ice in the dark. Immediately before analysis, 100 µL of the extract was diluted with 900 µL of the sodium acetate buffer.

A horseradish peroxidase stock solution was prepared using the above sodium acetate buffer and kept on ice. The peroxidase standard solutions (25 µL), control sample (25 µL) and extraction buffer blank (25 µL) were pipetted into the first, third and fifth columns of a 96-well microplate while the extracts (25 µL) were pipetted into the second, fourth and sixth columns. The Pierce 1-Step (product # 37615) ABTS substrate (150 µL)

was then pipetted into the wells containing the standard and the extracts. The timed reaction was stopped after exactly one minute by pipetting 100 µL of 2M sulphuric acid into each of the wells using an 8 channel micropipettor. The plate was placed immediately in the microplate reader where the plate was vortexed, and the absorbance read at 405 nm. The blank values were subtracted from the sample and standard values, and a linear regression analysis of the calibration standards was automatically defined, and individual well enzyme activity was calculated for each sample. All extracts were read in triplicate and the results were averaged for each extract.

3.2.3. Phenolic Extraction

Soluble and bound phenolic acids were extracted using acidified methanol and subsequent 16 hour basic hydrolysis using 10 M sodium hydroxide solution containing 2% ascorbic acid and 13.4 mM EDTA protectors in accordance to the method described by Ross *et al,* (2009). Samples were treated with acidified methanol (15:85 v/v glacial acetic acid to methanol) to release free phenolics, and then treated overnight with 10M NaOH to release both the insoluble and soluble bound phenolics. The solution was then treated with 6M HCl to increase the acidity of the suspension to pH 2, then a 1:1 diethyl ether:ethyl acetate was added to extract the phenolics from the suspension. The sample was then centrifuged 3 times at 6800 x G, vortexing for 45 seconds in prior to and between centrifuging. The supernatant on the top of the suspension was removed and evaporated to dryness using a Buchner rotary evaporator. The residue was re-dissolved in 1 mL 75% methanol and filtered through a 0.45 µL syringe filter before use.

Naringen, a flavonoid glycoside found primarily in grapefruit, was used as an internal standard. The free naringen was injected into a pea composite sample and subjected to the hydrolysis method of Ross *et al* (2009) as previously described. Test results showed that 85% of the naringen was recovered, which is in line with the amount of gallic acid recovered from the internal standard reported by Ross *et al* (2009). The results given in this paper have been adjusted to reflect the 85% recovery rate of this method.

3.2.4. Peroxidase-Catalyzed Enzymatic Method of TPC Analysis

The TPC of the field pea samples was first analyzed using a spectrophotometric peroxidase-catalyzed enzymatic (PE) method as described by Stevanato *et al* (2004) with modifications by Ma & Cheung (2007). An enzyme-reagent working solution of 3 mL – 30 mM 4-dimethylaminoantipyrine, 3 mL - 20 mM hydrogen peroxide and 1.5 mL 6.6 μM horseradish peroxidase was prepared, combined and made to 30 mL using 0.1 M potassium phosphate buffer (pH 8.0). This working reagent was kept on ice. In a 96-well microplate, 25 μL of the phenolic extract (or 25 μL of methanol used as a blank) was pipetted into each well, followed by 225 μL of the enzyme-reagent. The plate was shaken in the microplate reader for 30 seconds and the absorbance was read in kinetics mode at 500 nm every minute for 20 minutes. The reaction was considered complete when the absorbance plateaued on a plot of time vs. absorbance. The results are expressed as μM catechin equivalent (CE) per gram of sample.

3.2.5. Folin-Ciocalteu Method of Total Phenolic Content Analysis

The TPC analysis of the field pea samples using the Folin-Ciocalteu method was conducted as described by Singleton & Rossi (1965) using method variations as outlined by Hung & Morita (2008). The phenolic extract (0.5 mL) was oxidized with the Folin-Ciocalteu reagent (0.5 mL) in a 13 mL centrifuge tube. The reaction was stopped after one minute using 1.0 mL of a 14% sodium carbonate solution. The centrifuge tubes were then adjusted to 10 mL using Millipore filtered water and allowed to stand at ambient temperatures for 45 minutes. The centrifuge tubes were then centrifuged for 5 minutes at 4000 x G and the clear supernatant was pipetted into a cuvette and measured using a spectrophotometer at 725 nm. The total phenolic content was calculated based on a standard curve prepared using 5, 10, 15, 20, 25, 40, 60, 80, 100 and 150 µg/mL catechin standards. The results are expressed as µM CE per gram of sample.

3.2.6. Separation of Phenolic Components using UPLC

The field pea extract was analyzed for 10 different phenolics that were suspected in the samples. Standards were made for 4-hydroxybenzoic acid, protocatechuic acid, vanillic acid, caffeic acid, p-coumaric acid, syringic acid, sinapic acid, ferulic acid, quercetin and rutin to a concentration of 0.8 mg/mL for each phenolic (Table 3). The retention times for each component were recorded and a processing method was developed based on this information. Methanol was used as the solvent for all standards.

A Xorbax® Eclipse Plus RP C18 1.8 µm x 30 mm column was used to separate the components. The stationary phase consists of a silane ligand chemically bonded to porous silica (95 Å). The non-polar properties of both the silica and ligand allow for excellent separation of non-polar aromatics. The smaller 1.8 µm particle provides an

increased efficiency resulting in better resolution, while the 30 mm column size increases the elution speed

Two solvents were used in the mobile phase of the analysis. Solvent 'A' consisted of Millipore-filtered water containing 0.1% trifluoroacetic acid (TFA), while solvent 'B' consisted of acetonitrile containing 0.1% TFA. The solvent gradients are depicted in Table 4. The flow rate was set at 0.6 mL/minute and the injection volume was 3 µL. The column pressure was maintained at 15 000 psi and the temperature at 45ºC. The total run time was 5 minutes. The components were detected at 280 and 325 nm with a Waters Acquity™ photodiode array detector.

3.2.7. Antioxidant Activity using the Free Radical DPPH

DPPH radical scavenging capacity using cuvettes was analyzed according to the method described by Hung, Maeda, Miyatake & Morita (2008) with the following exceptions. A 0.0634 mM solution of DPPH in methanol was used, as this concentration resulted in an ideal change in colour when added to the extracts. The absorbance was measured at 517 nm, as per Anton, Ross, Lukow, Fulcher & Arntfield (2008). DPPH radical scavenging capacity using a 96 well microplate was also analyzed employing the same DPPH solution. The phenolic extracts (10 µL) were pipetted into the wells, and 200 µL of the DPPH solution was pipetted into each well. A 210 µL methanol blank with no DPPH added was also pipetted on to the microplate. The microplate containing the reagents was kept in the dark at ambient temperature for exactly 30 minutes.

Table 3. Polyphenols Used as Standards in the UPLC Analysis of the Field Pea Samples

	Chemicals
Sigma Aldrich Co. (Oakville, ON)	Naringen (internal standard) Sinapic acid trans-4-Hydroxy-3-methoxycinnamic acid (ferulic acid) Quercetin Syringic acid Caffeic acid Rutin 3,4-Dihydroxybenzoic acid (protocatechuic acid) 4-hydroxybenzoic acid
Fluka Scientific (Oakville, ON)	Cinnamic acid Vanillic acid p-Coumaric acid

Table 4. Mobile Phase Gradient Used for Phenolic Separation on the Reversed-Phase C-18 UPLC System.

	Solvent (%)	
Minutes	Millipore water with 0.1% TFA	Acetonitrile with 0.1% TFA
0 to 1.0	95	5
1.0 to 2.0	90	10
2.0 to 3.0	85	15
3.0 to 4.0	75	25
4.0 to 4.2	40	60
4.2 to 4.8	0	100
4.8 to 5.0	95	5

The microplate was then read on a microplate reader at 517 nm. For both the cuvette and microplate methods a methanol control, resulting in no colour change, was used. The scavenging capacity of DPPH was calculated as per Liyana-Pathirana & Shahidi (2006):

$\%DPPH\ scavenging = (Absorbance_{control} - Absorbance_{extract}) / Absorbance_{control} \times 100$

The amount of free radical scavenging is expressed in μM of Trolox equivalents (TE) per gram of sample.

3.2.8 Statistical Analysis

The experiment was carried out as a randomized complete block design. Environment and growing season were set as random effects. An analysis of variance (ANOVA) was carried out using SAS software v. 9.1 (Cary, NC) using PROC GLM. The results stated are significant to $p < 0.05$ unless otherwise stated.

4.0. RESULTS

4.1 Dietary Fibre Analysis

4.1.1. Weight Trials for the TDF Method

The initial phase of the TDF analysis involved investigating the influence that the amount of sample used in the AACC method had on analysis time and reproducibility to determine whether a smaller sample size could be used for analysis. Previous fibre testing of raw field pea samples by Canadian Grain Commission technicians had proven difficult, as the initial IDF filtrations were very time consuming and tedious. Where other types of pulses (i.e. lentils) and pulses subjected to processing treatments (dehulling, cooking) seemed to filter in reasonable time (<1 hour/sample), raw field peas tended to take an exceptionally long time to filter (>3 hours/sample). Such a delay is not conducive to large sample numbers, such as in this thesis project. Investigation of various minor modifications, such as applying back pressure to the crucibles, washing samples with hot deionized water, keeping filtration samples warm and light scraping of the celite bed were initially evaluated and aided in the filtration, they did not achieve a point where the filtrations could be done comfortably in one day. Therefore, analyses of 2 different sample sizes (0.5 & 0.75 g) in addition to the standard 1.0 g sample were undertaken to ascertain if using smaller sample sizes would yield statistically similar fibre results from the full 1.0 g sample required by AACC Method 32-07. Four different samples were used in this preliminary evaluation. An AACC oat bran check sample, a yellow pea composite check sample, a 2004 Cooper pea sample and a 2004 Cutlass pea sample. All pea samples were ground in an Udy Cyclone Mill in the same manner as the G x E test samples. The

results (Table 5) indicated that although the mean TDF results were similar for each sample at the various weights, the smaller sample sizes had significantly more variation than the 1.0 g sample. As well, the coefficient of variation (CV) for the SDF were extremely high for all samples at all weights. For the field pea samples, this could be accounted for by the low percentage of SDF in the samples (< 3%). Although the actual differences in SDF between the samples were negligible, the fact that such small amounts were found exaggerated the differences. Taking into account the CV of the TDF and IDF of the field pea samples at all three weights, the 1.0 g sample size seemed to be the most consistent with the least amount of variation. Therefore, a 1.0 g sample was used for the TDF analysis for this project.

Table 5. Mean Insoluble, Soluble and TDF Percentages (%) for 0.5, 0.75 and 1.0 g Samples and Corresponding Coefficient of Variation (CV)

Sample Wt. (g)	Sample	Insoluble Mean (%)	Soluble Mean (%)	TDF Mean (%)	Insoluble CV	Soluble CV	TDF CV
0.5	AACC Oat	7.8	7.8	15.6	4.8	26.6	13.1
0.75	AACC Oat	8.5	8.9	17.3	8.8	11.4	5.4
1.0	AACC Oat	10.8	9.7	20.5	12.1	15.7	9.9
0.5	Pea Check	11.0	2.3	13.3	2.8	31.6	6.0
0.75	Pea Check	10.5	2.0	12.5	4.6	58.6	11.7
1.0	Pea Check	11.0	1.8	12.7	6.5	22.0	6.4
0.5	Cooper	12.8	2.6	15.4	2.8	21.7	4.8
0.75	Cooper	12.9	2.1	15.0	4.5	51.9	10.2
1.0	Cooper	13.0	1.9	14.9	1.6	17.9	1.3
0.5	Cutlass	14.4	2.7	17.1	2.9	23.4	3.8
0.75	Cutlass	14.2	2.2	16.4	6.4	31.7	8.6
1.0	Cutlass	14.2	2.2	16.4	4.5	17.1	3.2

4.1.2. Re-evaluating the TDF Check Sample

While using the AACC oat bran check, it was found that the CV's for the IDF, SDF and TDF, were very high and variable, ranging between 4.8 to 12.1 for the IDF samples, 11.4 – 58.6 for the SDF samples and 5.4 – 13.1 for the TDF samples. Finding it quite unusual that the AACC standard check sample would have such high CV values, especially compared to the field pea samples, a small literature review on the dietary fibre analysis of oat bran was carried out. Lee *et al* (1992) found that oat bran samples had a high reproducibility standard deviation between laboratories and a high time-to-time variation in the percentage fibre values. Lee *et al* (1992) concluded that since oat bran had a tendency to separate and because its particle size is quite variable, an improved method sample preparation needed to be developed to minimize the issues with heterogeneity.

Upon comparing the AACC Fiber Reference Standard data sheet that was included with the AACC oat bran check sample with the study by Caldwell & Nelsen (1999) citing the TDF values for the oat bran check, more issues arose. The values that were used for the AACC oat bran check sample were derived by removing the results of 2 labs of 10 for the IDF-TRIS analysis, the results of 3 labs out of 9 for the SDF-TRIS analysis and the results of 2 labs out of 9 for the TDF-TRIS analysis due to Cochran or Grubbs outliers. Because of variability of the AACC oat bran check values as cited by Caldwell & Nelsen (1999) and the issues with homogeneity described by Lee *et al* (1992), it was decided that the yellow pea composite sample from the weight trials be used as a TDF check sample instead of the AACC oat bran check sample.

4.1.3. TDF, IDF and SDF Content of Field Pea

The total dietary fibre (TDF) component of the pea samples ranged from 10.7-14.8% of the dry matter (Figure 2). This differs from what Black *et al* (1998) found in Australian field peas, as TDF constituted 13.9-23.6% of the dry matter content. These differences were not unexpected as growing environments and genotypes differed. The insoluble dietary fibre (IDF) component comprised 8.7-12.9% of the dry matter content (Figure 3), making up the majority of the TDF content (72.2-94.2%). Soluble dietary fibre component accounted for only 0.6-3.7% of the dry matter content (Figure 4) representing only 5.8-27.8% of the TDF content. These results are in line with the results reported by Wang *et al* (2008), where IDF comprised 88.6-90.2% and SDF comprised 9.5-11.1% of the TDF component in raw field peas. Bednar *et al* (2001), working with pulses other than field peas, found that IDF comprised the majority of the TDF in pulse samples (black beans, red kidney beans, lentils, navy beans, black-eyed peas, split peas and northern beans), with IDF accounting for 92.2-100% and SDF accounting for 0-7.8% of the TDF content.

4.1.4. Influence of Genotype, Location, Growing Year and Cotyledon Colour on TDF

Significant differences in the TDF contents of pea genotypes were detected (Table 6). SW Sergeant had the highest mean TDF content at 13.1% in 2006 and 13.5% in 2007(Figure 2). Cooper had the lowest mean TDF content in 2006 (12.1%) while SW Marquee had the lowest mean TDF in 2007 (12.7%). This was consistent with the findings of Wang *et al* (2008) working with the 6 different Canadian field pea genotypes (Nitouche, Keoma, SW Parade, Eclipse, Delta and CDC Mozart) which showed

Figure 2. Total dietary fibre (TDF) Content (%) Found in the 6 Cultivars: CDC Striker (ST), Cooper (CO), Cutlass (CU), Eclipse (EC), SW Marquee (MA) and SW Sergeant (SE) Grown in Each of the 5 Growing Locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada Throughout the 2006-2007 Growing Seasons.

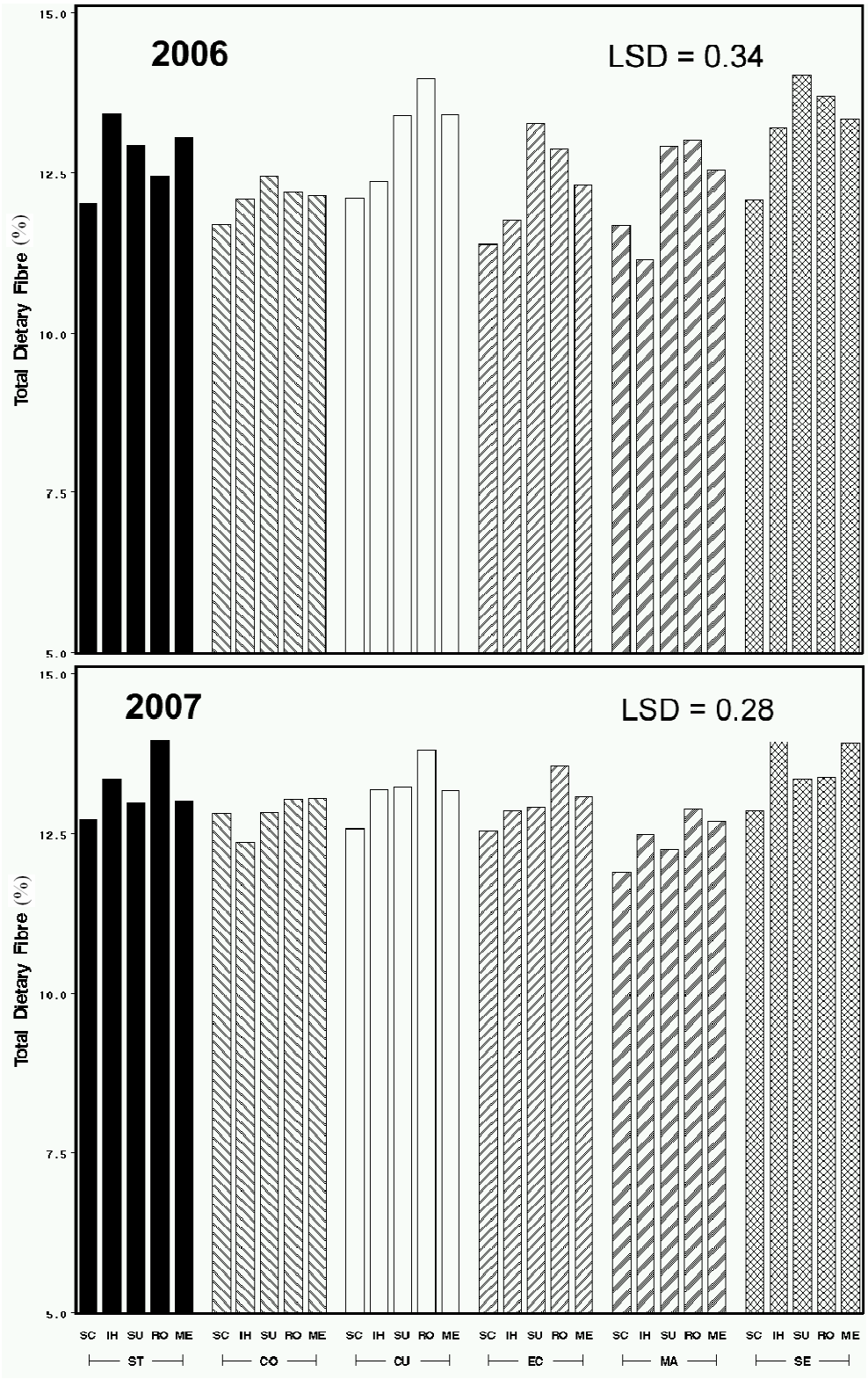


Figure 3. Insoluble Dietary Fibre (IDF) Content (%) of the 6 Cultivars: CDC Striker (ST), Cooper (CO), Cutlass (CU), Eclipse (EC), SW Marquee (MA) and SW Sergeant (SE) Grown in Each of the 5 Growing Locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada Throughout the 2006-2007 Growing Seasons**.**

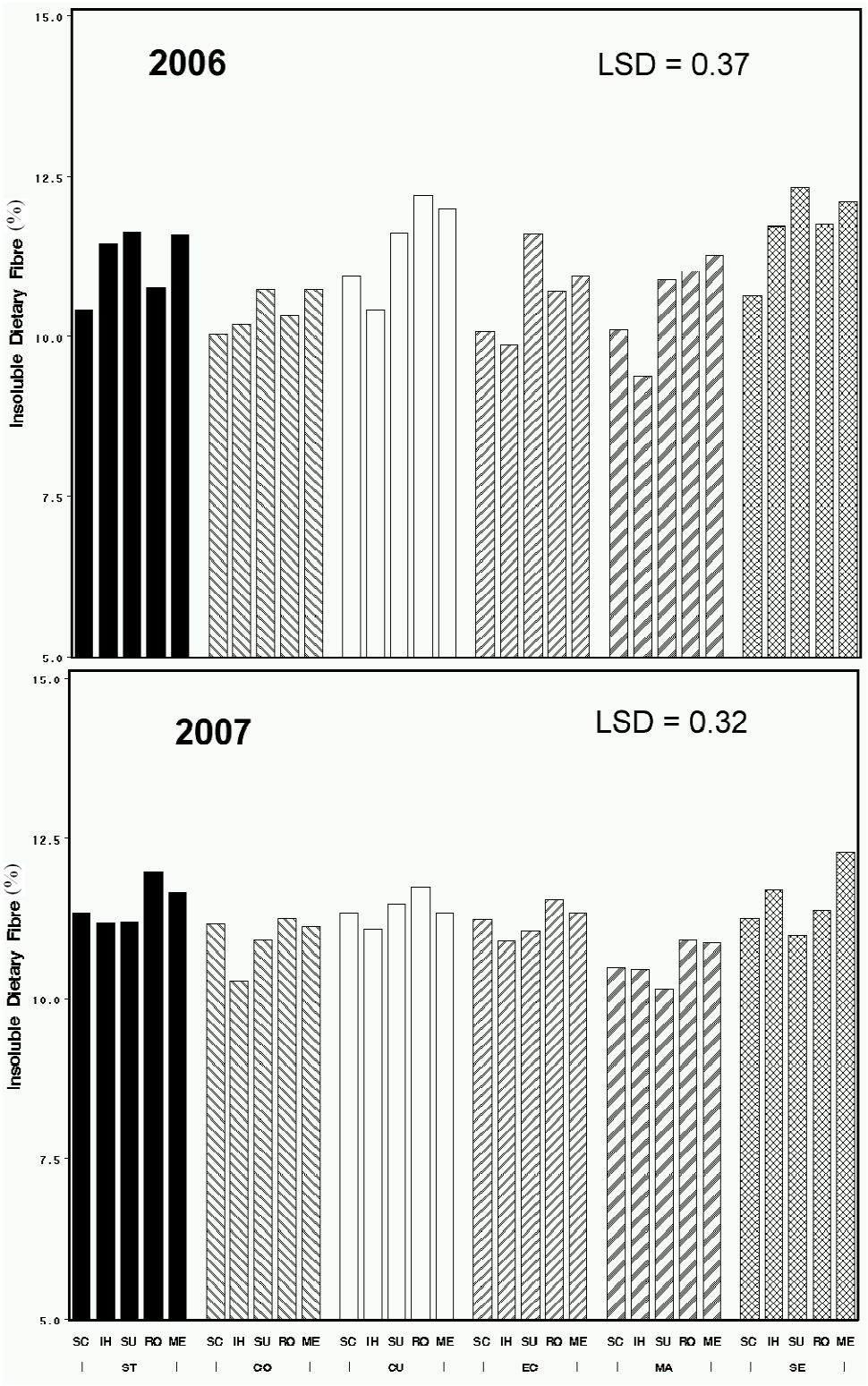
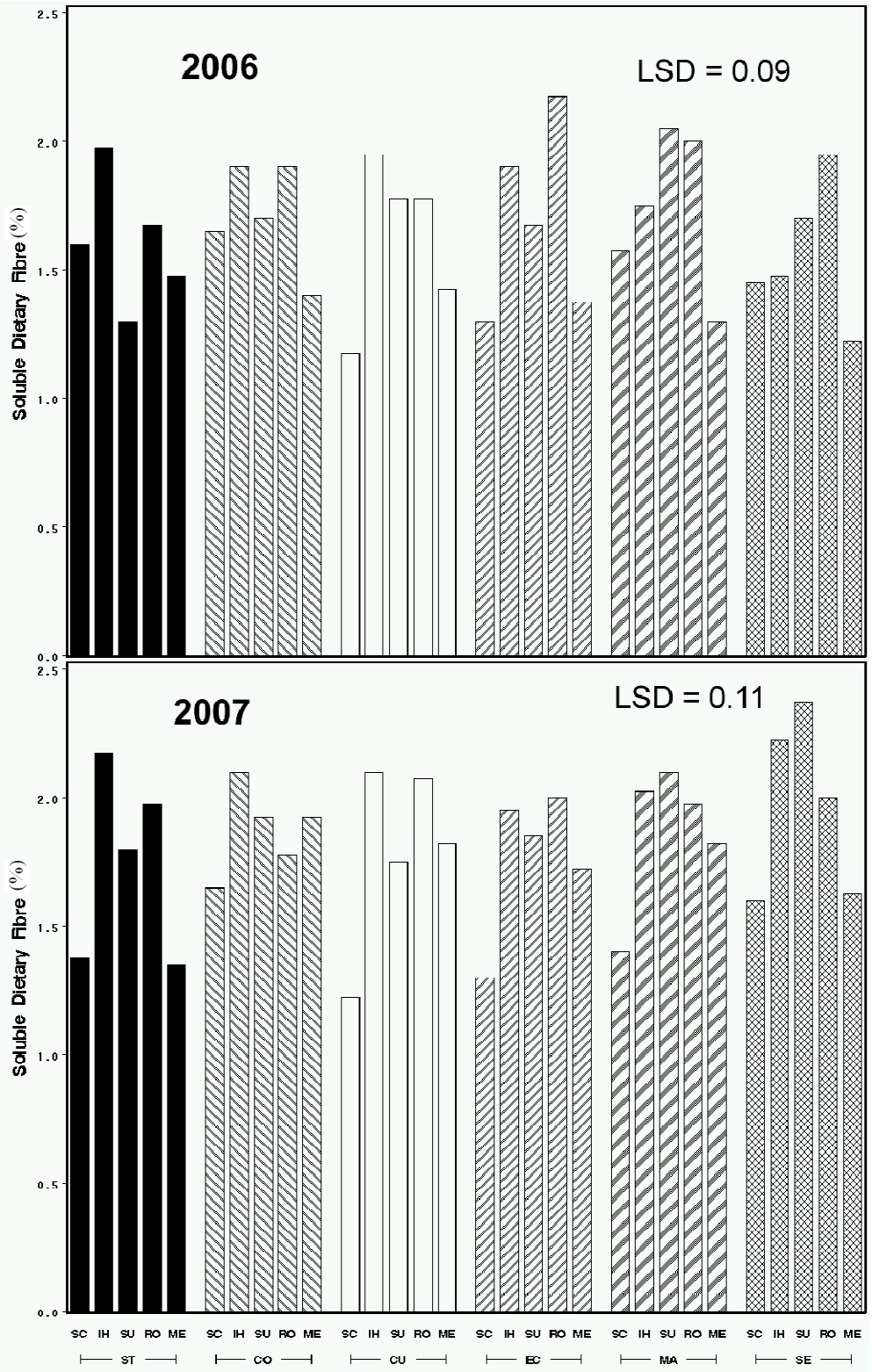


Figure 4. Soluble Dietary Fibre (SDF) Content (%) of the 6 Cultivars: CDC Striker (ST), Cooper (CO), Cutlass (CU), Eclipse (EC), SW Marquee (MA) and SW Sergeant (SE) Grown in Each of the 5 Growing Locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada Throughout the 2006-2007 Growing Seasons.



significant differences in TDF ($p < 0.001$) content. In this study, growing location was also found to have a significant effect on the TDF content ($p < 0.0001$). The highest mean TDF content was found in genotypes grown in Saskatoon (13.2%) in 2006 and Rosthern (13.5%) in 2007.

Genotypes grown in Swift Current consistently had the lowest TDF, with a mean of 11.8% in 2006 and 12.7% in 2007. This may be due to the generally warmer and drier climate that Swift Current experiences compared to the four other growing locations (Table 7). SW Marquee samples grown in Indian Head in 2006 had the lowest TDF content while SW Sergeant samples grown in Indian Head in 2007 had the highest TDF content.

Growing year had a significant effect on TDF content ($p < 0.0001$). The 2007 growing season yielded consistently higher mean TDF (13.0%) than 2006 (12.6%). The green field pea cotyledon genotypes had higher TDF contents ($p < 0.05$) than the yellow cotyledon genotypes (Table 8). There was a significant genotype x environment effect on TDF content ($p < 0.05$) that was not observed in either the IDF or SDF contents individually. As well, location x growing year had a significant effect on TDF ($p < 0.001$) content.

Table 6. The Effect of Genotype, Location, Cotyledon Colour and Growing Year on the Analysis of Variance (ANOVA) on Insoluble Dietary Fibre (IDF), Soluble Dietary Fibre (SDF) and Total Dietary Fibre (TDF) of Field Peas.

Attribute	**F value**					
	Genotype (G)	Cotyledon Colour	Location (E)	Year (Y)	G x E	E x Y
TDF	20.74***	10.86**	25.56***	28.76***	1.88*	5.88**
IDF	21.93***	10.20**	15.28***	7.46*	1.47	8.26***
SDF	1.03	0.00	26.92***	19.45***	1.58	3.11*

*Significant at $p<0.05$,
**Significant at $p<0.01$
***Significant at $p<0.001$

4.1.5. Influence of Genotype, Location, Growing Year and Cotyledon Colour on IDF

Significant differences in IDF content were detected among pea genotypes (Table 6). There were significant differences in IDF ($p<0.0001$) between all genotypes. In 2006, CDC Striker had the highest mean IDF (11.5%) across all sites while Cooper had the lowest mean IDF (10.4%) across all growing locations. This varied in 2007, as SW Sergeant had the highest mean IDF (11.5%) across all sites while SW Marquee had the lowest mean IDF (10.9%) across all growing locations, which is in agreement with the findings of Wang *et al* (2008), as the 6 different field pea genotypes tested showed significant differences in IDF ($p<0.01$). Growing location also had a significant effect on the total IDF content. In 2006, genotypes grown in Melfort had the highest mean IDF (11.6%) while in 2007 genotypes grown in Rosthern had the highest mean IDF (11.5%). In 2006, genotypes grown in Swift Current had the lowest mean IDF (10.4%) while in 2007 genotypes grown in Indian Head had the lowest mean IDF (10.9%). There were significant differences in the IDF contents of the different pea genotypes and the various growing environments separately (Table 6). SW Marquee samples grown in Indian Head in 2006 had the lowest IDF while SW Sergeant grown in Melfort in 2007 had the highest IDF content. Growing year also had a significant effect on IDF content ($p < 0.001$). The 2007 growing season yielded consistently higher mean IDF (11.21%) contents than 2006, where the mean IDF was lower at 11.0%. Green cotyledon genotypes exhibited higher IDF contents ($p = 0.0016$) than yellow cotyledon genotypes (Table 8). As well, there was a significant genotype x growing year effect in the TDF content ($p < 0.05$) which was not seen in SDF or IDF contents.

Table 7. Mean Temperature, Total Precipitation (May - August) and Soil Zones of 5 Locations of the Saskatchewan Regional Field Pea Trials[1]

Location	Soil zone	Year	Mean Temperature ºC	Total Precipitation (mm)
Swift Current	Brown	2006	17.2	184
		2007	16.9	122
Saskatoon	Dark Brown	2006	16.5	210
		2007	16.5	274
Melfort	Black	2006	15.8	221
		2007	14.7	259
Rosthern	Black	2006	15.8	320
		2007	15.0	346
Indian Head	Black	2006	15.6	135
		2007	15.0	206

[1]Data source: Environment Canada reporting site nearest to listed trial site (Environment Canada, 2006, 2007).

Table 8. Mean, Minimum and Maximum SDF, IDF and TDF Values (%) of Three Yellow and Three Green Cotyledon Field Pea Cultivars Evaluated in 2006 and 2007.

		Mean fibre values (%)			
Attribute	Year	green	range	yellow	range
TDF	2006	12.72	10.74 -14.50	12.54	10.10 -14.27
IDF		11.09	9.01 -12.87	10.86	8.70 -12.67
SDF		1.63	1.00 - 2.40	1.68	0.60 -2.60
TDF	2007	13.17	11.70 -14.81	12.87	11.57-14.43
IDF		11.31	9.63 -12.82	11.06	9.44 -12.43
SDF		1.86	1.00 – 3.70	1.81	1.00-2.70

4.1.6. Influence of Genotype, Location, Growing Year and Cotyledon Colour on SDF

Table 6 reveals significant differences in the SDF contents of the different pea genotypes grown at the various locations. In 2006, Eclipse had the highest mean SDF (1.8%) across all sites while SW Sergeant had the lowest mean SDF (1.6%) across all growing locations. This differs from 2007, where SW Sergeant displayed the greatest mean SDF (2.0%) across all growing locations while CDC Striker had the lowest mean SDF (1.7%) across all growing locations. Growing location also had a significant effect on the SDF. Genotypes grown in Rosthern exhibited the highest mean SDF (1.9%) in 2006 while genotypes grown in Indian Head had the highest mean SDF (2.1%) in 2007. Genotypes grown in Swift Current consistently had the lowest mean SDF in both 2006 (1.5%) and in 2007 (1.4%). Eclipse field pea samples grown in Swift Current in 2006 had the lowest SDF content while SW Sergeant field peas samples grown in Saskatoon in 2007 having the highest SDF content (Figure 4). Growing year was correlated with SDF, as the 2007 growing season yielded consistently higher mean SDF (1.8%) content than in 2006, where mean SDF was 1.7%. As well, location x growing year had a significant effect on SDF content ($p < 0.05$). However, cotyledon colour had no significant effect on SDF content.

4.2 Peroxidase Content Analysis

Initially, a peroxidase analysis was planned for this project using a rapid quantitative spectrophotometric method developed by Hatcher & Barker (2005). However, initial analyses on field pea check samples showed only negligible amounts of peroxidase activity. This was contrary to our initial hypothesis because, as was outlined

in the literature review, there are issues with enzymatic browning in pulses and cereal crops. Because there was little peroxidase activity in the check samples, this portion of the project was eliminated.

4.3 Total Phenolic Content Analysis

4.3.1. Peroxidase-Catalyzed Enzymatic Method of Total Phenolic Content Analysis

The first series of tests that were performed on the phenolic content in field peas was based on the peroxidase-catalyzed enzymatic (PE) method by Stevanato *et al* (2004) and Ma & Cheung (2007). Upon starting the PE analysis complications with the method arose. Table 9 shows the absorbance, concentration and total phenolic content (TPC) of a yellow pea composite sample. Three extracts were taken from a yellow field pea composite sample and over a 3 day period, the absorbances were taken using the PE method. The total phenolic concentration ranged from 87 µM to 223 µM. Not only was there significant variance between extractions and between test days, but in some cases there was a significant difference (>5%) between the same extractions on the same test day. Because the purpose of this study is to analyze for possible G x E effect and it would be impossible, with this level of variance, to safely assume that any significant differences were not from method variance, this method had to be abandoned.

4.3.2. Folin-Ciocalteu Method of Total Phenolic Content Analysis

As a result of the unacceptable variances determined in the PE experiment, TPC trials were conducted using the Folin-Ciocalteu method. Preliminary tests were carried out before testing the G x E samples using the same yellow pea composite sample as was

used as in the peroxidase-catalyzed enzymatic method. As outlined by Table 10, the CV between the various repetitions and extractions of the same yellow pea composite remained less than 4%, with the CV of all repetitions and extractions combined being 3.4%. Despite the possibility of inferences with the Folin-Ciocalteu method and other limitations as previously outlined, much more consistent results were attained. As well, the Folin-Ciocalteu method is one of the most commonly used and recognized methods for TPC analysis. For these reasons, the TPC of the samples was assessed using the Folin-Ciocalteu method as opposed to the PE method previously discussed.

Table 9. TPC of Yellow Pea Composite from 3 Different Extracts Over 3 Days using Peroxidase-Catalyzed Enzymatic Method

Date	Extract	Absorption (500 nm)	Concentration (uM)*	Phenolics (mg/g sample)	CV for Extract
14-Jul-08	1	0.0670	215.5	0.64	0.63
		0.0676	221.5	0.65	
	2	0.0564	165.5	0.48	6.23
		0.0616	193.0	0.56	
	3	0.0593	177.5	0.53	0.36
		0.0596	181.5	0.53	
16-Jul-08	1	0.0419	91.5	0.27	2.58
		0.0404	82.5	0.25	
	2	0.0447	106.0	0.31	6.97
		0.0405	85.0	0.25	
	3	0.0446	106.5	0.31	4.25
		0.0420	92.0	0.28	
17-Jul-08	1	0.0438	98.0	0.30	0.81
		0.0433	95.5	0.29	
	2	0.0503	132.5	0.40	7.24
		0.0454	106.5	0.33	
	3	0.0479	122.5	0.36	7.95
		0.0428	94.5	0.29	
Total SD:	0.01				
Total CV:	34.66				

*Concentration is expressed in uM catechin equivalents

Table 10. TPC of Yellow Pea Composite from 3 different extracts using Folin-Ciocalteu Method

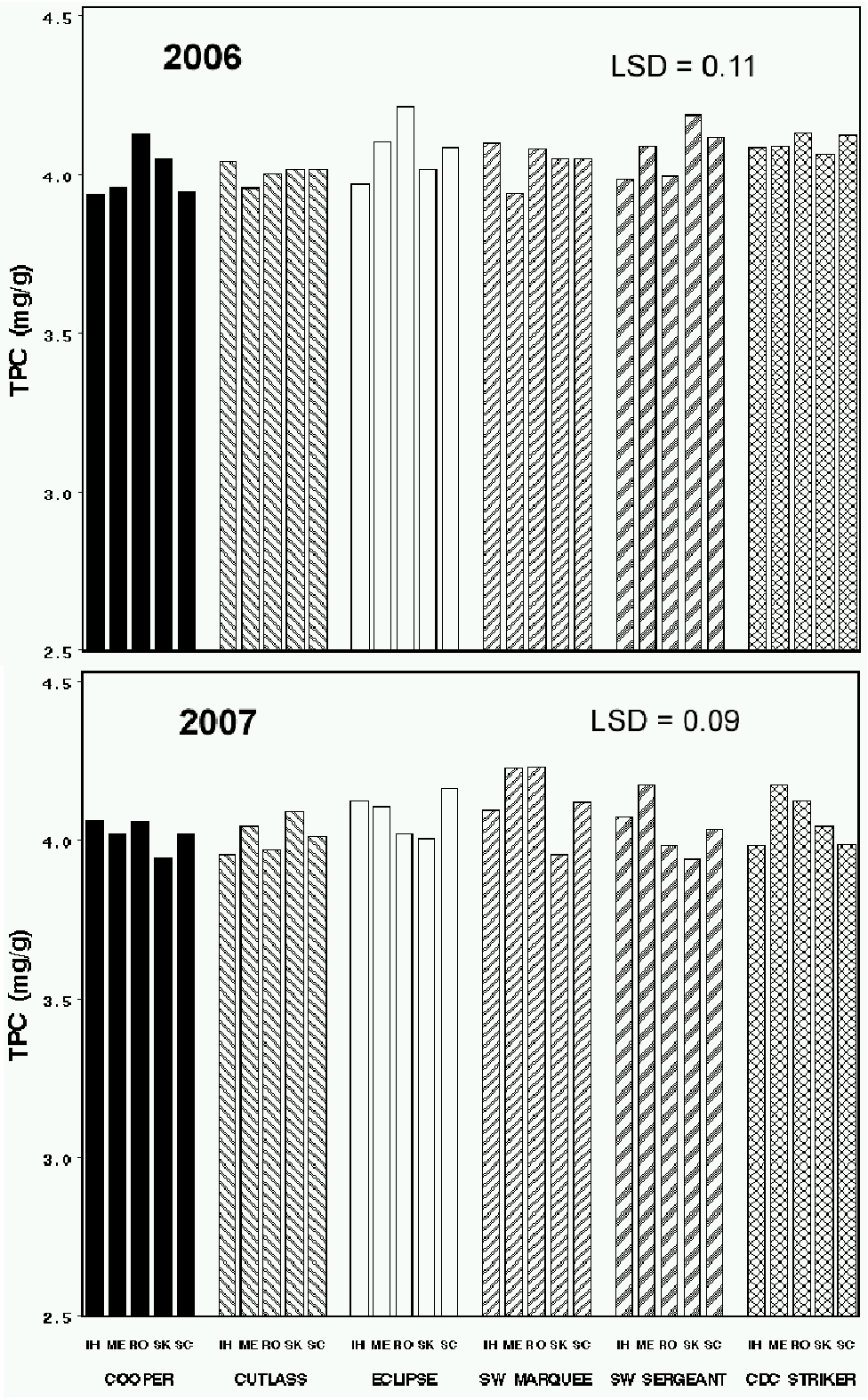
Date	Extract	Absorption (725 nm)	Concentration (uM)	Phenolics (mg/g sample)	CV for Extract
25-Jul-08	1	1.77	168.28	0.44	1.5
		1.75	166.36	0.44	
		1.81	172.12	0.45	
		1.78	169.24	0.45	
		1.81	172.12	0.45	
25-Jul-08	2	1.78	169.24	0.45	0.3
		1.79	170.24	0.45	
		1.78	169.24	0.45	
		1.78	169.24	0.45	
		1.78	169.24	0.45	
25-Jul-08	3	1.66	157.71	0.41	3.8
		1.74	165.40	0.43	
		1.67	158.68	0.42	
		1.61	152.91	0.40	
		1.77	168.28	0.44	
Total SD:	0.059				
Total CV:	3.4				

*Concentration is expressed in uM catechin equivalents

4.3.2.1. The TPC of Field Pea Samples based on the Folin-Ciocalteu Method.

The mean TPC for each genotype and growing location is shown in Figure 5. The TPC of the field pea samples ranged from 3.94 mg/g CE to 4.22 mg/g CE. The lowest TPC was found in the Cooper genotype grown in Indian Head in 2006 and the highest from the Eclipse genotype grown in Rosthern in 2006. The genotypes having the lowest overall TPC were the Cooper and the Cutlass, with a mean TPC of 4.01 mg/g, while the genotype with the highest overall TPC was the SW Marquee with a mean TPC of 4.09 mg/g. The growing locations that had the lowest overall TPC's were Saskatoon and Indian Head, having mean TPC's of 4.03 mg/. Genotypes grown in Rosthern had the highest overall TPC with a mean of 4.08 mg/g. The TPC values seen in this study were higher than in other published studies. Xu *et al* (2007) found that the TPC of field pea samples ranged from 0.65 mg/g gallic acid equivalents (GAE) (1.11 mg/g CE) in their Cooper genotype, to 1.14 mg/g GAE (1.95 mg/g CE) in their Golden genotype. Xu & Chang (2007) found that the TPC of their field pea samples ranged from 1.04 mg/g GAE to 1.67 mg/g GAE (1.77 to 2.86 mg/g CE). Wang *et al* (1998) found that the TPC of their field pea samples ranged from 0.162 mg/g CE to 0.325 mg/g CE, with the AC Tamor genotype having the lowest TPC, and the Richmond genotype having the largest. Although the TPC is substantially higher in this study as opposed to the research done by Wang *et al* (1998), Xu & Chang (2007) and Xu *et al* (2007), it was difficult to make proper comparisons, since the extraction methods in these three studies differed from how the phenolic components were extracted in this thesis. The results from Xu & Chang (2007) and Xu *et al* (2007) are also expressed in a different unit than CE, adding further

Figure 5. Total Phenolic Content (TPC), based on the Folin-Ciocalteu ethod, found in the 6 cultivars: CDC Striker , Cooper, Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons.



difficulty to direct comparisons. Amarowicz & Troszyńska (2003) found the TPC of low molecular weight phenolics in Polish-grown field pea extracts to be 11.2 mg/g CE using an 80% (v/v) acetone extraction and a 1:10 solid: liquid (w/v) solid to solvent ratio than a subsequent basic hydrolysis step. Amarowicz, Karamać & Weidner (2001) also found that Polish-grown field peas subjected to the same extraction method as stated in Amarowicz & Troszyńska (2003) had a TPC ranging from 2.7 – 9.2 mg/g CE in the low molecular weight phenolic extracts.

4.3.2.2. Influence of Genotype, Location, Growing Year and Cotyledon Colour on the TPC Based on the Folin-Ciocalteu Method.

There were very significant genotype ($p < 0.0001$) influences on the TPC. Interestingly, growing year, location and cotyledon colour had a significant effect on the TPC. In addition, there was a strong genotype by environment interaction effect ($p < 0.001$) detected on the TPC of our field pea samples. Wang *et al* (1998) also found a correlation between TPC and genotype, a correlation between TPC and environment, and a G x E interaction effect in the TPC of field pea. Xu *et al* (2007) also observed significant differences among the genotypes with green cotyledons and genotypes containing yellow cotyledons.

4.3.2.3. The Simple Phenolic Content of Field Pea.

Figure 6 depicts the mean total simple phenolic acid content for each genotype and growing location. The results from the UPLC analysis indicate a wide range in the phenolic content and composition of the field pea samples. The total simple phenolic

Figure 6. Total simple phenolic acid content, based on UPLC analysis, found in the 6 cultivars: CDC Striker, Cooper, Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons.

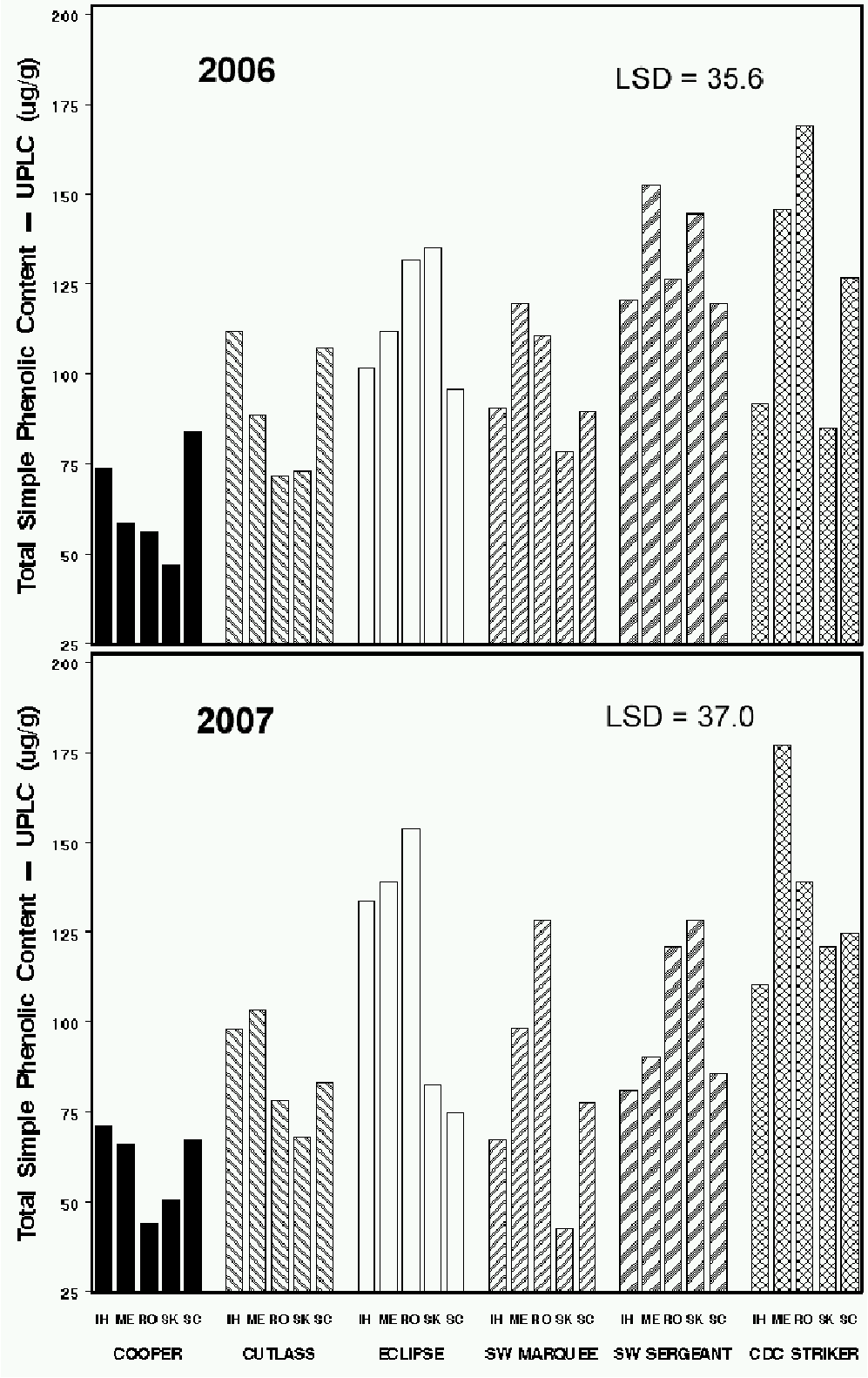


Figure 7. 4-hydroxybenzoic acid content, based on UPLC analysis, found in the 6 cultivars: CDC Striker , Cooper, Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons.

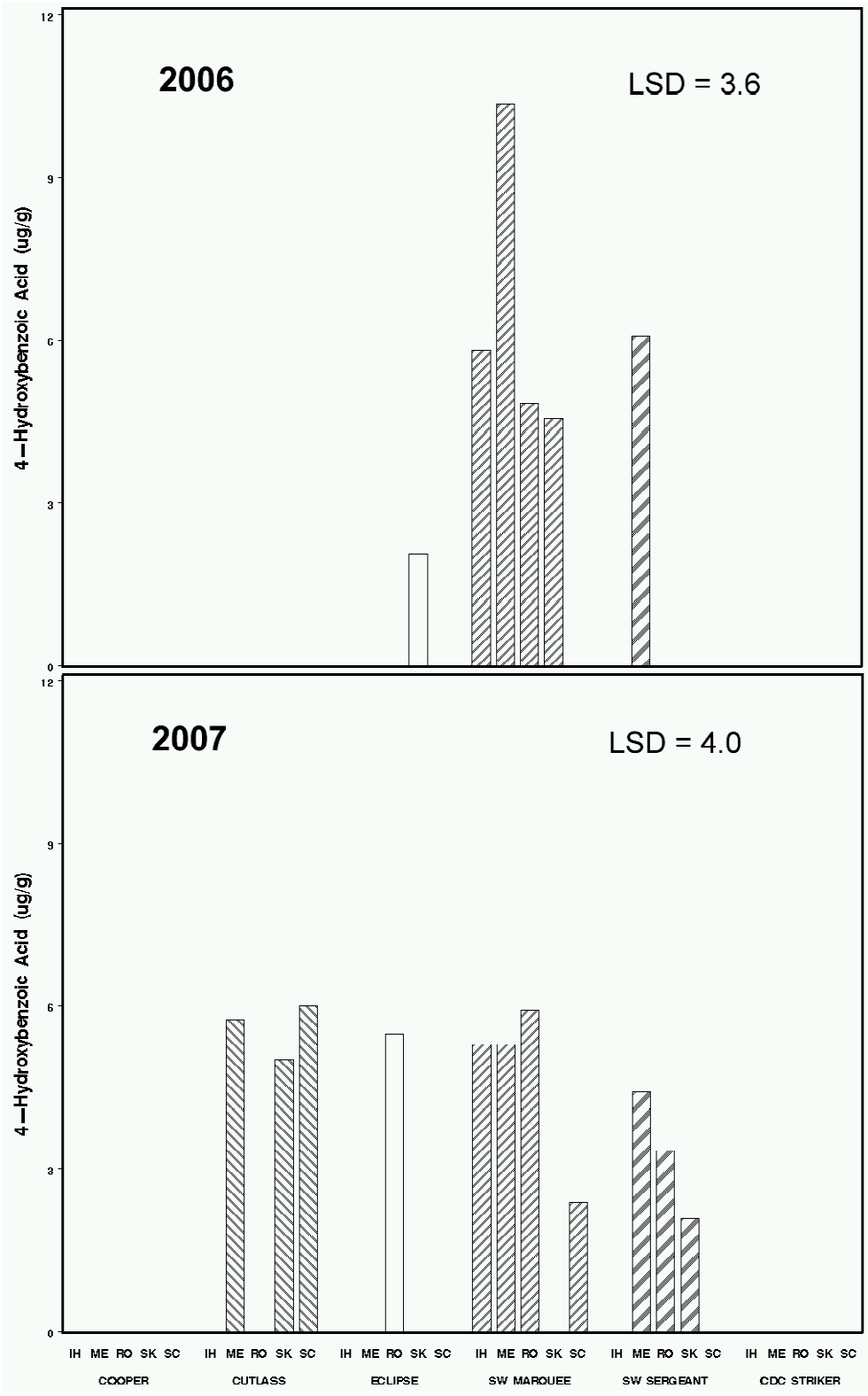


Figure 8. Caffeic acid content, based on UPLC analysis, found in the 6 cultivars: CDC Striker, Cooper, Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons.

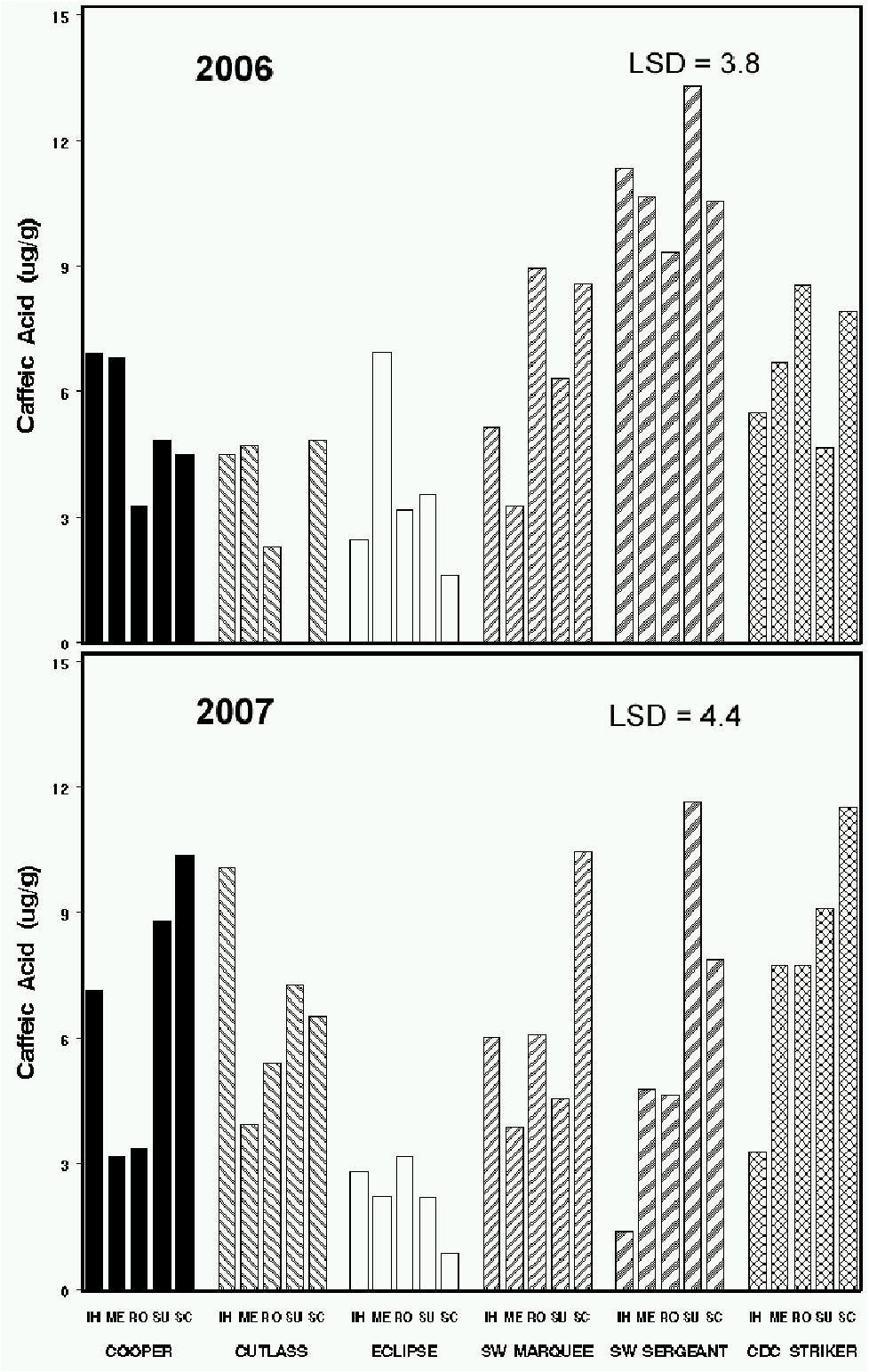


Figure 9. Ferulic acid content, based on UPLC analysis, found in the 6 cultivars: CDC Striker, Cooper, Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons.

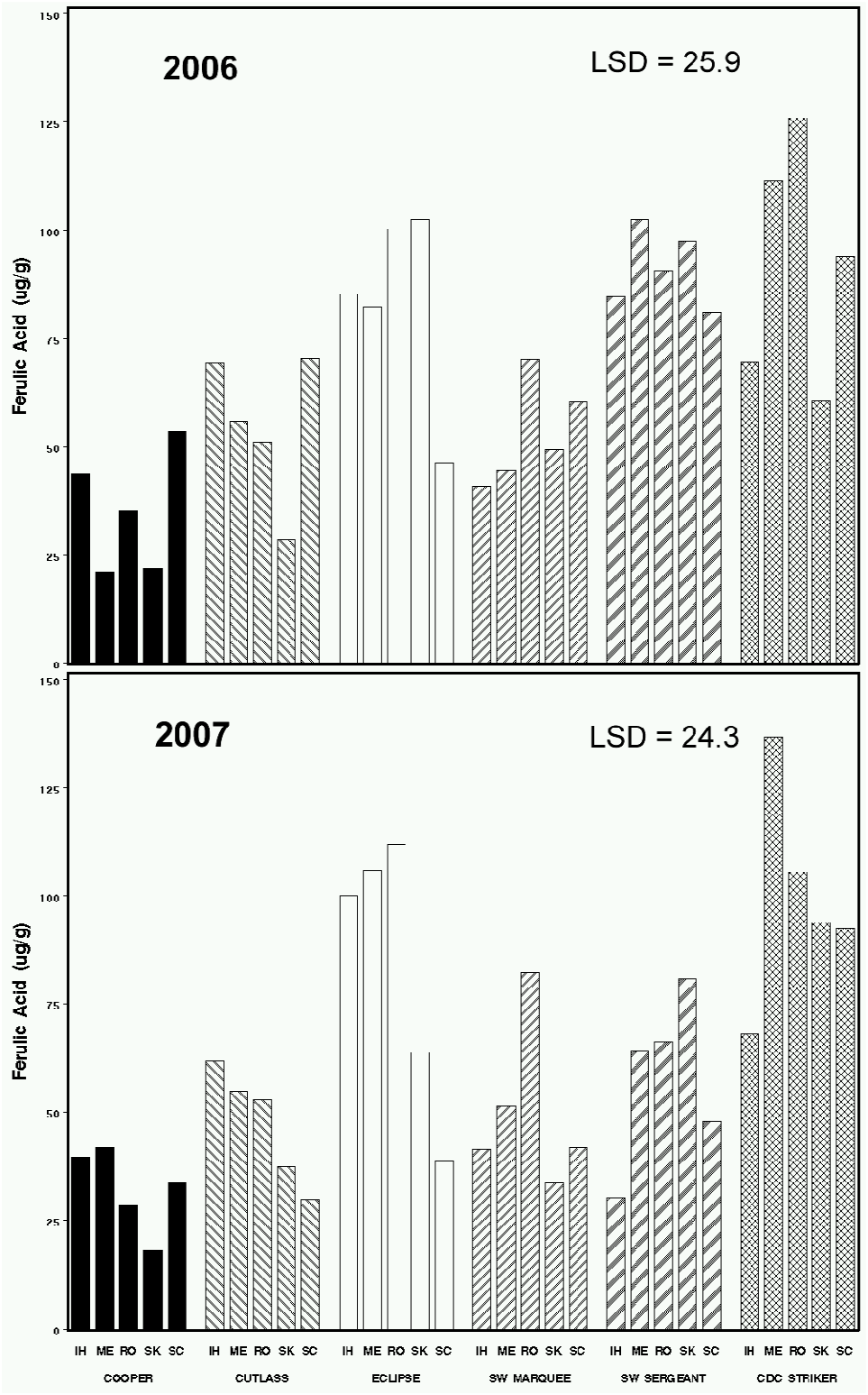


Figure 10. *p*-Coumaric acid content, based on UPLC analysis, found in the 6 cultivars: CDC Striker, Cooper, Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons**.**

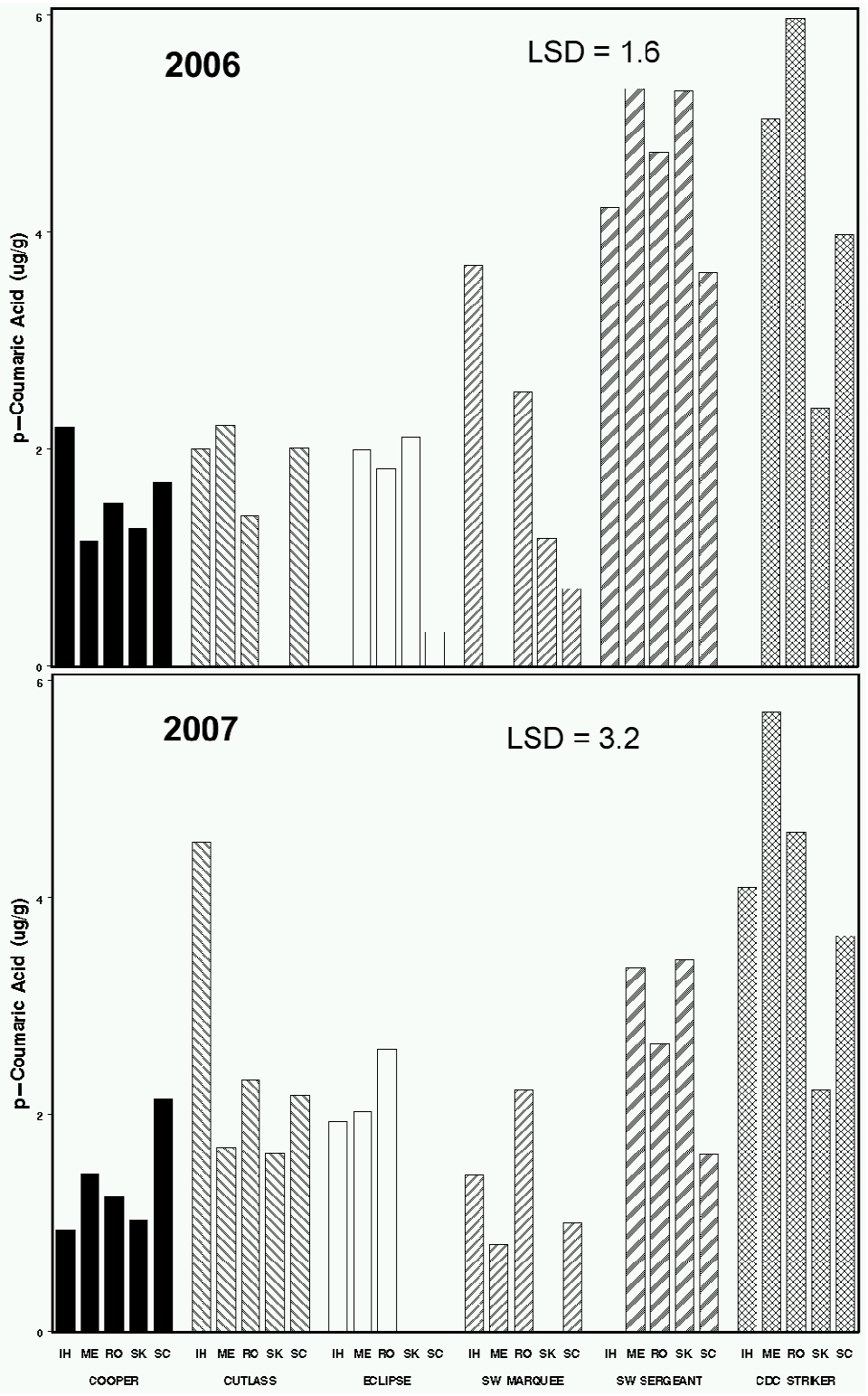


Figure 11. Protocatechuic acid content, based on UPLC analysis, found in the 6 cultivars: CDC Striker, Cooper, Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons.

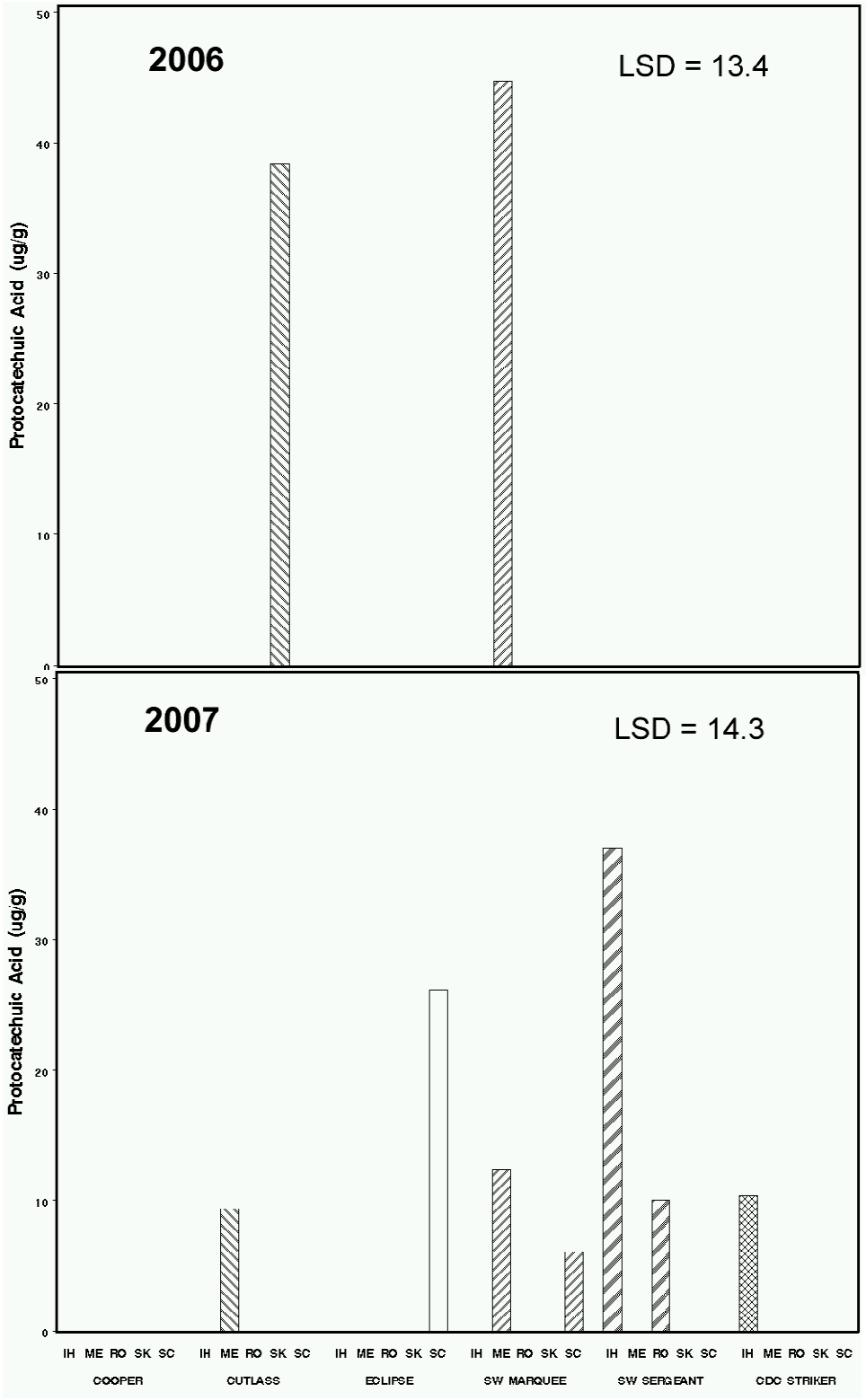


Figure 12. Quercetin content, based on UPLC analysis, found in the 6 cultivars: CDC Striker, Cooper, Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons.

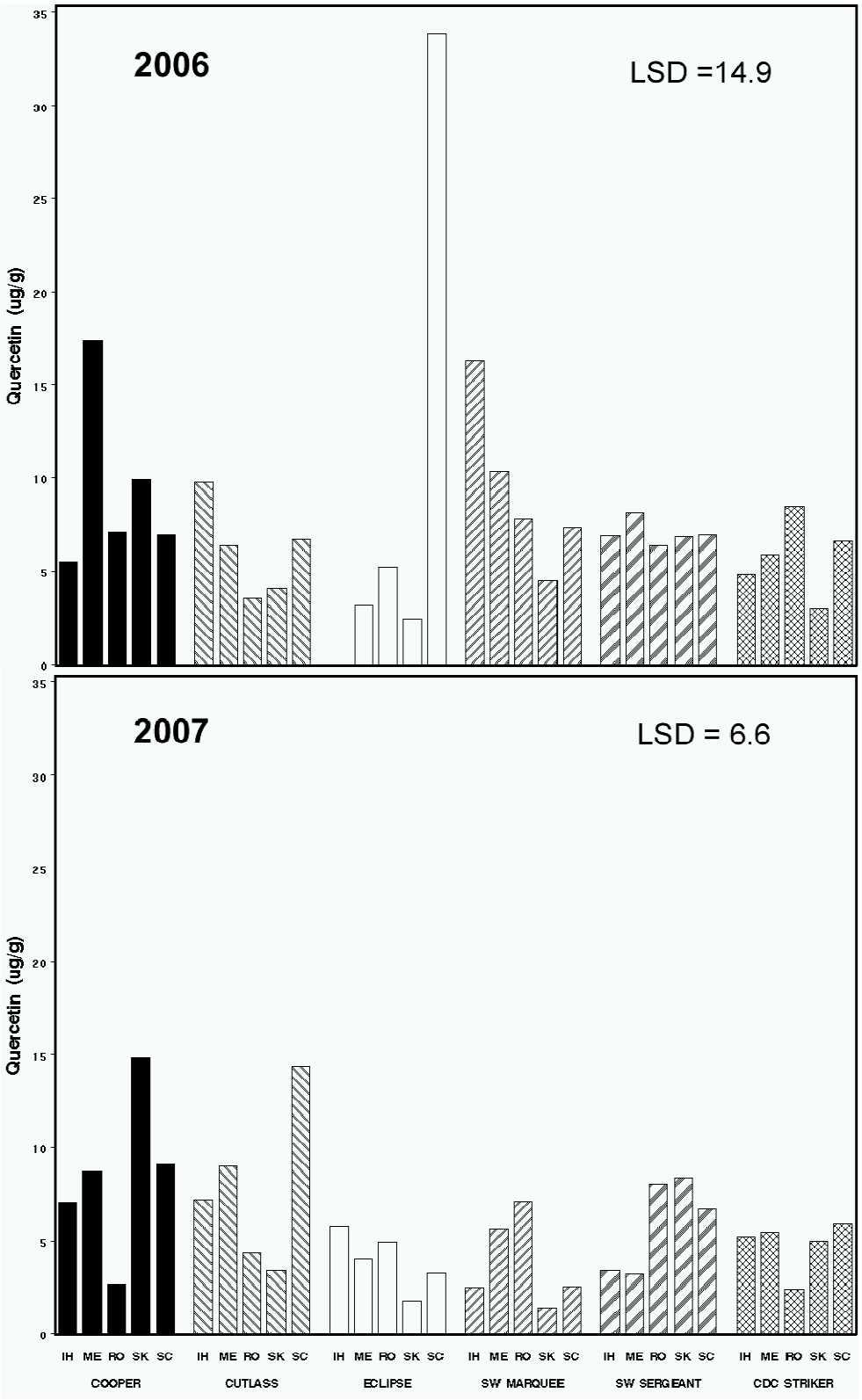


Figure 13. Rutin content, based on UPLC analysis, found in the 6 cultivars: CDC Striker, Cooper, Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons.

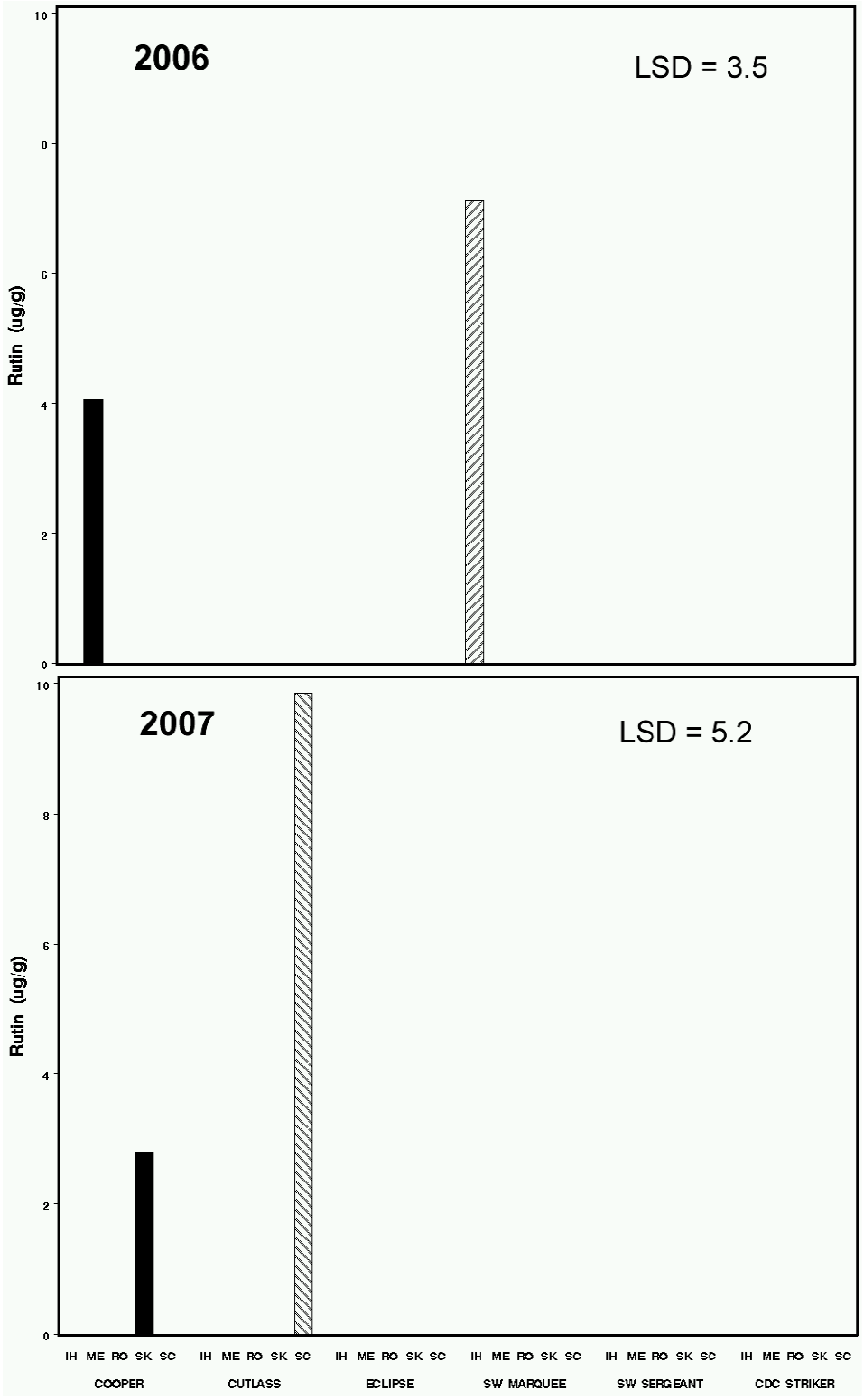


Figure 14. Sinapic acid content, based on UPLC analysis, found in the 6 cultivars: CDC Striker, Cooper, Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons.

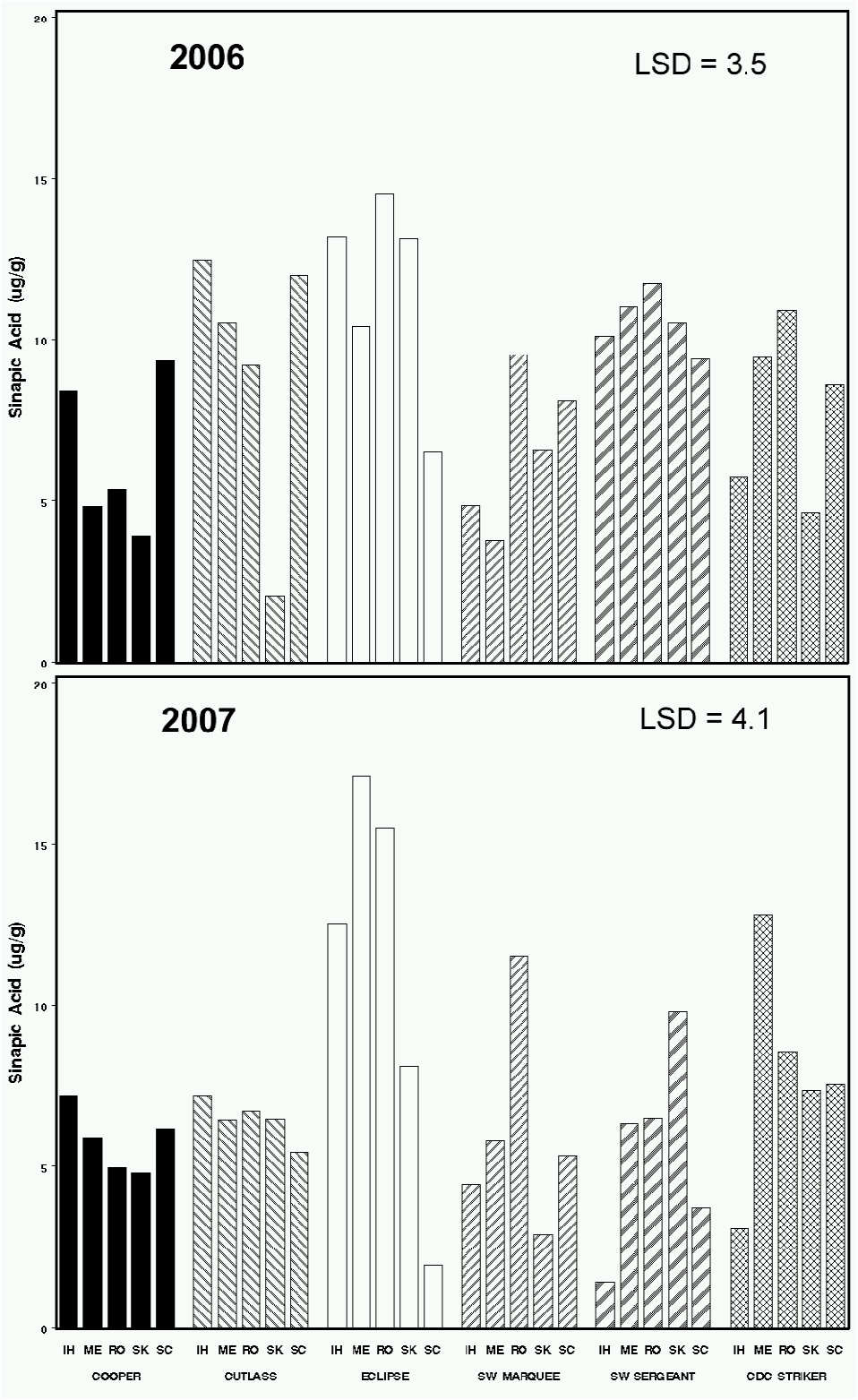


Figure 15. Syringic acid content, based on UPLC analysis, found in the 6 cultivars: CDC Striker, Cooper Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons.

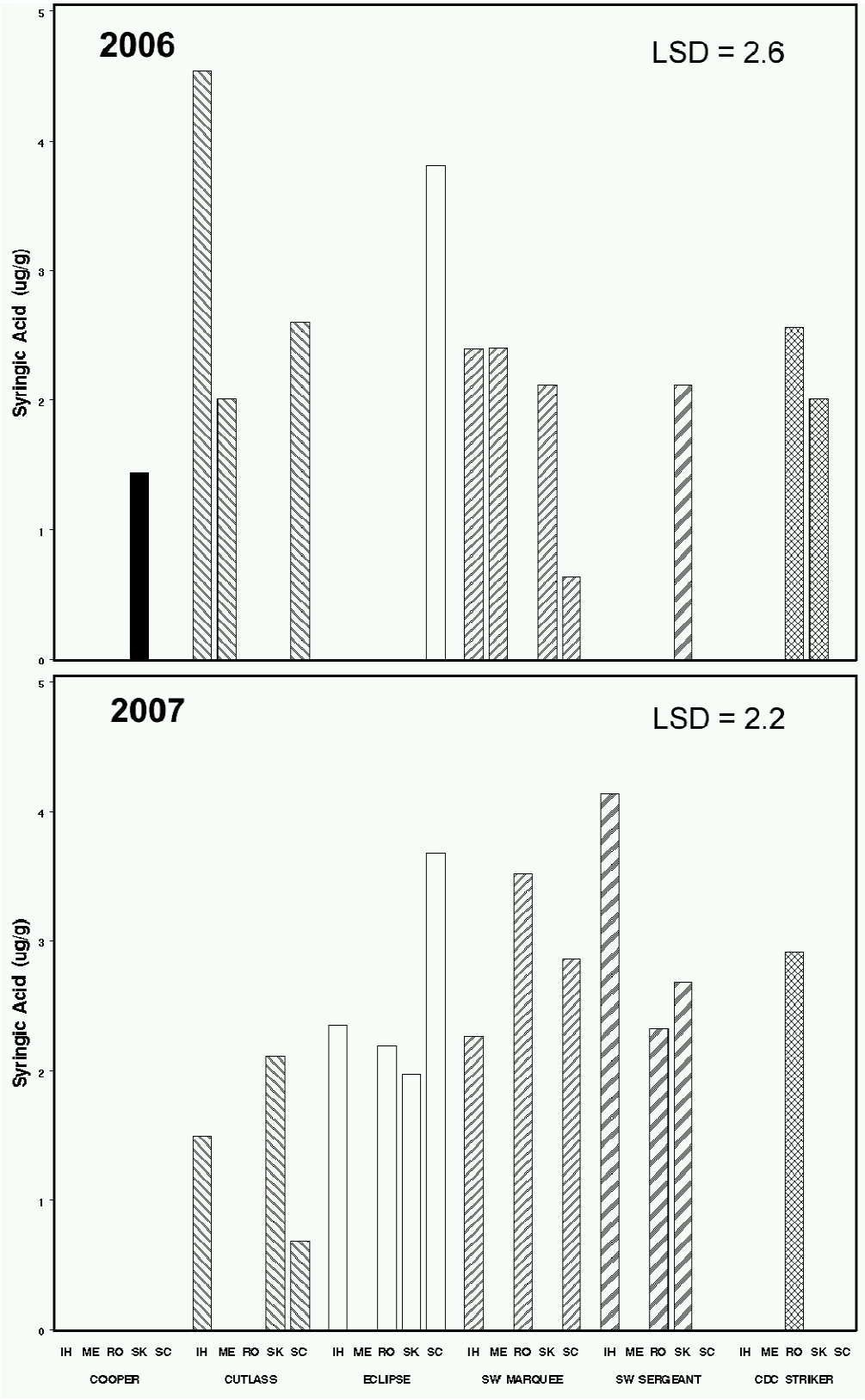
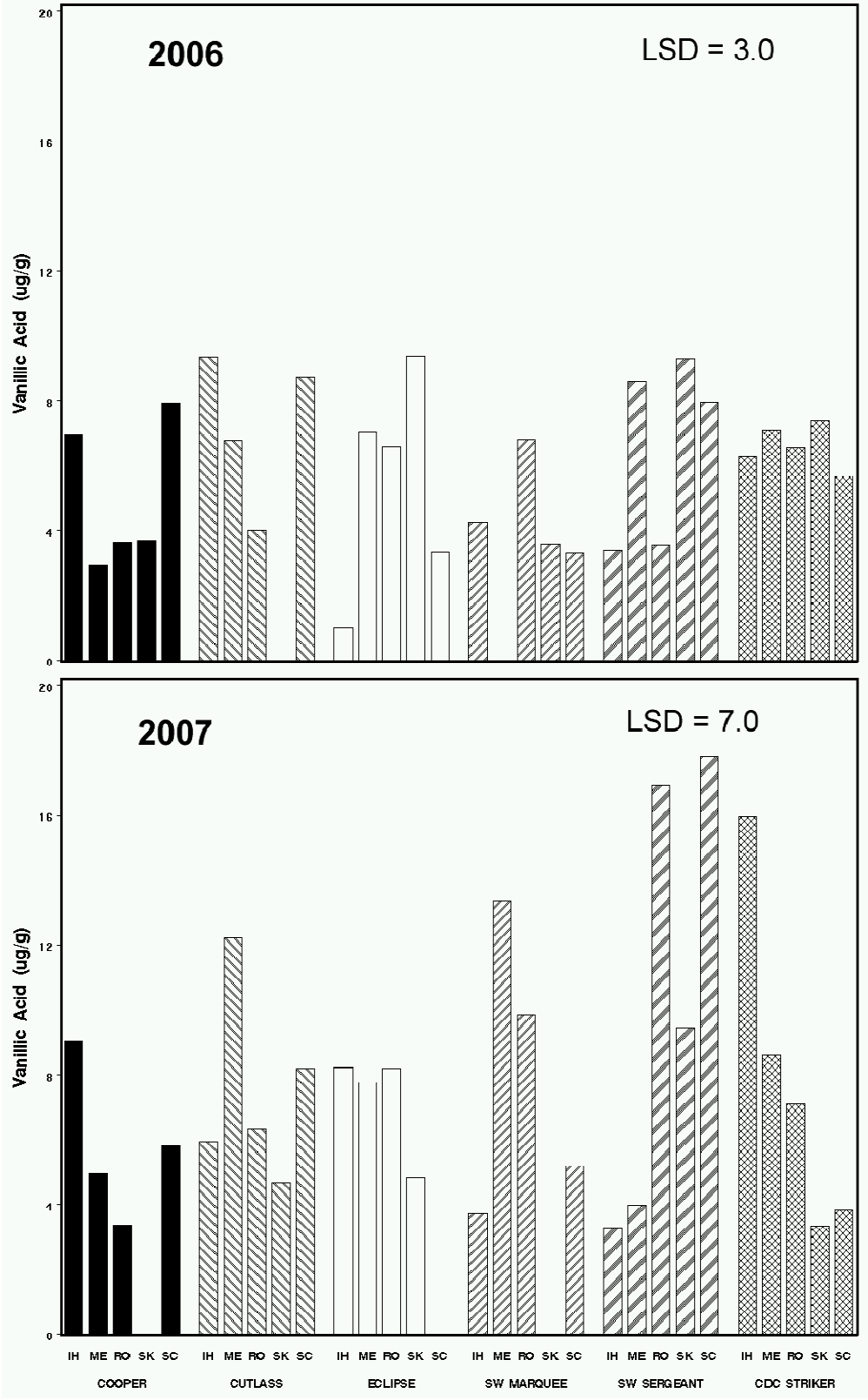


Figure 16. Vanillic acid content, based on UPLC analysis, found in the 6 cultivars: CDC Striker, Cooper, Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons.



content ranged from 8.6 – 172.0 μg/g. This is slightly higher than the findings of Sosulski & Drabowski (1984), as they found that Canadian field pea contained 2 – 3 mg/100g free and hydrolyzable phenolic acids, as quantified by GLC. Troszyńska & Ciska (2002) also found that Polish field peas with coloured cotyledons contained 78.53 μg/g phenolic acids. Ferulic acid was by far the most abundant phenolic acid present in all of the field pea samples analyzed, with a mean content of 65.3 μg/g. This result was verified by the work of Sosulski & Drabowski (1984), Amarowicz & Troszyńska (2003), and Ross *et al* (2009) who found that pinto beans, black beans and dark red kidney beans subjected to a basic hydrolysis showed high amounts of ferulic acid. However, Dueñas, Hernández and Estrella (2007) were not able to detect ferulic acid in their field pea samples. This may be the result of using an acidified methanol extraction method instead of a basic hydrolysis extraction, since a simple acidified methanol extraction could be insufficient for releasing bound phenolics such as ferulic acid.

Tables 11 and 12 depict the mean simple phenolic contents for each genotype and growing location, respectively. Genotypes grown in Melfort, followed closely by those in Rosthern, had the highest phenolic content, averaging 115.6 μg/g and 110.8 μg/g, respectively. Genotypes grown in Melfort contained the highest amounts of protocatechuic acid, vanillic acid and 4-hydroxybenzoic acid, while genotypes grown in Rosthern contained the highest average amounts of sinapic acid, p-coumaric acid and ferulic acid. Genotypes grown in Saskatoon had the lowest mean phenolic acid content, averaging 88.0 μg/g. Genotypes grown in Saskatoon also had the lowest amounts of vanillic acid, ferulic acid, quercetin, p-coumaric acid and sinapic acid.

Table 11. Mean Individual† Simple Phenolic Acid Content and TPC of the 6 Field Pea Genotypes

	Cooper	Cutlass	Eclipse	SW Marquee	SW Sergeant	CDC Striker
Protocatechuic	0.00	4.78	2.62	6.31	4.70	1.04
4-hydroxybenzoic	0.00	1.68	0.75	4.44	1.59	0.00
Vanillic	4.72	6.62	5.63	5.00	8.41	7.27
Caffeic	5.90	4.96	2.90	6.33	8.56	7.24
Syringic	0.14	1.34	1.40	1.62	1.13	0.75
p-Coumaric	1.46	2.00	1.28	1.36	3.42	3.79
Ferulic	33.73	51.25	83.72	51.65	74.63	96.90
Sinapic	6.10	7.84	11.29	6.27	8.05	7.89
Quercetin	8.88	6.88	6.44	6.55	6.48	5.27
Rutin	0.68	0.99	0.00	0.71	0.00	0.00
Total content†	61.62	88.33	116.03	90.25	116.99	130.16
TPC*	4.01	4.01	4.08	4.09	4.06	4.08

†Based on UPLC Analysis (μg/g)

*Based on Folin-Ciocalteu Analysis (mg/g) CE

Table 12. Mean Individual† Simple Phenolic Acid Content and TPC of Field Peas from Each Growing Location

	Saskatoon	Indian Head	Swift Current	Melfort	Rosthern
Protocatechuic	3.49	3.94	2.69	5.87	0.84
4-hydroxybenzoic	1.19	0.92	0.70	2.78	1.63
Vanillic	4.59	6.44	6.48	7.18	6.90
Caffeic	6.24	5.56	7.10	5.28	5.50
Syringic	1.08	1.43	1.19	0.40	1.13
p-Coumaric	1.63	2.09	1.93	2.68	2.80
Ferulic	56.76	61.25	58.40	75.91	76.78
Sinapic	6.65	7.54	7.04	9.02	9.58
Quercetin	5.41	6.20	9.15	6.43	5.67
Rutin	0.23	0.59	0.82	0.00	0.00
Total content†	87.26	95.97	95.50	115.56	110.83
TPC*	4.03	4.03	4.06	4.07	4.08

†Based on UPLC Analysis (μg/g)

*Based on Folin-Ciocalteu Analysis (mg/g) CE

The genotype with the lowest overall simple phenolic content was the Cooper genotype, with a mean phenolic content of 61.6 μg/g, while the genotype that had the highest overall total phenolic content was the CDC Striker with a mean phenolic content of 130.2 μg/g. The Cooper genotype contained the lowest amounts of ferulic acid, vanillic acid, syringic acid and sinapic acid and contained no protocatechuic acid or 4-hydroxybenzoic acid. Ferulic was the most prevalent phenolic acid in CDC Striker, which contained a mean ferulic acid content of 96.9 μg/g. Cutlass contained the highest amounts of sinapic acid, vanillic acid, caffeic acid and syringic acid, all of which were found in significantly lower quantities than ferulic acid.

4.3.2.4. Influence of Genotype, Location, Growing Year and Cotyledon Colour on the Simple Phenolic Acid Content.

Variations in genotype, growing locations, cotyledon colour and growing seasons resulted in significant differences in the phenolic composition and the amount of phenolic in the field pea samples, as depicted in table 13. Genotype and cotyledon colour had a significant effect on the total phenolic content ($p < 0.0001$) as did the growing season ($p < 0.05$). Figures 7-16 depict the mean content of the individual simple phenolic acids at different locations in different years.

Genotype had a significant effect on the 4-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid and sinapic acid content, but did not affect the protocatechuic acid, quercetin or rutin content. Cotyledon colour had a significant effect on the 4-hydroxybenzoic acid, caffeic acid, syringic acid, p-coumaric acid and sinapic acid, but not the protocatechuic acid, vanillic acid, ferulic acid, quercetin or rutin content.

Growing location had a significant effect on the 4-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid and sinapic acid that was not seen with protocatechuic acid, quercetin or rutin content. Growing season also had a significant effect on the vanillic acid, ferulic acid and sinapic acid that was not seen with the other phenolic acids analyzed. A significant G x E interaction effect was seen in all the individual phenolic acids analyzed with the exceptions of quercetin and rutin. Furthermore, there was a significant genotype, location, growing season and G x E interaction effect on the total phenolic content of the field pea samples as assessed using UPLC analysis.

4.4. AOA Analysis using the Free Radical DPPH

4.4.1. Genotype, Environment and Growing Season and their Effect on AOA: Cuvette method

Figure 14 depicts the AOA for each genotype in each growing location as measured using cuvettes. The DPPH values of the field pea samples as measured using a spectophotometer ranged from 20.5 to 114.9 μmol Trolox Equivalents (TE)/g. The lowest DPPH value was from Cutlass grown in Indian Head in 2007 while the highest DPPH value was from Eclipse grown in Rosthern in the 2006 growing season. The genotype exhibiting the lowest DPPH value was Cooper, with a mean AOA of 45.4 μmol TE/g while Eclipse had the highest mean AOA, with a mean of 63.8 μmol TE/g. Genotypes grown in Indian Head had the lowest mean AOA, averaging 44.2 μmol TE/g while genotypes grown in Melfort had the highest mean AOA, averaging 59.3 μmol TE/g. The values that are reported here are higher than reported by Xu & Chang (2007), who estimated the AOA of field peas to be 0.01 – 2.75 μmol TE/g and of Xu *et al* (2007), who

estimated the AOA of field peas to be 0.57 – 2.65 μmol TE/g. This may be because of the differences in the extraction method used in this study compared to that of Xu & Chang (2007) and Xu *et al* (2007). Xu *et al* (2007) used acetone/water (50:50 v/v) to extract the pea phenolics while Xu & Chang (2007) used a variety of solvent systems to extract the phenolics from the pea samples. Neither study used a basic hydrolysis method to extract the phenolics from the field peas as was used in this study. A basic hydrolysis method, such as the one described by Ross *et al* (2009), should be used when extracting phenolics from pulses and cereal grains as the majority of the phenolic acids found in such commodities are in bound form. For example, only 2 – 3.5% of the phenolic acids in wheat are free phenolic acids (Klepacka & Fornal, 2006). Free phenolic acids are easily extracted using only solvent; however, the remaining bound phenolic acids that comprise the vast majority of the phenolic composition and AOA of the sample must undergo a basic hydrolysis method to be released from the cell wall material.

Table 14 shows the ANOVA F-values and significance of the random variables. There was a very significant ($p < 0.01$) genotype effect on the AOA of field peas. This is consistent with the finding of Xu *et al* (2007), who found that there was a significant difference ($p<0.05$) in the AOA between all ten yellow pea genotypes and a significant difference ($p<0.05$) between most of the ten green pea genotypes. As well, there was a significant difference ($p<0.01$) between growing locations, and a significant ($p<0.0001$) genotype and environment interaction effect (G x E). The growing season did not have an effect on the AOA, nor did the colour of the cotyledon.

Table 13. The Effect of Genotype, Location, Cotyledon Colour and Growing Year on the Analysis of Variance (ANOVA) F-Values for the Individual Phenolic Acids, Total Simple Phenolic Acids and TPC

Attribute	**F value**					
	Genotype (G)	Location (E)	Year (Y)	Cotyledon Colour	G x E	L x Y
4-Hydroxybenzoic	14.46***	3.84**	2.61	19.19***	2.55**	1.21
Caffeic	17.05***	2.91*	0.07	30.21***	3.28***	4.15**
Ferulic	65.71***	11.03***	8.39**	2.83	6.09***	3.63**
***p*-Coumaric**	14.88***	2.93*	1.28	26.51***	2.38**	0.32
Protocatechuic	2.12	1.27	0.47	2.99	4.44***	3.89**
Quercetin	0.78	1.55	3.27	0.05	1.45	0.58
Rutin	0.78	0.47	0.01	0.66	1.16	1.39
Sinapic	17.29***	8.59***	18.63***	5.32*	4.66**	5.79**
Syringic	3.71**	2.53*	0.92	10.10**	2.67**	2.48*
Vanillic	4.60**	2.46*	7.70**	3.00	3.73***	3.35*
Total Simple	35.65***	7.66***	4.80*	1.08	3.89***	1.27
TPC	8.11***	2.15	1.43	3.47	2.91**	8.04***

*Significant at $p<0.05$

**Significant at $p<0.01$

***Significant at $p<0.001$

Table 14. The Effect of Genotype, Location, Cotyledon Colour and Growing Year on the Analysis of Variance (ANOVA) F-Value for the AOA Based on the Microplate Method and AOA Based on the Cuvette Method.

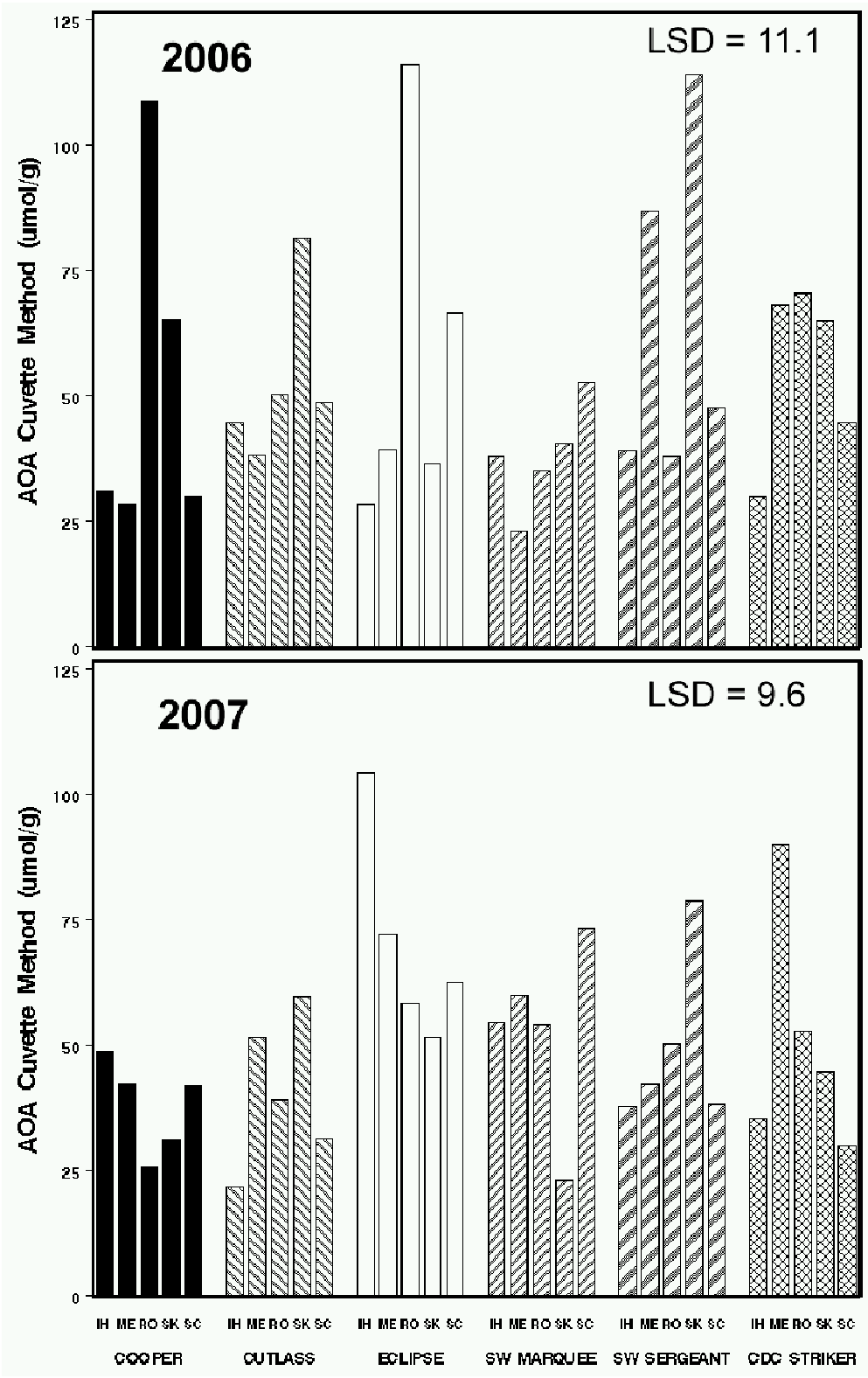
Attribute	**F value**					
	Genotype (G)	Location (E)	Year (Y)	Cotyledon Colour	G x E	E x Y
AOA (Cuvette)	3.35*	2.71*	0.98	0.01	2.7***	4.42**
AOA (Microplate)	2.69*	2.26*	0.75	0.01	3.11***	3.74*

*Significant at $p<0.05$

**Significant at $p<0.01$

***Significant at $p<0.001$

Figure 17. AOA based on the reduction of DPPH using cuvettes, found in the 6 cultivars: CDC Striker, Cooper, Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons.



4.4.2. Genotype, Environment and Growing Season and their Effect on AOA: Microplate Method

Figure 18 depicts the AOA for each genotype in each growing location as measured using a microplate. The DPPH values of the field pea samples as measured using a microplate reader ranged from 14.6 to 76.7 μmol Trolox Equivalents (TE)/g. The lowest DPPH value was from Cutlass grown in Swift Current 2007 while the highest DPPH was from Cooper grown in Rosthern in the 2006 growing season. The genotype with the lowest DPPH value was Cooper, with a mean AOA of 31.8 μmol TE/g while Eclipse had the highest mean AOA, with a mean of 43.6 μmol TE/g. Genotypes grown in Indian Head had the lowest mean AOA, averaging 29.2 μmol TE/g while genotypes grown in Melfort had the highest mean AOA, averaging 42.0 μmol TE/g.

Like the cuvette method, there was a significant ($p<0.05$) genotype effect, a significant difference ($p<0.05$) between growing locations, and a significant ($p<0.0001$) genotype by environment interaction effect. As well, the growing season did not have an effect on the AOA, nor did the colour of the cotyledon; consistent to what was found using the cuvette method.

Figure 18. AOA based on the reduction of DPPH using microplates, found in the 6 cultivars: CDC Striker, Cooper, Cutlass, Eclipse, SW Marquee and SW Sergeant grown in each of the 5 growing locations: Swift Current (SC), Indian Head (IH), Saskatoon (SK), Rosthern (RO) and Melfort (ME), Saskatchewan, Canada throughout the 2006-2007 growing seasons.

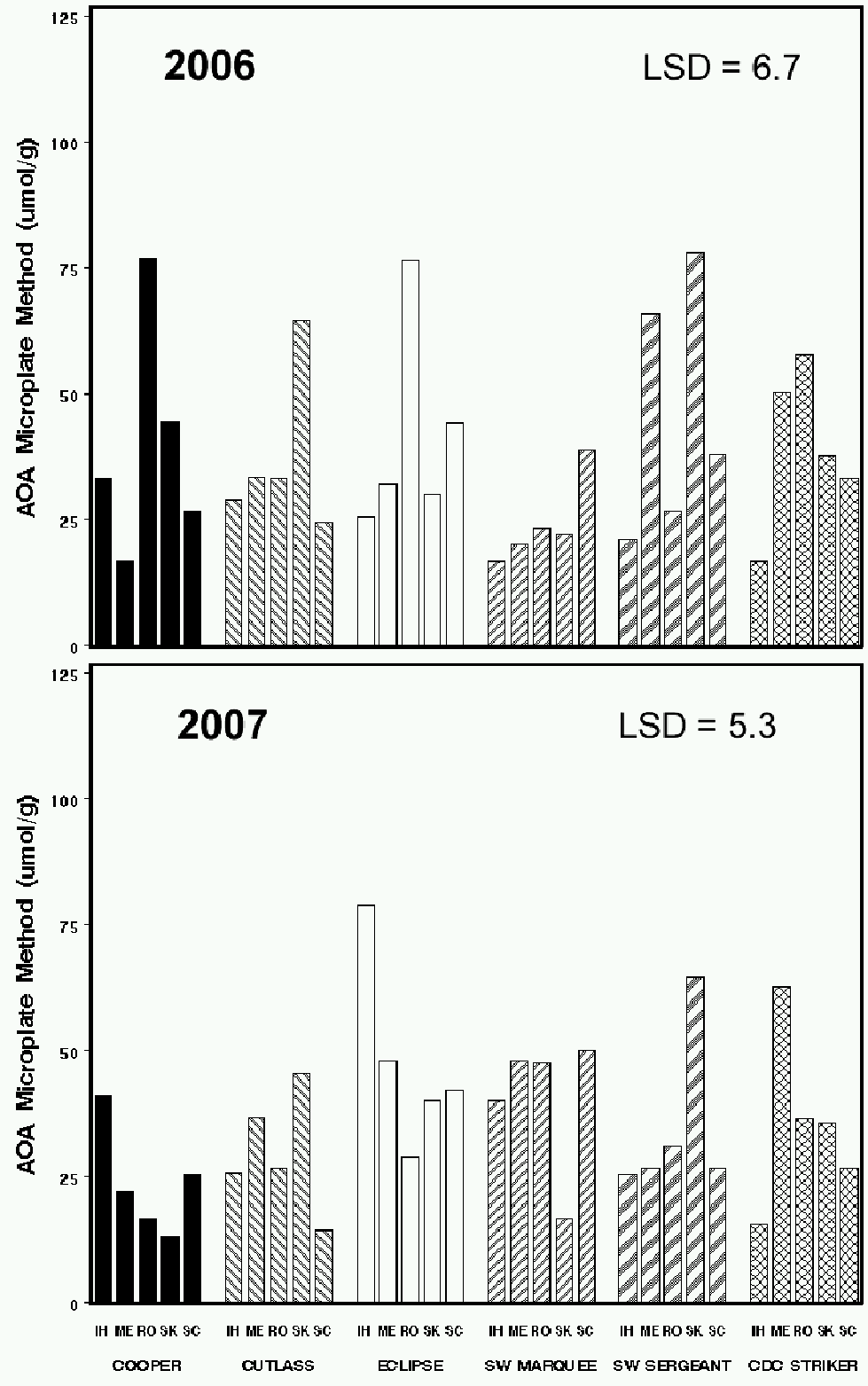


Table 15. Mean Cuvette and Microplate AOA of Field Peas from Each Genotype Grown in 2006 and 2007 (µmol TE/g)

2006	cuvette	microplate
Cooper	53.00[ab]	39.42[ab]
Cutlass	49.50[ab]	35.45[ab]
Eclipse	56.51[ab]	41.44[ab]
SW Marquee	37.52[b]	24.51[b]
SW Sergeant	60.92[a]	44.39[a]
CDC Striker	57.10[ab]	40.36[ab]
Standard deviation	*4.17*	*3.60*
2007		
Cooper	36.72[a]	21.67[a]
Cutlass	51.06[a]	36.97[ab]
Eclipse	66.26[b]	43.88[c]
SW Marquee	51.88[ab]	38.74[bc]
SW Sergeant	53.89[a]	35.48[abc]
CDC Striker	47.72[a]	36.52[abc]
Standard deviation	*4.17*	*2.94*

Means with the same letter are not significantly different (LSD $p < 0.05$)

Table 16. Mean Cuvette and Microplate AOA of Field Peas from Each Growing Location Grown in 2006 and 2007 (µmol TE/g)

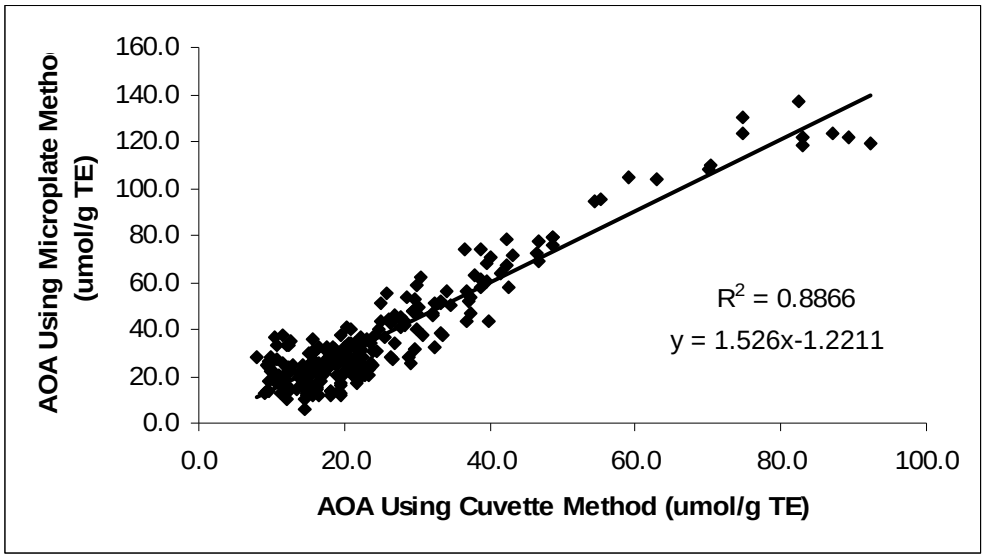
2006	cuvette	microplate
Indian Head	34.89[a]	23.57[a]
Melfort	52.49[ab]	39.85[ab]
Rosthern	62.17[c]	44.87[b]
Saskatoon	58.32[bc]	40.42[b]
Swift Current	54.50[ab]	38.92[ab]
Standard deviation	*3.64*	*3.17*
2007		
Indian Head	50.04[a]	35.46[a]
Melfort	59.64[a]	40.36[a]
Rosthern	47.71[a]	32.62[a]
Saskatoon	50.52[a]	36.40[a]
Swift Current	47.91[a]	33.20[a]
Standard deviation	*2.86*	*2.79*

Means with the same letter are not significantly different (LSD $p< 0.05$)

4.4.3. Comparison of DPPH Free Radical Scavenging Ability between Cuvettes and Microplates

As mentioned previously, alterations of the original Brand-Williams *et al* (1995) DPPH method to incorporate a microplate reader were based on more time consuming methods such as antiradical power (Fukumoto & Mazza, 2000). In this study, AOA was read after a set time of 30 minutes, allowing for rapid results. The results from the microplate reader tended to be slightly lower than the results from the cuvette reader, and had slightly smaller standard deviations. However, because the AOA in the field pea samples is so minute, the differences between the methods appear to be larger than they are and are not significantly different as determined by an F-test, as well as a t-test, performed on the differences in AOA between the two methods. Differences in the reduction of DPPH between cuvette and microplate methods were also reported by Fukumoto & Mazza (2000). Both methods found similar trends in AOA, and there was a high degree of correlation ($r^2 = 0.89$) between the two methods (Figure 19). This suggests that the rapid 30 minute DPPH scavenging ability method for use in a spectrophotometer can be transferred to a microplate reader, thereby reducing test time and solvent usage.

Figure 19. Correlation (r^2) Between AOA Using the Cuvette Method vs. AOA Using the Microplate Method



5.0. DISCUSSION

5.1. Genotype, Environment and their Effect on Fibre Content

The results of this study indicate that genotype has a very significant effect on TDF and IDF content in field pea. Genotype had a significant effect on TDF, IDF and SDF in Australian field peas as well, as Black *et al* (1998) found significant differences ($p<0.001$) in fibre content among 61 field pea genotypes arranged into the 4 Australian field pea categories (dun, white, blue and mottled). Although the results of this study indicate that growing season had an effect on TDF, IDF and SDF contents, this contradicted Black *et* al (1998), as no significant differences were detected in fibre content between the two years in which their study was conducted. The genotype x environment effect on TDF content corresponds to the findings of other studies. Wang & Daun (2004) found that ADF and NDF were affected significantly by a genotype x environment (as indicated by protein content) effect. Black *et al* (1998) similarly found a significant genotype x environment effect on the TDF content of Australian field pea.

The cotyledon colour of the genotype also influences the amount of fibre, as green-coloured genotypes contained higher mean levels of TDF and IDF in 2006 and 2007 (Table 8). The differences found between growing locations and growing years may, in part, be accounted for by differences in climate as well as soil conditions. Table 7 outlines the mean monthly temperatures, precipitation levels as well as the soil types between growing locations in the 2006-2007 growing seasons. Wang & Daun (2004) used crude protein content as an environmental indicator when studying ADF and NDF content of field peas because previous research indicated that protein content in field pea

was affected by environmental conditions such as growing location, agronomic factors, precipitation and temperature. Wang & Daun (2004) found that ADF and NDF were positively correlated with protein content, thus indicating that the IDF, SDF and TDF content would vary with such stresses as well. Table 7 shows that the 2007 growing year was a slightly cooler growing year and had more precipitation on average than the 2006 growing year. Field peas produced under cooler and moister conditions tend to have increased fibre content. Field pea is also best suited to the Black soil zones (AAFC, 2008), which include Rosthern, Melfort and Indian Head. These three locations had higher fibre content in field pea. It appears that although some genotypes will produce higher fibre contents than others, all genotypes will yield higher fibre contents in such growing conditions.

5.2. Genotype, Environment and their Effect on TPC and Simple Phenolic Acid Content

In order to understand how genotype and environment affect the phenolic content of field pea, the role of phenolics in vascular plants must be examined. Not only are plant phenolics beneficial to the health of the humans consuming them, they are essential to the health of the plants that contain them. As cited previously, phenolic components are part of the defence mechanism for plants against adverse conditions or physical damage by pathogens such as insects, moulds, fungi, bacteria and viruses. Phenolics are produced as a by-product of carbohydrate metabolism in the shikimate pathway, which is responsible for the biosynthesis of carbohydrates to aromatic compounds in plants (Herrmann & Weaver, 1999). The enzyme deoxyarabinoheptulosonate 7-phosphate, which is synthesized following carbohydrate metabolism and glycolysis, controls the carbon flow

into the shikimate pathway. As the pathway progresses, the aromatic amino acid phenylalanine is produced. The phenylalanine is later deaminated by the phenylalanine ammonialyase (PAL) and catalyzes the formation of *trans*-cinnamic acid, and subsequently other phenolic acids, flavonoids and phenolic compounds. During a resistant response due to a pathogenic attack, reactive oxygen species (ROS), such as hydrogen peroxide, are formed during an oxidative burst. The ROS catalyze the reaction between polyphenol oxidase and polyphenols to form *o*-diphenols, and the subsequent oxidation of *o*-diphenols to *o*-quinones. These *o*-quinones react with cell wall proteins resulting in protein cross-linking, forming an insoluble barrier to the pathogen (Greenberg, 1997).

Increased resistance against disease has been correlated with the phenolic content and phenolic-related enzyme activities in cereal crops, and altering the level of phenolics in plants results in a change in their susceptibility to disease (Sahoo, Kole, Dasgupta & Mukherjee, 2009). This may explain, in part, the effect of genotype on the phenolic content of field peas. CDC Striker, which was shown to have the highest phenolic content in this study, also had one of the lowest incidence of ascochyta leaf and pod spot (11.7%) in a study done by Dokken, Banniza, Warkentin & Morrall (2006), while Cooper, which had the lowest phenolic content in this study, had the highest incidence of ascochyta blight (28.7%) in the same study done by Dokken *et al* (2006). Eclipse, which had a phenolic content of 116.1 µg/g, had a disease incidence of 15.3 %, while SW Marquee, which had a phenolic content of 90.3 µg/g, had a disease incidence of 18.0%. The anomaly was Cutlass, which had the highest disease resistance of the genotypes examined in this research (11.2%) but had a lower amount of total phenolics (88.3 µg/g).

This phenomenon may perhaps be because the Eclipse genotype uses other non-phenolic mechanisms to defend itself against the ascochyta pathogen.

The effect of growing location on the polyphenolic content of field peas may be due to the growing temperature and precipitation levels received in each location. Field peas tend to prefer semiarid growing environments and need only 266 mm precipitation throughout the growing season (Cutforth, M^{c}Ginn, M^{c}Phee & Miller, 2007). Melfort and Rosthern were the growing locations with the highest phenolic contents as well as receiving some of the highest precipitation levels (Table 7). Incidentally, the 2006-2007 growing seasons were unusually wet for the Rosthern and Melfort areas. While other areas had average or below average precipitation levels, Melfort received 134% of its mean growing season precipitation levels in 2006 and 119% of the mean growing season precipitation levels in 2007. Rosthern received 119% of its mean growing season precipitation levels in 2006 and 121% of the mean growing season precipitation levels in 2007 (Saskatchewan Crop Insurance, 2009).This excessive precipitation, although not necessarily excessive for field pea production, may have been excessive for the soil type and resulted in water logging in these regions. Previous studies have found that excessive precipitation has an effect on the polyphenolic content of plants. Cheruiyot *et al* (2008) found that an increase in soil water content and water stress index is linked to an increase in total catechin content in tea leaves. Hwang, Lin, Chern, Lo & Li (1999) found that sweet potato plants experienced an increase in peroxidase activity during water logging periods. The increase in phenolics during periods of high precipitation is linked to the increase in ROS that occurs. During flooding conditions, the stomata of the plant closes, causing a reduction of the CO_2 concentration, a reduction in photosynthesis and an

increase in the ROS present in the plant. The presence of ROS results in the production of superoxide dismutase (SOD), an enzyme which converts the oxygen radicals to hydrogen peroxide. The hydrogen peroxide is then decomposed by peroxidase (Hwang *et al,* 1999), which was catalyzed by the polyphenols produced in the shikimate pathway (as mentioned previously). The high levels of polyphenols that were produced in the wetter growing areas, particularly Rosthern and Melfort, could be explained as a response to the increase in ROS due to the reduced photosynthesis in the plants.

5.3. Genotype, Environment and their Effect on AOA

It is not surprising that genotype has a strong effect on the AOA of field pea, as genotype effects the phenolic composition of field peas and other pulses. Troszyńska & Ciska (2002) found significant differences in the phenolic composition between the Kwestor and Fidelia genotypes of *Pisum sativum* L. Dueñas, Hernández & Estrella (2006) found significant differences between the percentage of groups of phenolic compounds as compared to the TPC in the Fidelia and ZP-849 genotypes of *Pisum sativum* L. Dueñas *et al* (2006) also found that higher AOA correlated with higher concentrations of gallocatechins and dimer prodelphinidins in the ZP-849 genotype than in the Fidelia genotype.

As previously stated, the structural composition of phenolic acids dictates the AOA exhibited. Brand-Williams *et al* (1995) found that caffeic acid had an anti-radical power (ARP) of 9.1, while its monophenol counterpart, coumaric acid, had an antiradical power of 0.02; gallic acid had an ARP of 12.5 while its diphenol counterpart, protocatechuic acid, had an ARP of 7.14. Hence, two field pea extracts could potentially

have the same TPC value but have very different AOA. For example, if caffeic acid comprised the majority of the phenolic composition of one sample and coumaric acid comprised the majority of the phenolic composition of the other sample, this results in a different AOA for each sample.

Information on the effect of growing location on the AOA of leguminous crops is limited. Riedl *et al* (2007) reported that the total isoflavone content of soybeans correlated with the amount of precipitation received in the growing location. Tsukamoto *et al* (1995) reported that higher growing temperatures were inversely correlated with isoflavone content in soybeans. There is some information available on the effect of growing location on the AOA of other crops. Mpofu, Sapirstein & Beta (2006) found that growing location had a significant effect on the AOA of hard spring wheat. Yu & Zhou (2004) found that growing location had a significant effect on the AOA of the bran extracts of 'Platte' hard winter wheat. Oomah & Mazza (1996) found that location had a significant effect on the antioxidant activity of buckwheat.

Melfort and Rosthern tended to have higher AOA than other growing locations. This may be due to the fact that both growing regions had significantly higher than average precipitation levels in 2006 and 2007. Past research has indicated that excessive precipitation and water logging may stimulate the production of phenolics and phenolic-catalyzed enzymes involved in the defence mechanism of plants. Increased resistance against disease has been correlated with the phenolic content and phenolic-related enzyme activities in cereal crops, and altering the level of phenolics in plants results in a change in the susceptibility to disease in plants (Sahoo, Kole, Dasgupta & Mukherjee, 2009). Since the antioxidant activity of food is in part due to its phenolic

content (Xu *et al*, 2007), it is likely that changes in the phenolic composition would affect the AOA of field peas.

5.4. Comparison of the Cuvette Method vs. the Microplate Method

Although there is no statistically significant difference between the cuvette and microplate method, it is apparent that the values between the methods show some variance. However, when measuring the AOA in µmol/g, the resulting differences in AOA are very small and therefore not significantly different. The high correlation (R^2 = 0.8866) between the two methods indicates that the microplate method is able to detect the same differences between samples as the cuvette method. Moreover, the microplate method was able to detect the same significant differences between genotypes, environments and G x E interaction influences. At this point in time, the microplate method may be used to detect trends and list samples in order of their AOA.

5.5. Correlation between TPC, Simple Phenolic Acid Content and AOA

Surprisingly, there did not appear to be any relationship between the TPC, total simple phenolic acid content and AOA. There was no correlation between TPC vs. simple phenolic content via UPLC analysis nor was there a correlation between AOA vs. simple phenolic content via UPLC analysis. However, the UPLC analysis only tested for ten specific simple phenolic acids and did not analyse for other phenolic components that comprise much of the AOA, such as the flavonoids and other compounds. The lack of correlation between the TPC and the AOA was of special interest. This may be due to interferences with the method used for determining TPC using the Folin-Ciocalteu

reagent. Literature review of this method revealed the issue of interferences as prevalent. Some studies cited the use of reversed-phase column chromatography on phenolic extraction samples prior to TPC analysis employing the Folin-Ciocalteu reagent, to separate the ascorbate and reducing sugars from the extract to eliminate interferences that would overestimate the TPC (Georgé, Brat, Alter & Amiot, 2005; Gancel, Alter, Dhuique-Mayer, Ruales & Vaillant, 2008; Fukushima *et al*, 2009). A few studies revealed interference issues with the use of NaOH and with sugars while using Folin-Ciocalteu reagent for the Lowry assay for protein detection. Wessels (1965) found that when *Schizophyllum commune* homogenates were heated in alkali, the Lowry method detected erroneously high amounts of protein. Bonitati, Elliot & Miles (1969) found that carbohydrate contamination and interference was likely when the sample was exposed to alkali hydrolysis and possible after acid hydrolysis. Since our extraction method involved the addition of 10 N NaOH and a subsequent 16 hour hydrolysis, followed by the addition of HCl to reduce the pH to approximately 2, it is very likely that reduced sugars from our field pea samples caused interference during our TPC analysis. A reversed-phase column chromatography separation beforehand may be needed on the extract in order to properly analyze for the TPC using the Folin-Ciocalteu reagent.

5.6. Genotype, Environment, their Interaction Effect and how they affect the Dietary Fibre and Phenolic Content of Field Pea

Dietary fibre and phenolic acids have a common link besides their nutraceutical functionality. Both dietary fibre and phenolic acids function in the protective mechanism of plants against pathogenic attack by strengthening plant cell wall structure. As mentioned previously, phenolic acids react with polyphenol oxidase in the presence of

ROS to form *o*-quinones which react with cell wall proteins, forming an insoluble barrier against pathogen attack. These phenolic acids, along with polysaccharides, glycoproteins and trace minerals, comprise the primary wall structure of most flowering plants. The primary wall structure is relatively thin and allows for wall reorganization and expansion during periods of plant growth. The secondary cell wall structure consists of cellulose, hemicellulose, pectins and lignins - the dietary fibre component of the plant. These plant cell structures provide stability and support to the plant. The dietary fibre components are resistant to physical, chemical or enzymatic degradation (Guillon, Saulnier, Robert, Thibault & Champ, 2007), providing defence and stability to a plant during a pathogenic attack, as many pathogens secrete polysaccharide degrading enzymes to utilize the plant cell walls as a source of nutrition (Juge, 2006).

Cell wall components are also able to respond to external stimuli, resulting in changes in cellular structure. Reductions in the cellulose content due to biotic and abiotic stressors result in the production of enzymes as well as the expression of genes that are involved in the synthesis of a series of hormones that are associated with plant tolerance against pathogens and environmental stressors. This in turn results in the excessive production of other dietary fibre components such as pectins, lignans and β-glucans. Mechanical stimuli can also induce reaction wood formation, resulting in the formation of cellulose (Humphrey, Bonetta & Coring, 2008). The capacity of cell wall signalling is not yet well understood, nor is the exact mechanism for signalling due to plant stressors.

The defence response mechanism in plants may explain why certain field pea genotypes and growing locations tended to have higher amounts of the nutraceutical components examined in this study. Not only did genotypes grown in Rosthern and

Melfort contain high amounts of dietary fibre, they tended to have a high phenolic contents and AOA. As Rosthern and Melfort tended to have higher than normal precipitation levels, this stress may have induced a defensive response by the plants to increase the production of cell wall structure components and phenolic components. A similar trend was found with the phenolic content of field pea genotypes. Not only did the CDC Striker and SW Sergeant genotypes tend to have higher TDF and IDF contents, but they also had the highest phenolic contents and AOA. Not only did the Cooper genotype have the lowest TDF and IDF contents, it tended to have the lowest phenolic content and AOA. As mentioned previously, CDC Striker was found to have a high resistance level to pathogens while Cooper was found to have the lowest resistance against pathogens. It may be that genotypes with higher resilience and pathogen defence genetically contain higher amounts of the nutraceutical components that also act as defence components in plants.

6.0. CONCLUSIONS AND RECOMMENDATIONS

Genotype and environmental factors have a significant effect on the nutritional composition of field pea. There is a very strong genotype effect and environment effect on dietary fibre content in field pea. Growing location has a strong effect on dietary fibre content. Genotype has a strong effect with IDF and TDF content, while having no significant effect with SDF content. Growing season has a strong effect with SDF and TDF content and a statistically significant effect on the IDF content. Green cotyledon genotypes have greater amounts of IDF and TDF than yellow cotyledon genotypes. Genotype x environment has a significant effect on TDF while environment x year has a very significant effect on TDF content. There is a very strong genotype, environment and genotype x environment effect on the TPC and total simple phenolic acid content of field peas. As well, individual phenolic acids respond differently to the influence of genotype and environment. The AOA of field peas is affected by genotype, environment and genotype x environment interaction according to both the cuvette and microplate methods.

The increase in fibre and phenolic content, as well as the AOA, appears to be related to the disease resistance properties of the variety as well as the plant's response to adverse growing conditions. Wetter zones, such as Rosthern and Melfort, tended to produce field peas with higher levels of dietary fibre and simple phenolics. Drier, semi-arid growing regions, such as Swift Current and Indian Head, tended to produce field peas with the lowest dietary fibre and simple phenolic content. Genotypes with lower disease resistance also tended to have lower dietary fibre and phenolic contents. Cooper,

which was shown to have the least resistance against ascochyta blight, also has the lowest amount of dietary fibre, simple phenolics and AOA. CDC Striker, Eclipse and SW Sergeant genotypes tended to have the highest dietary fibre, phenolic content and AOA, while also having the most resistance against ascochyta blight. The stress that the plant is exposed to during growth, along with how the plant is genetically disposed to cope with stress, tends to dictate the fibre content and phenolic content of field pea.

Research into the methodologies used in this study has shed light on their actual analytical capacities. Reduction of sample weight for the TDF enzymatic-gravitational test produces similar results to the full 1.0 g sample size, but results in a large variance. Although basic hydrolysis is essential for liberating the bound phenolic acids in field pea for analysis, it may also result in the production of reduced sugars which interfere with the TPC method of phenolic analysis. A reversed-phase column chromatography separation after extraction may be necessary to separate the sugars from the phenolics before TPC analysis. Finally, a high degree of correlation between the traditional cuvette method of AOA analysis using the free radical DPPH and the modified method using a microplate indicates that the method can be transferred to a microplate and used to track trends in AOA in field pea.

The research findings resulting from this study have a wide application for the agriculture and agrifood industry in Canada. Plant breeders can use this information as a basis to see which genotypes have the highest nutraceutical potential, so that future genotypes can be manipulated to produce higher amounts of dietary fibre and simple phenolics. Agronomists can use this information to work with pulse producers to maximize the functionality of their pea crops, especially for producers who sell their pea

crops to mills for fractionation. Functional food manufacturers who wish to integrate whole pea or pea fractions into their products now have a clearer idea of the fibre and phenolic content of Canadian-grown field peas. Nutritionists and pulse marketers can use this information to promote the nutraceutical content of this crop. Future studies could expand the phenolic analysis to include the polyphenols, tannins and flavonoid antioxidants. As well, it would be interesting to research whether heat/drought stress had a similar, or greater, effect on the fibre and phenolic content of field pea as wet growing conditions.

7.0. REFERENCES

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8.0. APPENDICIES

Appendix 1. Mean Daily Temperatures (°C) of the Six Locations in Saskatchewan Throughout the 2006-2007 Growing Season

		Growing Location				
Year	Month	Melfort	Swift Current	Rosthern (Carlton)	Saskatoon	Indian Head
2006	May	11.1	12.3	11.4	12.5	11.2
	June	16.7	16.2	16.1	n/d	16.0
	July	18.3	21.0	19.2	21.0	17.9
	August	17.1	19.1	16.5	19.3	17.4
2007	May	9.6	11.4	10.9	11.9	9.6
	June	14.4	15.7	14.7	15.8	15.0
	July	20.1	22.6	20.2	21.8	19.9
	August	14.7	17.7	14.3	16.7	15.5

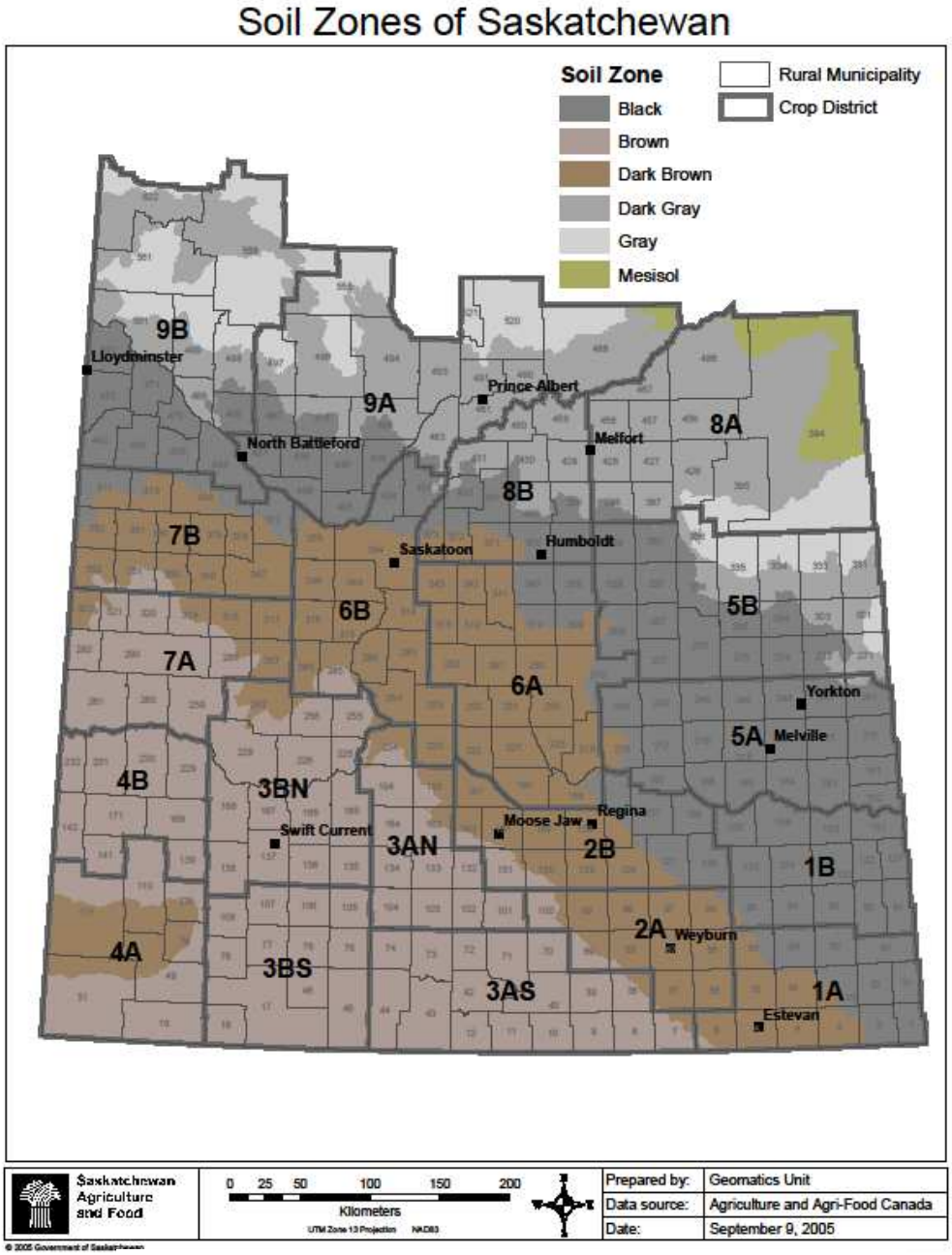
n/d = no data available (Environment Canada, 2006, 2007)

Appendix 2. Mean Precipitation Levels (mm) in the Six Locations in Saskatchewan Throughout the 2006-2007 Growing Season

		Growing Location				
Year	Month	Melfort	Swift Current	Rosthern (Carlton)	Saskatoon	Indian Head
2006	May	63.0	34.9	92.2	46.8	39.0
	June	73.6	96.8	98.2	n/d	80.4
	July	38.6	30.6	72.8	39.8	4.4
	August	45.4	20.6	59.8	38.4	11.6
2007	May	54.0	37.1	49.0	44.0	46.0
	June	119.0	56.0	151.4	109.0	46.2
	July	46.8	9.8	58.0	16.4	50.6
	August	39.2	19.0	87.8	104.6	62.8

n/d = no data available (Environment Canada, 2006, 2007)

Appendix 3. Soil Types and Conditions of the Six Growing Locations in Saskatchewan (Saskatchewan Ministry of Agriculture, 2005)



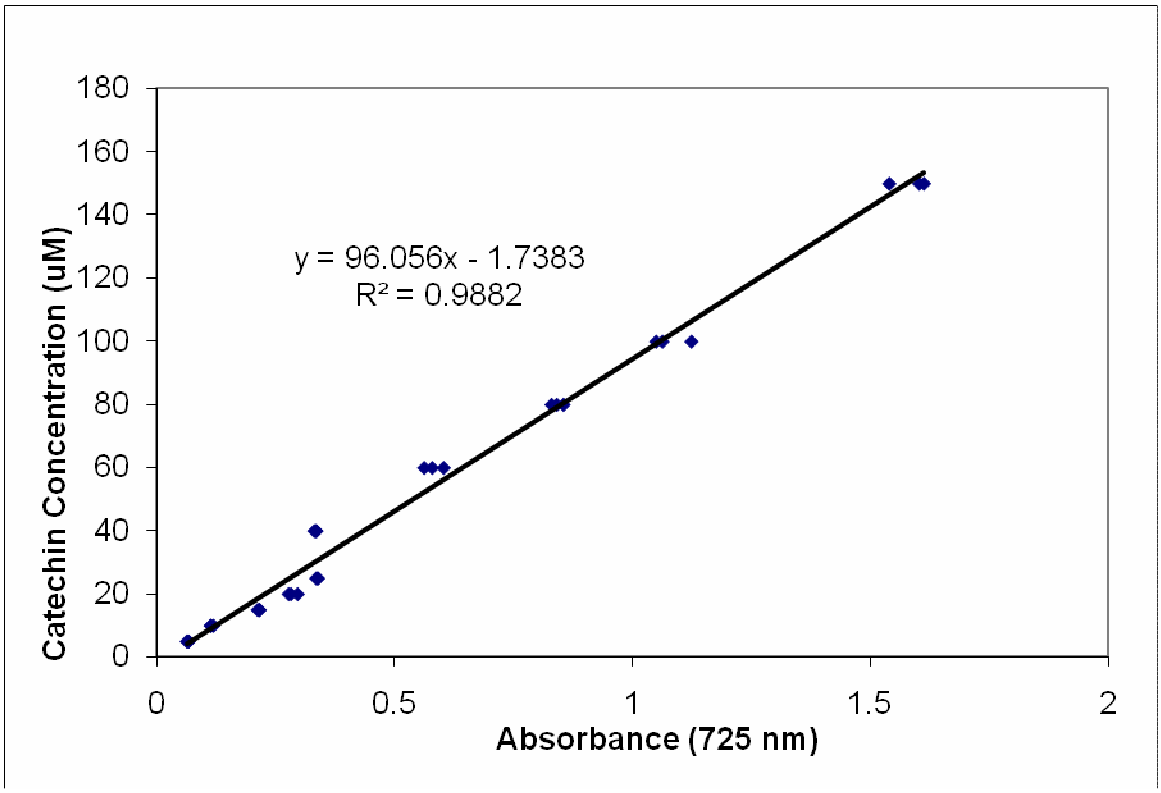
Location	Crop District	Soil Zone
Indian Head	2B	Black
Melfort	8A/8B	Dark Grey
Rosthern	6B	Black
Saskatoon	6B	Dark Brown
Swift Current	3BN	Brown

Appendix 4. Historical Mean Temperature (°C) of Growing Locations

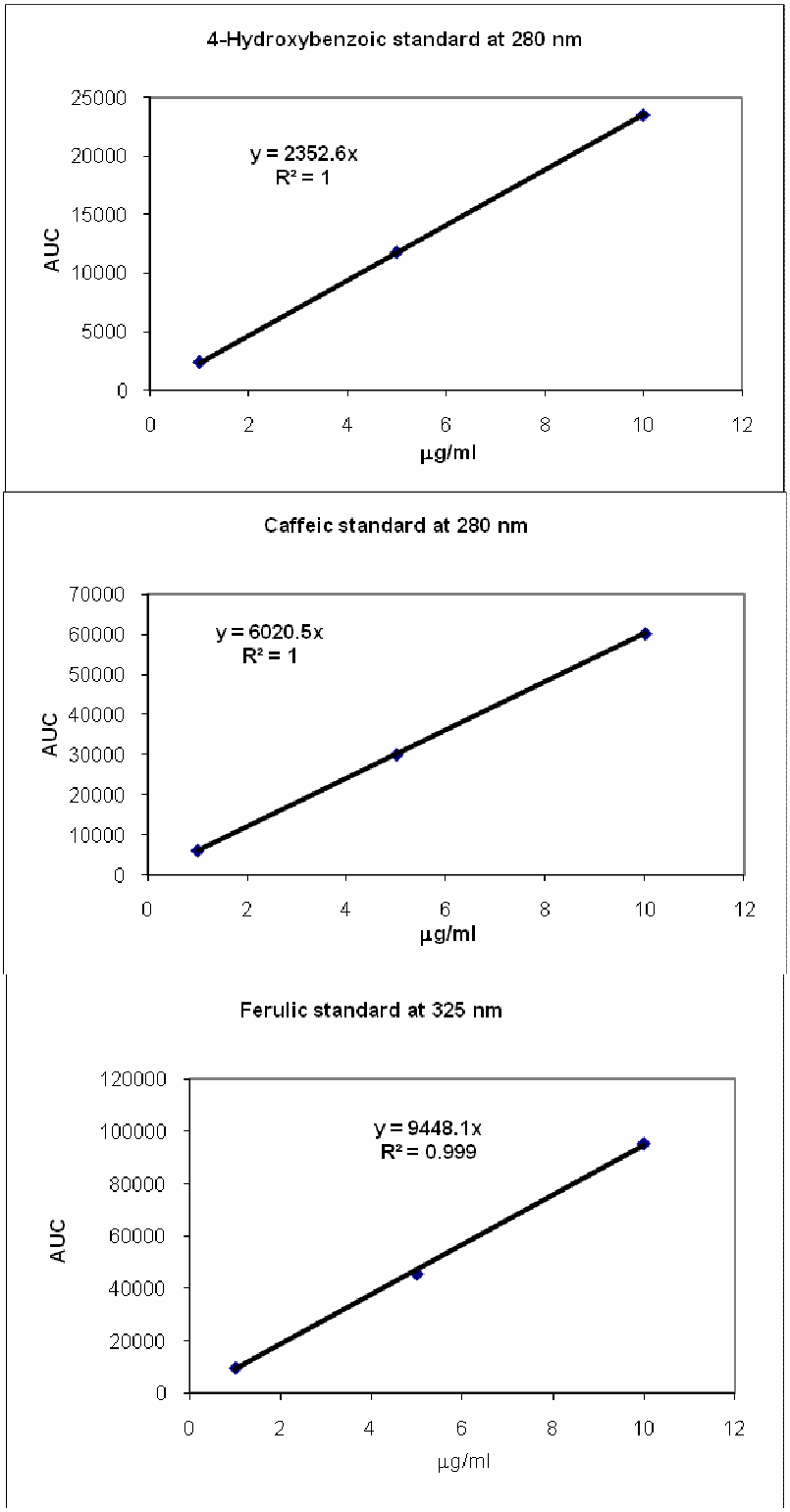
2006										
	Melfort		Swift Current		Rosthern		Saskatoon		Indian Head	
	high	low	high	low	high	low	high	low	high	low
May	16.9	5.3	18.7	5.9	17.8	4.9	20.6	14.6	17.9	4.4
June	22.0	11.2	22.2	10.1	21.9	10.5	23.9	18.2	22.1	9.9
July	24.5	12.3	28.8	13.3	26.1	12.5	29.5	22.7	25.7	10.2
August	23.8	10.5	27.3	11.0	24.1	9.4	28.1	20.6	26.3	8.4
2007										
May	15.5	3.6	18.0	4.7	17.4	4.3	20.4	7.5	15.7	3.3
June	20.5	8.3	22.5	8.9	20.9	8.3	23.9	12.4	21.9	8.1
July	26.1	14.1	30.5	14.7	26.6	14.0	29.5	15.9	27.4	12.4
August	20.4	9.0	25.0	10.3	20.6	8.5	28.1	13.1	22.4	8.5

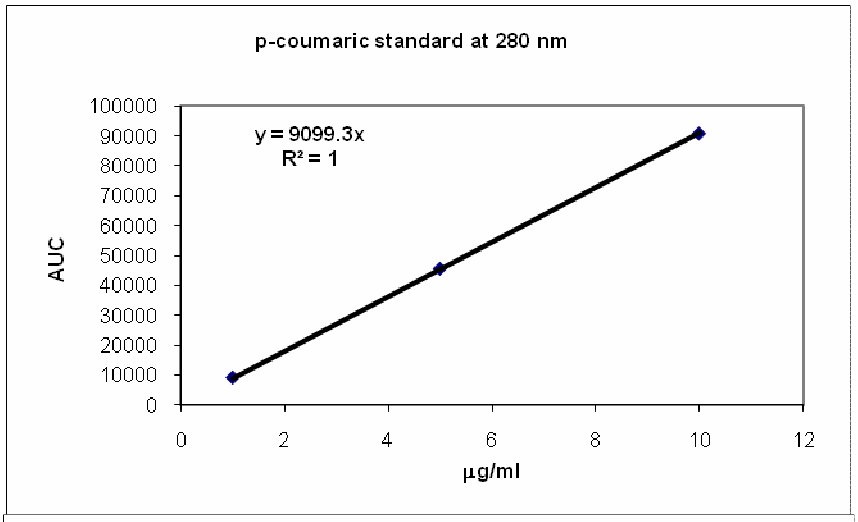
(Environment Canada, 2006; 2007)

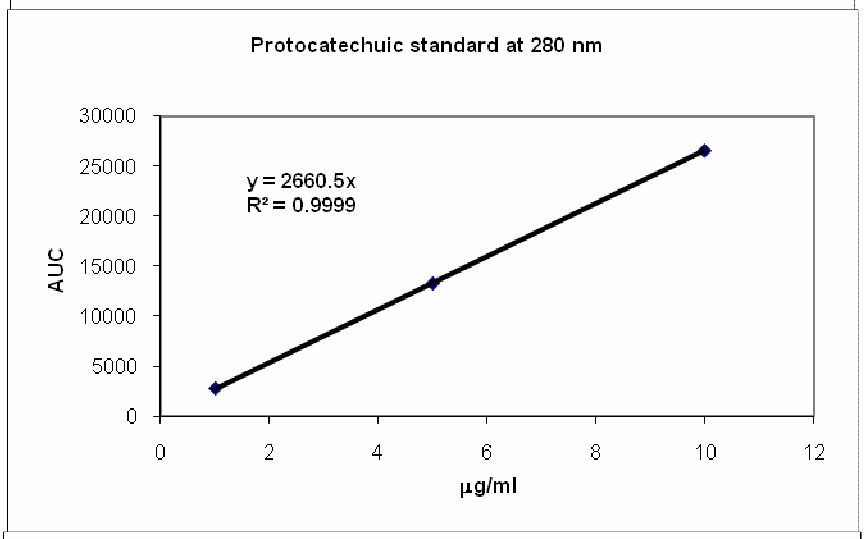
Appendix 5. Calibration Curve used to Assess TPC in the Folin-Ciocalteu Method

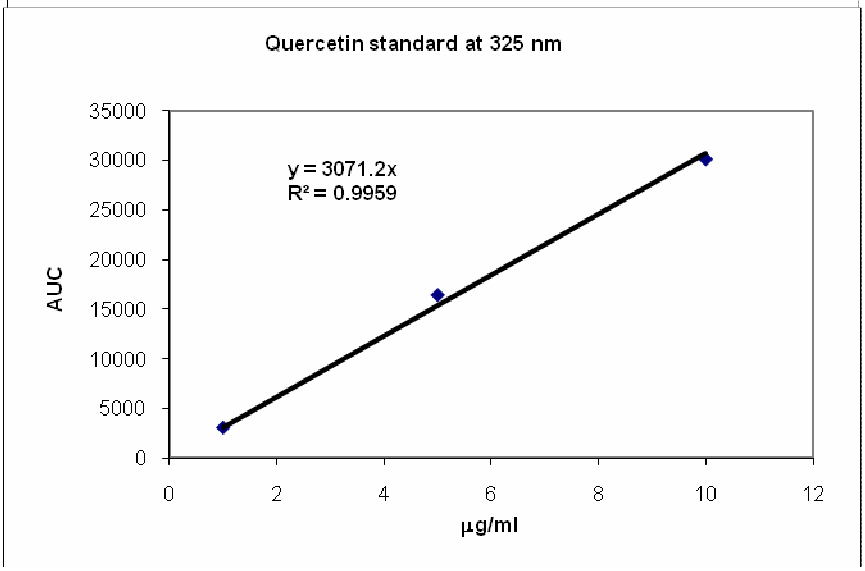


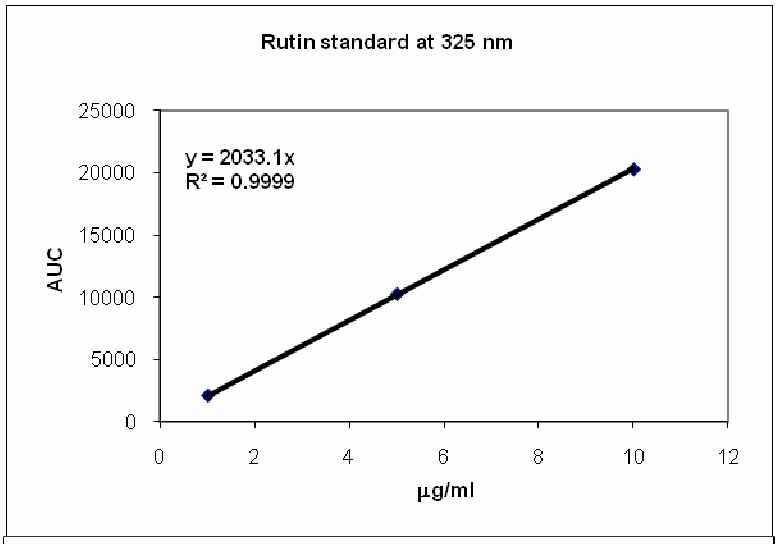
Appendix 6. Calibration Curves used to calculate the simple phenolic acid content from the area under the curve (AUC) derived from UPLC analysis

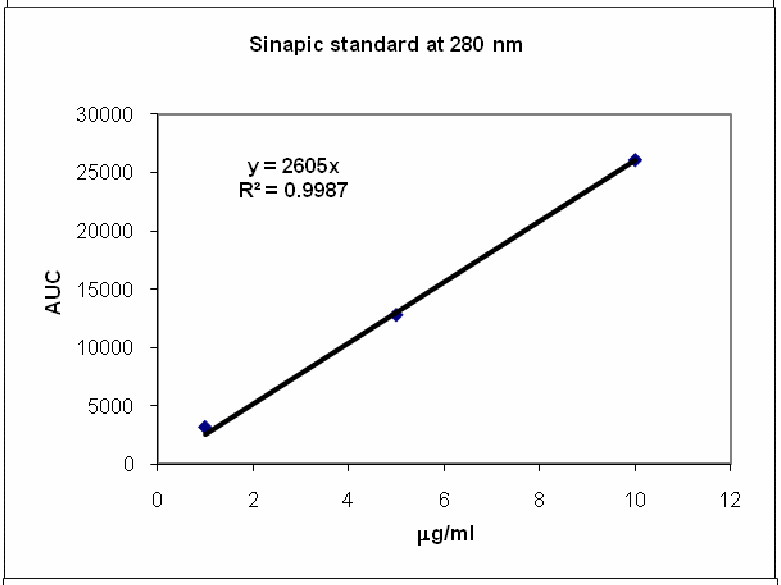


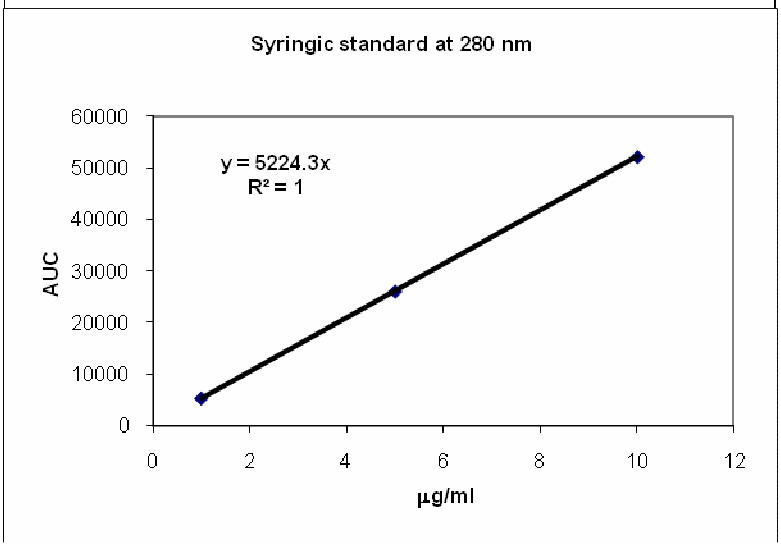

p-coumaric standard at 280 nm
y = 9099.3x
R² = 1
AUC
µg/ml
0
10000
20000
30000
40000
50000
60000
70000
80000
90000
100000
0
2
4
6
8
10
12

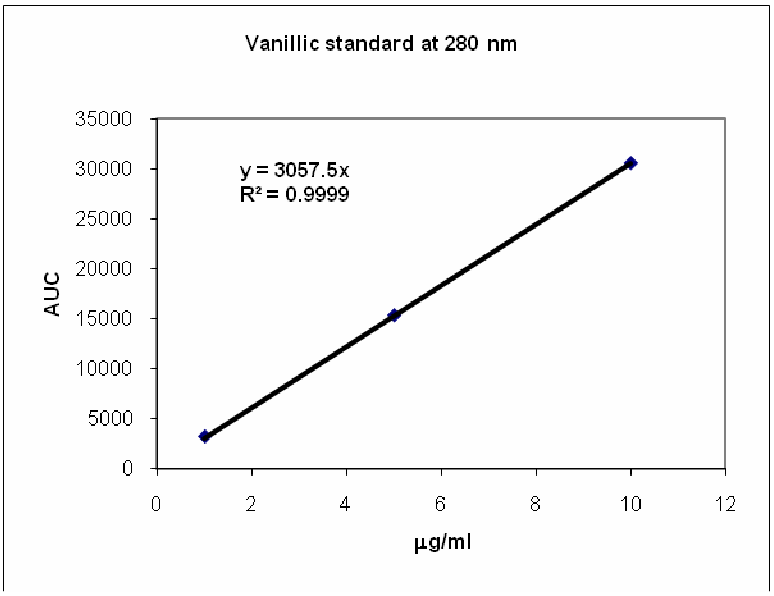

Protocatechuic standard at 280 nm
y = 2660.5x
R² = 0.9999
AUC
µg/ml
0
5000
10000
15000
20000
25000
30000
0
2
4
6
8
10
12


Quercetin standard at 325 nm
y = 3071.2x
R² = 0.9959
AUC
µg/ml
0
5000
10000
15000
20000
25000
30000
35000
0
2
4
6
8
10
12


Rutin standard at 325 nm
y = 2033.1x
R² = 0.9999
AUC
µg/ml
0
5000
10000
15000
20000
25000
0
2
4
6
8
10
12


Sinapic standard at 280 nm
y = 2605x
R² = 0.9987
AUC
µg/ml
0
5000
10000
15000
20000
25000
30000
0
2
4
6
8
10
12


Syringic standard at 280 nm
y = 5224.3x
R² = 1
AUC
µg/ml
0
10000
20000
30000
40000
50000
60000
0
2
4
6
8
10
12


Vanillic standard at 280 nm
y = 3057.5x
R² = 0.9999
AUC
µg/ml
35000
30000
25000
20000
15000
10000
5000
0
0
2
4
6
8
10
12

Appendix 7. Calibration Curve used to Assess AOA Using the Free Radical DPPH via Cuvette and Microplate Methods