Effects of genotype and environment variation on wheat secondary metabolites and human health properties

By

Maryam Shamloo

A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

Department of Food and Human Nutritional Sciences

University of Manitoba

Winnipeg

ABSTRACT

Regular daily intakes of whole grain products are associated with reduced risk of several diseases including type 2 diabetes. The health benefits of whole grains are linked to the existence of secondary bioactive metabolites including phenolic acids (PAs), flavonoids, and phytosterols. The production of these secondary metabolites in plants and the associated health properties is strongly dependent on the plant's genetic and agro-climate environmental variations. Temperature as an abiotic factor has a powerful effect on a plant's response to produce the secondary metabolites. Yet, how global warming influences wheat and other major crops' secondary metabolite profiles and the associated health-promoting benefits remain unknown. The objective of this study was to investigate the genotypic and environmental factors that affect the production of wheat secondary metabolites and their associated health benefits. In the first phase of the present study, wheat varieties, representing different commercial classes, were grown at different geographical locations over two consecutive crop years. Genotypes and environment variations resulted in significant changes in wheat secondary metabolites' levels and composition. Additionally, the impact of genotype, growth year and location variations on physiologic relevance was studied via investigating the ability of wheat's phenolic acids to inhibit glucose uptake in a cell model of small intestinal transport. The degree of inhibition was defined by wheat genotype and growing environment conditions. In the second phase, we investigated if different growing temperatures could alter the accumulation of these secondary metabolites in different wheat varieties. Increased levels of phenolic acids and flavonoids were observed for wheat grains grown at higher temperatures. Extracts of wheat phenolics inhibited glucose accumulation in the CaCo-2 model of intestinal uptake, and this effect positively correlated with the phenolic contents and growth temperatures. The results offer a possible

mechanism on how the addition of whole wheat products in the human diet improves postprandial glycemic responses through blunted glucose absorption.

ACKNOWLEDGEMENTS

There are several people to whom I would like to express my appreciation for their support and inspiration throughout my PhD program. I am sincerely honored to have had Dr. Peter J.H. Jones and Dr. Peter K. Eck as my co-supervisors. Throughout the duration of my doctoral journey at the University of Manitoba's Richardson Centre for Functional Foods and Nutraceuticals, my supervisory team provided me with excellent supervision, outstanding support, exemplary encouragement and exceptional opportunities. Dr. Jones, your crucial supervision, and expertise have always guided me since I began this PhD journey and I truly thank you for always believing in me, and for teaching me how to be a better scientist. Dr. Eck, I have learned more from you than I ever could have imagined. You taught me how to think outside the box, and how to be stronger. I have learned and benefited so much from your leadership, friendship, and exemplary support during my whole academic adventure. It has been an incredible journey and I truly thank you! I would also like to extend my appreciation to my committee members, Dr. James House, for your remarkable mentorship and advise, and Dr. Susan Arntfield, for your valuable knowledge and directions and insightful support. I would like to sincerely thank my external examiner, Dr. Zohar Kerem for agreeing to review and evaluate this thesis.

I would also like to thank Drs. Susan Arntfield and Nancy Ames, for their assistance with finding the wheat samples through Cereal Research Centre, Canadian Wheat Board and Agriculture and Agri-Food Canada, and the Agri-Food Canada research' team particularly; Tracy Exley, Kim Kuzminski and Camille Rhymer for their technical assistance. I also would like to thank Dr. Robert Henry, Dr. Agnelo Furtado from the University of Queensland, for providing the Australian wheat varieties and for their kind advice on the design of the growth chamber

experiment, Dr. Rale Gjuric, Dr. Debbie Jones, Jill Lockery for their assistance with growing and harvesting the wheat grains, Dr. Parimalan Rangan and Dennis Labossiere, for their technical assistance. I would also like to acknowledge the financial support provided to me by the NSERC Food Advancement through Science and Training (FAST) scholarship and in part to my PhD studies by Manitoba Science and Technology International Collaboration Fund (STIC), Mitacs Accelerate Graduate Research Internship Program, and Haplotech Inc. Thank you to the staff and faculty of the Department of Food and Human Nutritional Sciences at the University of Manitoba, specifically Mrs. Jennifer Palichuk, Mrs. Jennifer McLaren and Mrs. Janelle Ritchot. Lastly, no words can described how special and blessed I feel to have been able to meet wonderful people and lab-colleagues during my PhD journey. Sara, Behzad, Siavash, Rojiar, Farnaz, Navid, Mandana, Jyoti, Dylan, Rebecca, Jennifer, John, Yanan, Mohammad, Lovemore, Shatha, Sujith, Alie, Yongbo, and Nooshin, thank you all for your supports and for being such a beautiful part of this amazing journey. Above all, this academic journey, and life, could not have been possible without the never-ending love and continuous encouragement, support, and prayers, from my beloved husband, mother, father and sister, and the rest of my family members back home. I am very thankful to all of you and I love you all more than anything.

DEDICATIONS

I dedicate this work to my family.

To my husband and best friend **Babak**.

To my daughter **Isla**.

To my mother **Zahra** and my father **Mohammadbagher**.

To my sister **Mahsa**.

To my mother-in-law **Mehrangiz**.

And, to my late father-in-law **Hamzehali.**

I would not have been able to do this without your continuous love and undying support.

FOREWORD

This thesis is written in manuscript style and is composed of seven chapters and five manuscripts. The first chapter includes "Overall Introduction" followed by manuscript 1, which will explain the existing body of evidence surrounding the influence of genotype and environment on wheat secondary phytochemicals; phenolic acids and flavonoids. Research manuscripts, addressing specific objectives, will be presented in the following chapters 3 to 6. A one-page transition bridge statement links the chapters at the end of each manuscript. The final chapter discusses an "Overall Conclusion" of the thesis with concluding remarks of the work, its limitations, and proposed future directions of the research project.

List of Authors of each manuscript

- Manuscript 1 (Under revision: Journal of Cereal Science)

  Maryam Shamloo[a,c], Peter K Eck[d]*, and Peter JH Jones[a,c]*

- Manuscript 2 (Under review: Journal of Agriculture and Food Chemistry)

  Maryam Shamloo[a,c], Peter K Eck[d]*, and Peter JH Jones[a,c]*

- Manuscript 3 (Accepted: Journal of Nutrition and Metabolism)

  Maryam Shamloo[a,c], Peter K Eck[d]*, and Peter JH Jones[a,c]

- Manuscript 4 (Published: Scientific Reports 7, 9133, (2017))

  Maryam Shamloo[a,c], Elizabeth A. Babawale[a], Agnelo Furtado[d], Robert J. Henry[d], Peter K. Eck[b] and Peter J. H. Jones[a,c]*

- Manuscript 5 (Submitted: Global Cell Biology)

Maryam Shamloo[a,c], Peter K Eck[d]*, and Peter JH Jones[a,c]

Affiliations: [a]Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB R3T 2N2, Canada. [b]Department of Human Nutritional Sciences, W569 Duff Roblin Building, 190 Dysart Road, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada. [c]Department of Food Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada. [d]Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia QLD 4072, Australia.

Corresponding authors for Manuscripts 1 & 2: Peter JH Jones, Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB R3T 6C5, Canada Phone: +1(204) 474 8883, E-mail: Peter.Jones@umanitoba.ca and Peter K Eck, Department of Human Nutritional Sciences, W569 Duff Roblin Building, 190 Dysart Road, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada. Tel: +1(204) 291 2917, E-mail: Peter.Eck@umanitoba.ca

Corresponding author for Manuscripts 4: Peter JH Jones, Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB R3T 6C5, Canada Phone: +1(204) 474 8883, E-mail: Peter.Jones@umanitoba.ca

Corresponding author for Manuscripts 3 and 5: Peter K Eck, Department of Human Nutritional Sciences, W569 Duff Roblin Building, 190 Dysart Road, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada. Tel: +1(204) 291 2917, E-mail: Peter.Eck@umanitoba.ca

List of financial sources for all the manuscripts: Maryam Shamloo: Food Advancement through Science and Training (FAST) program (NSERC-FAST-PhD fellowship). Peter Jones and Peter Eck: Manitoba Science and Technology International Collaboration Fund *(*STIC) and Peter Jones: Mitacs Accelerate Graduate Research Internship Program.

TABLE OF CONTENTS

APENDICES

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AB	Alberta
ANOVA	Analysis of variance
BSTFA	Bis(trimethylsilyl) trifluoroacetamide
C3H	P-coumarate 3-hydroxylase
Caco-2	Heterogeneous human epithelial colorectal adenocarcinoma Cells
CHD	Coronary heart disease
CV	Coefficient of variation
DHC	Dihydrocholesterol
DF	Degrees of freedom
DM	Dry matter
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
E	Environment
FBS	Fetal bovine serum
FID	Flame ionization detection
G	Genotype
GC	Gas chromatography
G×E	Genotype × environment Interaction
G×Y	Genotype × year interaction
G×Y×E	Genotype × year × environment interaction
GWAS	Genome-wide association study
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography

IPCC	Intergovernmental Panel on Climate Change
LDL	Low-density lipoprotein
M	Molar
MB	Manitoba
MetS	Metabolomic syndrome
µg	Microgram
µg rutin eq	Microgram rutin equivalent
MUFA	Monosaturated fatty acids
N	Nitrogen
PA	Phenolic acid
PAL2	Phenylalanine ammonia-lyase
PBS	Phosphate buffer saline
PDA	Photodiode array detector
PPF	Photosynthetic photon flux
PUFA	Poly saturated fatty acids
QTL	Quantitative trait loci
ROS	Reactive oxygen species
SD	Standard deviations
SDS	Sodium dodecyl sulfate
SFA	Saturated fatty acids
SPE	Solid phase extraction
SK	Saskatchewan
T2D	Type 2 diabetes
TFC	Total flavonoid content
TMS	Trimethylsilyl

TMCS	Trimethylchlorosilane
UFA	Unsaturated fatty acids
USDA	United States department of agriculture
UV	Ultraviolet
WG	Whole grain
Y×E	Year × environment interaction

CHAPTER 1

OVERALL INRTRODUCTION

1.1 INTRODUCTION

Plant secondary metabolites useful in maintaining the health of both animals and people are usually synthesized in response to environmental factors such as temperature, humidity, light intensity, the supply of water, CO_2 levels, minerals and microbial or insect attack [1]. These factors will change as a function of growing locations and climate. The ability of plants to adapt to this environmental dynamism is governed by genes that determine the degree of responsiveness to environmental stress [2,3]. Genotypic variation between and within the plant species rules the production of secondary metabolites. Therefore, these metabolite content and levels will vary based on plant genotypes (G), growing environment (E) and their interaction (G*E) [4,5]. Phenolic compounds represent one important group of the secondary metabolites and they are characterized by at least one aromatic ring with one or more hydroxyl groups attached [6]. Phenolic acids, flavonoids, lignans, and stilbenes are the most important polyphenols found in any plant and play a significant role in the defense mechanism against abiotic stresses [7]. For instance; polyphenols play major roles in abiotic stress responses associated with heat tolerance [8,9], and higher amounts of these compounds were reported in different plants subjected to the increased temperature [8,10-12].

Polyphenol-rich foods such as fruits, vegetables, and whole grains are known to be beneficial to human health [13]. Grains as important staple foods are consumed around the world and in all dietary guidelines, they include the fundamental key element of daily intake [14]. Staple foods are

those that are eaten regularly and constitute the dominant part of a diet. The most recent United States Department of Agriculture (USDA) food pattern recommends choosing 100 percent whole grain (WG) instead of refined products for at least half of all grain consumed to align with a healthy eating pattern [15]. Wheat is globally a critically important crop, and figures among the three most produced cereal grains in the world, along with corn and rice [16]. Epidemiological and clinical research associates WG wheat polyphenols with several health benefits including but not limited to reduced risk of type 2 diabetes, cardiovascular diseases, obesity, and metabolomic syndrome (MetS) [17-19] . The WG wheat consumption reduced inflammation in overweight and obese subjects with unhealthy dietary and lifestyle behaviors [17]. Antioxidant or anti-inflammatory mechanisms have been proposed for the health benefits of WG wheat polyphenols [20], however, additional mechanisms of action regarding these health-promoting agents are emerging.

On the other hand, IPCC's fourth assessment predicted that the global temperature will increase by 1.8 to 4.0°C by 2100 [21]. Evidence is mounting that climate change has a profound impact on plant's secondary metabolites [9,22-24], including wheat phytochemicals. An enhanced level of polyphenols as a response to increased growing temperature has been reported for sugarcane [8], strawberry [25], sorghum [26], lettuce [11], and tomatoes [27]. Yet, how global warming influences wheat and other major crops' secondary metabolite profiles and the associated health properties remains unknown.

In this research project, we speculated that variation in genotypes and growing environmental conditions of wheat grains would alter the wheat secondary metabolites and associated health

benefits. The results will address shortfalls in better understanding of how levels of health-promoting wheat bioactives are influenced by environment and genetic changes.

1.2 RATIONALE

The role of genotypes and climate change on the wheat grains response to produce secondary metabolites, including phenolic acids, flavonoid, fatty acids and plant sterols, is a neglected area of research. There have been no controlled experimental attempts to correlate the variations in genotypic and environmental factors with changes in wheat secondary metabolites and their impact on health outcomes. This particular aspect of health properties of wheat grains grown in different conditions, therefore, merits additional study, to take advantage of altered wheat grain's nutritional values.

To investigate the hypothesis that genotype and environmental changes affect content and composition of secondary bioactive compounds in wheat grains and their health beneficial properties, we firstly examined whether different wheat genotypes and/or growing temperatures could alter the accumulation of secondary metabolites in wheat grains differing in their genetic background. Secondly we studied if the wheat's polyphenols inhibit glucose uptake in a CaCo-2 model of intestinal absorption, to take advantage of their health properties impact on the glycemic index.

1.3 OBJECTIVES

The present research has 4 specific objectives:

1. To explore the effects of genotype, growth year and location on accumulation of secondary metabolites in Canadian wheat varieties.
2. To evaluate how genotype, growth year and location variations translate into physiologic relevance by investigating cellular glucose transporter inhibitions.
3. To investigate whether different growing temperatures could alter production of secondary metabolites in different wheat grains grown in controlled environments.
4. To determine glucose inhibitory effect of polyphenols extracts of different wheat grains grown in controlled environments.

1.4 HYPOTHESES

The hypotheses to be tested include:

1. Genotypic variation of wheat grains affects contents and compositions of secondary metabolites.
2. Growing environmental factors such as location and year influence the levels of secondary metabolites of wheat.
3. Polyphenols extracts of wheat inhibit glucose uptake in a CaCo-2 model of intestinal absorption.
4. Genotypic variation and growing environmental factors alter the degree of inhibition of glucose uptake of polyphenols extracts of wheat.

5. Elevated growth temperatures shift the production of secondary metabolites in wheat grains.
6. Elevated growth temperatures alter the ability of wheat phenolics to inhibit cellular glucose uptake.

1.5 REFERENCES

1 Ramakrishna, A. & Ravishankar, G. A. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior* **6**, 1720-1731, doi:10.4161/psb.6.11.17613 (2011).

2 Palmieri, L. *et al.* Genotype-by-environment effect on bioactive compounds in strawberry (Fragaria x ananassa Duch.). *Journal of the Science of Food and Agriculture* **97**, 4180-4189, doi:10.1002/jsfa.8290 (2017).

3 Bradshaw, A. D. Unravelling phenotypic plasticity – why should we bother? *New Phytologist* **170**, 644-648, doi:10.1111/j.1469-8137.2006.01761.x (2006).

4 Baye, T. M., Abebe, T. & Wilke, R. A. Genotype–environment interactions and their translational implications. *Personalized medicine* **8**, 59-70, doi:10.2217/pme.10.75 (2011).

5 Shamloo, M. *et al.* Effects of genotype and temperature on accumulation of plant secondary metabolites in Canadian and Australian wheat grown under controlled environments. **7**, 9133, doi:10.1038/s41598-017-09681-5 (2017).

6 Crozier, A., Clifford, M. & Ashihara, H. *Plant secondary metabolites: occurrence, structure and role in human diet*. 14-21 (Blackwell Publishing Ltd, 2006).

7 Seigler, D. *Plant Secondary Metabolism. Boston, MA, 1998; 711.*, 711 (Chapman and Hall (Kluwer Academic Publishers), 1998).

8 Wahid, A. Physiological implications of metabolite biosynthesis for net assimilation and heat-stress tolerance of sugarcane (Saccharum officinarum) sprouts. *Journal of plant research* **120**, 219-228, doi:10.1007/s10265-006-0040-5 (2007).

9 Bita, C. E. & Gerats, T. Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. *Frontiers in Plant Science* **4**, 273, doi:10.3389/fpls.2013.00273 (2013).

10 Wang, S. Y. & Zheng, W. Effect of plant growth temperature on antioxidant capacity in strawberry. *Journal of agricultural and food chemistry* **49**, 4977-4982, doi:10.1021/jf0106244 (2001).

11 Jeong, S. W. *et al.* The effects of different night-time temperatures and cultivation durations on the polyphenolic contents of lettuce: Application of principal component analysis. *Journal of Advanced Research* **6**, 493-499, doi:http://dx.doi.org/10.1016/j.jare.2015.01.004 (2015).

12 Wu, G. *et al.* Growth temperature and genotype both play important roles in sorghum grain phenolic composition. *Scientific Reports* **6**, doi:10.1038/srep21835 (2016).

13 Jonnalagadda, S. S. *et al.* Putting the whole grain puzzle together: health benefits associated with whole grains—summary of American aociety for nutrition 2010 Satellite symposium. *The Journal of nutrition* **141**, 1011S-1022S, doi:10.3945/jn.110.132944 (2011).

14 Flight, I. & Clifton, P. Cereal grains and legumes in the prevention of coronary heart disease and stroke: a review of the literature. *Eur J Clin Nutr* **60**, 1145-1159 (2006).

15 U.S. Department of Health and Human Services and U.S. Department of Agriculture. 2015–2020 Dietary Guidelines for Americans. 8th Edition. December 2015. Available at http://health.gov/dietaryguidelines/2015/guidelines/.

16 Shiferaw, B. *et al.* Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security. *Food Security* **5**, 291-317, doi:10.1007/s12571-013-0263-y (2013).

17 Vitaglione, P. *et al.* Whole-grain wheat consumption reduces inflammation in a randomized controlled trial on overweight and obese subjects with unhealthy dietary and lifestyle behaviors: Role of polyphenols bound to cereal dietary fiber. *American Journal of Clinical Nutrition* **101**, 251-261, doi:10.3945/ajcn.114.088120 (2015).

18 Zhu, Y. & Sang, S. Phytochemicals in whole grain wheat and their health-promoting effects. *Molecular Nutrition and Food Research* **61**, doi:10.1002/mnfr.201600852 (2017).

19 Aune, D. *et al.* Whole grain consumption and risk of cardiovascular disease, cancer, and all cause and cause specific mortality: systematic review and dose-response meta-analysis of prospective studies. *Bmj* **353**, i2716, doi:10.1136/bmj.i2716 (2016).

20 Poudel R & M, B. Review of Nutraceuticals and Functional Properties of Whole Wheat. *J Nutr Food Sci* **7**, 571, doi:10.4172/2155-9600.1000571 (2016).

21 *IPCC. Contribution of Working Group I To The Fourth Assessment Report of The Intergovernmental Panel On Climate Change. Cambridge University Press, Cambridge, United Kingdom (2007).*

22 García-Mier, L., Guevara-González, R. G., Mondragón-Olguín, V. M., Verduzco-Cuellar, B. d. R. & Torres-Pacheco, I. Agriculture and bioactives: achieving both crop yield and phytochemicals. *International Journal of Molecular Sciences* **14**, 4203-4222, doi:10.3390/ijms14024203 (2013).

23 Gray, S. B. & Brady, S. M. Plant developmental responses to climate change. *Developmental Biology* **419**, 64-77, doi:https://doi.org/10.1016/j.ydbio.2016.07.023 (2016).

24 Ahmed, S. & Stepp, J. R. Beyond yields: Climate change effects on specialty crop quality and agroecological management. *Elementa* **2016**, doi:10.12952/journal.elementa.000092 (2016).

25 Wang, S. Y. & Zheng, W. Effect of plant growth temperature on antioxidant capacity in strawberry. *J Agric Food Chem* **49**, 4977-4982 (2001).

26 Wu, G. *et al.* Growth temperature and genotype both play important roles in sorghum grain phenolic composition. *Scientific reports* **6**, 21835, doi:10.1038/srep21835 (2016).

27 Klunklin, W. & Savage, G. Effect on Quality Characteristics of Tomatoes Grown Under Well-Watered and Drought Stress Conditions. *Foods (Basel, Switzerland)* **6**, doi:10.3390/foods6080056 (2017).

CHAPTER 2

MANUSCRIPT 1

This manuscript is submitted to the Journal of Cereal Science

WHEAT GENOTYPE AND GROWING ENVIRONMENT CONTRIBUTES TO PHENOLIC ACIDS VARIATIONS AND THEIR HEALTH BENEFICIAL PROPERTIES: A MINI-REVIEW

Maryam Shamloo[a,c], Peter JH Jones[a,c] , and Peter K Eck[b]*

Affiliations: [a]Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB R3T 2N2, Canada. [b]Department of Human Nutritional Sciences, W569 Duff Roblin Building, 190 Dysart Road, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada. [c]Department of Food Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada.

*Corresponding coauthor: Peter K Eck[b]

2.1 ABSTRACT

Many plants secondary metabolites present in foodstuffs modulate health in humans. The levels of these compounds are influenced by the plant species or variety and the environmental exposure. The main secondary metabolites in cereals include the phenolic acids and the flavonoids, which are found in the bran fraction. The health benefits of phenolic acids and the flavonoids are in large part attributed to their antioxidant properties.

Wheat is the major agricultural and dietary commodity worldwide. The present review summarizes the knowledge how genotype and environment interact to modulate the phenolic acids and the total flavonoids contents in wheat grains.

The derived information provides research leads in the modification and optimization of the phenolic acids and flavonoid contents of food products, using either conventional plant breeding or manipulation of agronomic practices. Given this background, there may be favorable consequences for the general health of cereal consuming populations as a result of optimizing the phenolic and flavonoid content of diets.

2.2 INTRODUCTION

Common wheat, consisting of the polyploid *Triticum aestivum L*. subsp. Aestivum (genome 2n = 6x = 42) and durum *Triticum turgidum L*. subsp. durum (genome AABB, 2n = 4x = 28) are grown globally and are major sources of dietary carbohydrates and proteins. Wheat also contains

secondary plant metabolites, such as sterols, tocopherols, folates, fibers, alkylresorcinols, and phenolic acids [1].

Wheat is the major food commodity in the world grain market [2]. Numerous epidemiological studies have associated whole wheat consumption with improved health outcomes [3-6]. These studies indicate that whole wheat may act as a suitable functional food or nutraceutical ingredient source as it contains several antioxidants, most notably phenolic compounds, that may protect against reactive oxygen species (ROS) involved in different diseases such as cancers [7], diabetes [3], and coronary heart disease [5].

The production of secondary plant metabolites is encoded in a plant species' genetic background [8]; however, biochemical changes as a reaction to environmental factors such as light, temperature, and geographical locations also influence the levels of secondary plant metabolites [9]. As a consequence, both genetic and environmental influences determine the accumulation of plant bioactives.

This paper reviews the knowledge how the genotype and environment determine the concentrations of polyphenols in wheat and elaborates on their antioxidants function in human health.

2.3 PHYTOCHEMICALS IN WHOLE GRAINS

Phytochemicals (from the Greek word *phyto,* meaning plant) are biologically active chemical compounds found in plants that, when ingested, provide functional benefits beyond basic nutrition [7]. Many phytochemicals have strong antioxidant activity which would protect plants tissues against destructive free radicals. Two main classes of phytochemicals in grains include two major sub-classes: a) phenolic acids and b) flavonoids.

2.3.1 PHENOLIC ACIDS

Phenolic acids are aromatic secondary plant metabolites that contain a phenolic ring and an organic carboxylic acid function (C6-C1 skeleton). There are two classes of phenolic acids: hydroxybenzoic acids and hydroxycinnamic acids [10] **(Figure 1)**. Hydroxybenzoic acids include gallic, p- hydroxybenzoic, vanillic, syringic, and protocatechuic acids. The hydroxycinnamic acids include ferulic, coumaric, caffeic, and sinapic acids [11].

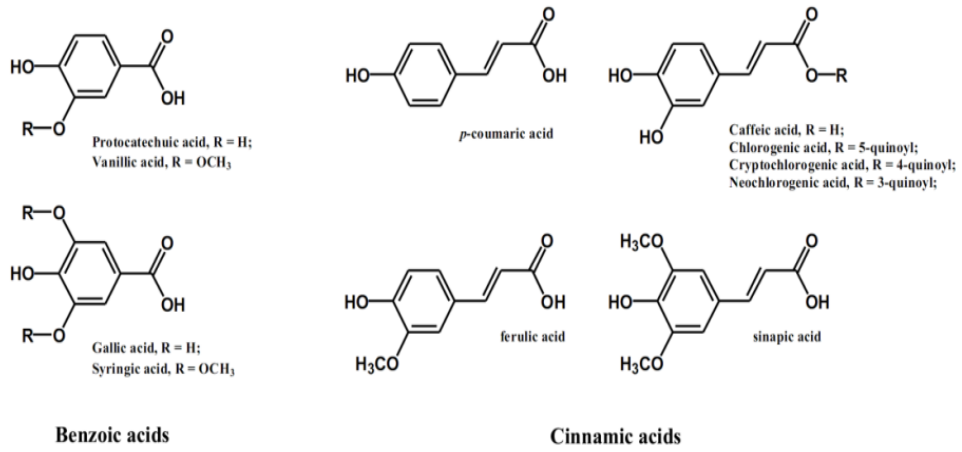


Figure 2.1. Structures of phenolic acids [12]

In grains, phenolic acids exist as free, soluble conjugates and insoluble bound forms [13], and are found as monomers, dimers, trimers, and tetramers. The free phenolic acids and the soluble conjugates can be extracted with organic solvents. The bound phenolic acids are esterified to the cell walls and therefore are not freely available. To extract them they require prior acid or base hydrolysis to release them from the cell matrix [14]. Phenolic acids are not equally distributed in the grain. The highest proportion is found in the outer layers, namely, the aleurone layer, testa, and pericarp, which form the main components in the bran fraction [15-17]. During milling, the endosperm is usually separated from the germ and the bran in order to obtain the white flour. Hence, a major proportion of phenolic compounds are lost in white flour during processing to remain in the bran.

2.3.2 FLAVONOIDS

Flavonoids are mostly compounds with a 15 (C6-C3-C6) carbon skeleton, which consists of two phenyl ring (A and B) and heterocyclic ring as shown in **Figure 2**. They have different distributions of hydroxyl groups (-OH) in their B ring. More than 10,000 flavonoids have been identified, which are divided into distinctive structural classes including flavonols (myricetin, kaempferol and quercetin), flavanones (pentahydroxyflavanone, naringenin and eriodictyol), flavanols (leucodelphinidin, leucopelargonidin and leucocyanidin), anthocyanins (delphinidin, pelargonidin and cyaniding), and isoflavones according to the chemical modifications of the central C-ring [18,19].

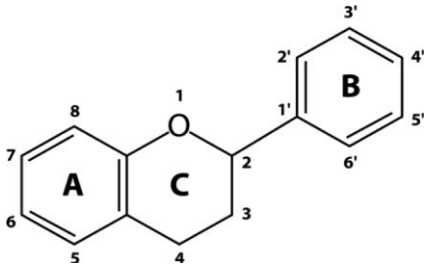


Figure 2.2. Basic chemical structure of flavonoids [20]

The basic structure of flavonoids is the aglycone; however, in plants, most of them exist as glycosides.

Flavonols, anthocyanins, and proanthocyanidins are the major types of flavonoids found in cereal grains [19,21]. Glycoside derivatives of flavonols and anthocyanins, including cyanidin-3-glucoside, penidin-3-glucoside, and delphinidin-3-glucoside, have been identified in pigmented cereal grains [22-24]. The biological activities of these compounds, including the antioxidant activity,

depend on the structure and the glycosylation patterns [25]. In cereal grains, the content of flavonoids is proportional to the degree of color and most are located in the pericarp, which contributes to grain pigmentation and protection against ROS[19].

2.4 DETERMINANTS OF THE PHENOLIC ACID CONTENT IN WHEAT

2.4.1 GENETIC DETERMINANTS OF PHENOLIC ACID CONTENT IN WHEAT

Variation in phenolic acid levels had been studied in wheat cultivars [1,26-33]. The total and individual phenolic acid levels are complex traits determined by the genotype and environmental factors [34]. While the levels of some secondary metabolites are mainly determined by the genotype (e.g. sterols), phenolic acids levels are highly variable depending on growing conditions [1]. Since the variance of individual genotypes at different locations is very high, the genotypic effects on phenolic acid concentrations are very hard to determine under field conditions. On the other hand, a recent study on tetraploid genotypes showed a higher ratio of genotypic variance to total variance for individual and total phenolic acids, emphasizing a stronger role of genetics compared to environmental factors [27].

Basic free phenolics acid concentrations of wheat were reported to range from 3 to 30 microgram per gram (μg/g) dry matter (dm), while bound phenolic acids ranged from 208 to 934 μg/g dm and total phenolic acids ranged from 326 to 1171 μg/g dm as previously reported in the HEALTHGRAIN Diversity Screen investigating 130 winter wheat genotypes, over 20 spring wheat varieties and 10 durum wheat varieties [35]. These ranges were in line with other reports

[32,33]. Mpofu et al, however, reported a higher range (1709 to 2009 μg/g) of total phenolic acids in six western Canadian wheat genotypes grown at four locations.

There is emerging evidence on the exact genetic determinants of phenolic acids in tetraploid wheat grains. Recently, a genome-wide association study (GWAS) genotyping a tetraploid wheat collection detected 22 quantitative trait loci (QTL) distributed on almost all durum wheat chromosomes [34]. Two QTL for p-coumaric acid covered the phenylalanine ammonia-lyase (PAL2) and p-coumarate 3-hydroxylase (C3H) genes on chromosome arms 2AL and 1AL, respectively. The seven candidate genes PAL1, PAL2, C4H, C3H, COMT1, and COMT2 were proposed to be determinants in the biosynthesis of hydroxycinnamic acid derivatives. However, current evidence does not allow a definite conclusion on all genetic factors determining phenolic acids levels in wheat grains.

2.4.2 ENVIRONMENTAL DETERMINANTS OF PHENOLIC ACID IN WHEAT

Mpofu *et al*. [36], determined the effects of genotype and growing environment on the phenolic acid contents of alcohol-soluble extracts from six red- and white-grained hard spring wheat genotypes grown at four diverse locations in Western Canada during the 2003 crop year [36]. Phenolic acids levels differed between all genotypes and environmental conditions [36]. Variations due to environmental factors were considerably larger than the genotypic determinants for vanillic acid, syringic acid and ferulic acid [36], and the genotype-by-environment interaction determined the total phenolic acid contents [36]. Menga *et al*. [37] corroborated these results in Italian

soft and durum wheat; in this study the growing location had the highest impact on phenolic acid contents.

Investigating the influence of environmental shifts versus genotypic variations, HEALTHGRAIN Diversity Study reported that free phenolic acids were largely susceptible to the environmental changes whereas bound PAs were less influenced by environmental shifts, and more by genotypic differences [38]. Later findings of Martini et al [39] confirmed those results showing that free phenolic acids in durum wheat appeared most influenced by growing conditions (ranged from 2.8 to 9.6 μg/g dm), whereas bound phenolic acids were most influenced by the genotype (ranged from 626.4 to 964.3 μg/g dm). Similarly, Sukalovic *et al*.[40], concluded that environmental factors generally had a larger impact on total phenolic content of durum and bread wheat genotypes than genetic factors.

Overall, contents of free and conjugated phenolics in wheat varieties were strongly influenced by the environment, while bound phenolics, which comprise the greatest proportion of total phenolic acids in wheat, are mostly stable across different growing conditions and are genetically determined.

This research confirms direct influences of genotype, environment and their interactions on the phenolic acids of wheat, suggesting the possibility of breeding wheat varieties with higher nutritional value matched to the growing conditions for a genotype. This would be an efficient way to maximize health benefits associated with wheat-based products.

2.5 HEALTH BENEFITS OF PHENOLIC ACIDS FROM WHEAT MIGHT BE RELATED TO BLOOD GLUCOSE CONTROL

Epidemiologic and clinical studies show whole grain consumption associated with reduced incidence of metabolic diseases including cardiovascular disease [41], type 2 diabetes [42], metabolic syndrome (MetS) [43] and some cancers [44]. The mechanisms of disease prevention and or treatment are still being discussed, but the proposed health benefits may be caused by phytochemicals including the phenolic compounds [36]. It had been proposed, that wheat phenols exhibit their beneficial effects through antioxidant or anti-inflammatory mechanisms [36], however, other mechanisms of action have emerged through recent research.

Polyphenols are substantially lost during the refining processing; for instance: 93% of ferulic acid and 79% of flavonoids are lost in refined products compared to whole grain products due to processing [45,46].

Phenolic acids in plasma can be increased through whole grain consumption. Specifically, dihydroferulic acid levels were increased after an 8-week intervention with whole grain products, demonstrating bioavailability of the major phenolic acid from wheat [47]. The whole grain wheat consumption reduced inflammation in overweight and obese subjects with unhealthy dietary and lifestyle behaviors [47]. These types of controlled feeding trials indicate that replacement of refined wheat products with whole grain wheat may reduce the risk of developing obesity-related inflammatory disease over the long period. This is further supported by a more recent intervention study, where effects on gut microbiota and improved immune and inflammatory markers in healthy adults had been reported after 6 weeks of whole wheat consumption[46].

Furthermore, replacing refined with whole grain wheat products modestly improved markers of MetS. Specifically, normalized blood glucose concentrations in pre-diabetes individuals has been reported [48]. There are even indications that genetically determined factors in wheat bioactives differentially affect health outcomes upon consumption. For example, a replacement diet with ancient Khorasan wheat improved risk profile of patients with type 2 diabetes (T2D) compared to a diet with products made with modern wheat [49]. Khorasan wheat has elevated protein levels and based on its color is expected to have elevated phenolic acid levels compared to modern commercial varieties [50]. It is therefore likely that the genetically determined phenolic acid levels enhance the positive health outcomes even further, which might be even more pronounced under environmental conditions favoring increased phenolic acid synthesis [8,51].

The notion that improved control of blood glucose levels, specifically postprandial hyperglycemia, can be related to inhibition of intestinal glucose transporters by phenolic compounds is currently emerging. For example, *in vitro* cell culture and animal studies established a proof of principle showing that some flavonoids inhibit glucose transport of intestinal membrane transporters, and also enhance the control of postprandial blood glucose levels in a diabetic rat model [52,53]. The same phenomenon was observed for extracts of phenolic acids from whole wheat [54], proving the possibility that elevated levels of phenolic acids in whole wheat have the potential to contribute to the control of diabetes and potential obesity.

2.6 CONCLUSIONS

The amounts of phenolics acids in wheat are highly affected by environmental conditions and their contents will vary by region and year. Rising global temperatures will cause rising levels of plant phenols in wheat, which could be used by food processors to improve the products health benefits. To capitalize on this, simple and economical methods for determining compositions of phenolic acids need to be established to monitor grain intake and facilitate product development.

2.7 REFERENCES

1 Shewry, P. R. *et al.* The HEALTHGRAIN Wheat Diversity Screen: Effects of Genotype and Environment on Phytochemicals and Dietary Fiber Components. *Journal of Agricultural and Food Chemistry* **58**, 9291-9298, doi:10.1021/jf100039b (2010).

2 FAO Statistical Pocketbook World Food and Agriculture 2015, available at http://www.fao.org/3/a-i4691e.pdf.

3 Montonen, J., Knekt, P., Jarvinen, R., Aromaa, A. & Reunanen, A. Whole-grain and fiber intake and the incidence of type 2 diabetes. *The American journal of clinical nutrition* **77**, 622-629 (2003).

4 Venn, B. J. & Mann, J. I. Cereal grains, legumes and diabetes. *European Journal of Clinical Nutrition* **58**, 1443-1461, doi:10.1038/sj.ejcn.1601995 (2004).

5 Slavin, J., Jacobs, D. & Marquart, L. Whole-grain consumption and chronic disease: Protective mechanisms. *Nutrition and Cancer* **27**, 14-21, doi:10.1080/01635589709514495 (1997).

6 Behall, K. M., Scholfield, D. J. & Hallfrisch, J. Whole-Grain Diets Reduce Blood Pressure in Mildly Hypercholesterolemic Men and Women. *Journal of the American Dietetic Association* **106**, 1445-1449, doi:http://dx.doi.org/10.1016/j.jada.2006.06.010 (2006).

7 Liu, R. H. Whole grain phytochemicals and health. *Journal of Cereal Science* **46**, 207-219, doi:http://dx.doi.org/10.1016/j.jcs.2007.06.010 (2007).

8 Ramakrishna, A. & Ravishankar, G. A. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior* **6**, 1720-1731, doi:10.4161/psb.6.11.17613 (2011).

9 Pek, Z., Daood, H., Nagyne, M., Nemenyi, A. & Helyes, L. Effect of environmental conditions and water status on the bioactive compounds of broccoli. *Central European Journal of Biology* **8**, 777-787, doi:10.2478/s11535-013-0172-7 (2013).

10 Pereira, D., Valentão, P., Pereira, J. & Andrade, P. Phenolics: From Chemistry to Biology. *Molecules* **14**, 2202-2211 (2009).

11 Robbins, R. J. Phenolic acids in foods: an overview of analytical methodology. *J Agric Food Chem* **51**, 2866-2887, doi:10.1021/jf026182t (2003).

12 Tsao, R. Chemistry and Biochemistry of Dietary Polyphenols. *Nutrients*, 1231-1246, doi:10.3390/nu2121231 (2010).

13 Hatfield, R. D., Ralph, J. & Grabber, J. H. Cell wall cross-linking by ferulates and diferulates in grasses. *Journal of the Science of Food and Agriculture* **79**, 403-407 (1999).

14 Abdel-Aal, E. S. M., Hucl, P., Patterson, C. A. & Gray, D. Phytochemicals and heavy metals content of hairless canary seed: A variety developed for food use. *LWT - Food Science and Technology* **44**, 904-910 (2011).

15 Mattila, P., Pihlava, J. M. & Hellstrom, J. Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. *Journal of Agricultural and Food Chemistry* **53**, 8290-8295, doi:10.1021/jf051437z (2005).

16 Barberousse, H. *et al.* Analytical methodologies for quantification of ferulic acid and its oligomers. *Journal of the Science of Food and Agriculture* **88**, 1494-1511, doi:10.1002/jsfa.3242 (2008).

17 Naczk, M. & Shahidi, F. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis* **41**, 1523-1542, doi:10.1016/j.jpba.2006.04.002 (2006).

18 Orlikova, B., Tasdemir, D., Golais, F., Dicato, M. & Diederich, M. Dietary chalcones with chemopreventive and chemotherapeutic potential. *Genes and Nutrition* **6**, 125-147, doi:10.1007/s12263-011-0210-5 (2011).

19 Liu, Z. *et al.* Regulation, evolution, and functionality of flavonoids in cereal crops. *Biotechnol Lett* **35**, 1765-1780, doi:10.1007/s10529-013-1277-4 (2013).

20 Jia, L. & Liu, F.-T. Why bortezomib cannot go with 'green'? *Cancer Biology & Medicine* **10**, 206-213, doi:10.7497/j.issn.2095-3941.2013.04.004 (2013).

21 Falcone Ferreyra, M. L. *et al.* Evolution and expression of tandem duplicated maize flavonol synthase genes. *Front Plant Sci* **3**, 101, doi:10.3389/fpls.2012.00101 (2012).

22 Abdel-Aal el, S. M., Young, J. C. & Rabalski, I. Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *J Agric Food Chem* **54**, 4696-4704, doi:10.1021/jf0606609 (2006).

23 Khlestkina, E., Tereshchenko, O. & Salina, E. Anthocyanin biosynthesis genes location and expression in wheat–rye hybrids. *Molecular Genetics and Genomics* **282**, 475-485, doi:10.1007/s00438-009-0479-x (2009).

24 Zilic, S., Serpen, A., Akillioglu, G., Gokmen, V. & Vancetovic, J. Phenolic compounds, carotenoids, anthocyanins, and antioxidant capacity of colored maize (Zea mays L.) kernels. *J Agric Food Chem* **60**, 1224-1231, doi:10.1021/jf204367z (2012).

25 Tsao, R. Chemistry and Biochemistry of Dietary Polyphenols. *Nutrients* **2**, 1231-1246, doi:10.3390/nu2121231 (2010).

26 Gawlik-Dziki, U., Świeca, M. & Dziki, D. Comparison of phenolic acids profile and antioxidant potential of six varieties of spelt (Triticum spelta L.). *Journal of Agricultural and Food Chemistry* **60**, 4603-4612, doi:10.1021/jf3011239 (2012).

27 Laddomada, B. *et al.* Genetic variation for phenolic acids concentration and composition in a tetraploid wheat (Triticum turgidum L.) collection. *Genetic Resources and Crop Evolution* **64**, 587-597, doi:10.1007/s10722-016-0386-z (2017).

28 Li, L., Shewry, P. R. & Ward, J. L. Phenolic acids in wheat varieties in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry* **56**, 9732-9739, doi:10.1021/jf801069s (2008).

29 Narwal, S. *et al.* Antioxidant activity and phenolic content of the Indian wheat varieties. *Journal of Plant Biochemistry and Biotechnology* **23**, 11-17, doi:10.1007/s13562-012-0179-1 (2014).

30 Pasqualone, A., Delvecchio, L. N., Mangini, G., Taranto, F. & Blanco, A. Variability of total soluble phenolic compounds and antioxidant activity in a collection of tetraploid wheat. *Agricultural and Food Science*, 307-316%V 323 (2014).

31 Vaher, M., Matso, K., Levandi, T., Helmja, K. & Kaljurand, M. Phenolic compounds and the antioxidant activity of the bran, flour and whole grain of different wheat varieties. *Procedia Chemistry* **2**, 76-82, doi:https://doi.org/10.1016/j.proche.2009.12.013 (2010).

32 Verma, B., Hucl, P. & Chibbar, R. N. Phenolic content and antioxidant properties of bran in 51 wheat cultivars. *Cereal Chemistry Journal* **85**, 544-549, doi:10.1094/CCHEM-85-4-0544 (2008).

33 Kurasiak-Popowska, D., Stuper-Szablewska, K., Nawracala, J., Tomkowiak, A. & Perkowski, J. Phenolic acid content in wheat grain *(triticum spp)* of different genotypes. *Revista de la Facultad de Ciencias Agrarias* **48**, 1-7 (2016).

34 Nigro, D. *et al.* Genome-wide association mapping of phenolic acids in tetraploid wheats. *Journal of Cereal Science* **75**, 25-34, doi:https://doi.org/10.1016/j.jcs.2017.01.022 (2017).

35 Li, L., Shewry, P. R. & Ward, J. L. Phenolic acids in wheat varieties in the HEALTHGRAIN Diversity Screen. *J Agric Food Chem* **56**, 9732-9739, doi:10.1021/jf801069s (2008).

36 Mpofu, A., Sapirstein, H. D. & Beta, T. Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. *J Agric Food Chem* **54**, 1265-1270, doi:10.1021/jf052683d (2006).

37 Menga, V., Fares, C., Troccoli, A., Cattivelli, L. & Baiano, A. Effects of genotype, location and baking on the phenolic content and some antioxidant properties of cereal species. *International Journal of Food Science & Technology* **45**, 7-16, doi:10.1111/j.1365-2621.2009.02072.x (2010).

38 Fernandez-Orozco, R., Li, L., Harflett, C., Shewry, P. R. & Ward, J. L. Effects of environment and genotype on phenolic acids in wheat in the HEALTHGRAIN diversity screen. *J Agric Food Chem* **58**, 9341-9352, doi:10.1021/jf102017s (2010).

39 Martini, D. *et al.* Effects of Genotype and Environment on Phenolic Acids Content and Total Antioxidant Capacity in Durum Wheat. *Cereal Chemistry Journal* **91**, 310-317, doi:10.1094/CCHEM-09-13-0201-CESI (2014).

40 Sukalovic, V. H.-T. *et al.* Genotypic and Environmental Variation of Bread and Durum Wheat Proteins and Antioxidant Compounds *Romanian Agricultural Research* **30**, 125-134 (2013).

41 Jacobs, D. R., Jr., Meyer, K. A., Kushi, L. H. & Folsom, A. R. Whole-grain intake may reduce the risk of ischemic heart disease death in postmenopausal women: the Iowa Women's Health Study. *The American journal of clinical nutrition* **68**, 248-257 (1998).

42 Liu, S. *et al.* A prospective study of whole-grain intake and risk of type 2 diabetes mellitus in US women. *American journal of public health* **90**, 1409-1415 (2000).

43 Sahyoun, N. R., Jacques, P. F., Zhang, X. L., Juan, W. & McKeown, N. M. Whole-grain intake is inversely associated with the metabolic syndrome and mortality in older adults. *The American journal of clinical nutrition* **83**, 124-131 (2006).

44 Jonnalagadda, S. S. *et al.* Putting the whole grain puzzle together: health benefits associated with whole grains--summary of American Society for Nutrition 2010 Satellite Symposium. *The Journal of nutrition* **141**, 1011s-1022s, doi:10.3945/jn.110.132944 (2011).

45 Adom, K. K., Sorrells, M. E. & Liu, R. H. Phytochemical profiles and antioxidant activity of wheat varieties. *J Agric Food Chem* **51**, 7825-7834, doi:10.1021/jf030404l (2003).

46 Vanegas, S. M. *et al.* Substituting whole grains for refined grains in a 6-wk randomized trial has a modest effect on gut microbiota and immune and inflammatory markers of healthy adults. *The American journal of clinical nutrition* **105**, 635-650, doi:10.3945/ajcn.116.146928 (2017).

47 Vitaglione, P. *et al.* Whole-grain wheat consumption reduces inflammation in a randomized controlled trial on overweight and obese subjects with unhealthy dietary and lifestyle behaviors: role of polyphenols bound to cereal dietary fiber. *The American journal of clinical nutrition*, doi:10.3945/ajcn.114.088120 (2015).

48 Harris Jackson, K. *et al.* Effects of whole and refined grains in a weight-loss diet on markers of metabolic syndrome in individuals with increased waist circumference: a randomized controlled-feeding trial. *The American journal of clinical nutrition*, doi:10.3945/ajcn.113.078048 (2014).

49 Whittaker, A. *et al.* A khorasan wheat-based replacement diet improves risk profile of patients with type 2 diabetes mellitus (T2DM): a randomized crossover trial. *European journal of nutrition* **56**, 1191-1200, doi:10.1007/s00394-016-1168-2 (2017).

50 Abdel-Haleem, M. A., Seleem, A. S. & Galal, K. W. Assessment of Kamut® wheat quality. *World Journal of Science, Technology and Sustainable Development* **9**, 194-203, doi:doi:10.1108/20425941211250543 (2012).

51 Belobrajdic, D. P. & Bird, A. R. The potential role of phytochemicals in wholegrain cereals for the prevention of type-2 diabetes. *Nutrition Journal* **12**, 62, doi:10.1186/1475-2891-12-62 (2013).

52 Kwon, O. *et al.* Inhibition of the intestinal glucose transporter GLUT2 by flavonoids. *Faseb Journal* **21**, 366-377, doi:10.1096/fj.06-6620com (2007).

53 Song, J. *et al.* Flavonoid inhibition of sodium-dependent vitamin C transporter 1 (SVCT1) and glucose transporter isoform 2 (GLUT2), intestinal transporters for vitamin C and Glucose. *J Biol Chem* **277**, 15252-15260, doi:10.1074/jbc.M110496200 (2002).

54 Malunga, L. N., Eck, P. & Beta, T. Inhibition of intestinal α-glucosidase and glucose absorption by feruloylated arabinoxylan mono- and oligosaccharides from corn bran and wheat aleurone. *Journal of Nutrition and Metabolism* **2016**, 9, doi:10.1155/2016/1932532 (2016).

TRANSITION STATEMENT 1

Whole grains foods include a wide range of secondary metabolites with several health benefits. Composition and levels of these secondary metabolites vary within and among the major cereal crops. Genotypic and environmental differences rule those variations. Limited information on how environment and genotypes influence the production of secondary metabolites in wheat grains was been previously reported, however, this area of research needs further investigation.

In the following manuscript, we determined how levels of four main classes of wheat secondary metabolites, including phenolic acids, flavonoids, plant sterols, and fatty acids are influenced by environment and genetic changes. The results would create a valuable foundation for wheat breeders to select and grow wheat grains containing higher amount of health-promoting secondary metabolites, which will then increase the intakes of these compounds by consuming natural whole wheat products.

CHAPTER 3

MANUSCRIPT 2

This manuscript is submitted to the Journal of Agricultural and Food Chemistry

EFFECTS OF GENOTYPE AND ENVIRONMENT ON SECONDARY METABOLITES OF CANADIAN WHEAT GRAINS

Maryam Shamloo[a,c], Peter K Eck[b]*, and Peter JH Jones[a,c]*

Affiliations: [a]Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB R3T 2N2, Canada. [b]Department of Human Nutritional Sciences, W569 Duff Roblin Building, 190 Dysart Road, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada. [c]Department of Food Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada.

*Corresponding coauthors: Peter JH Jones[a,c] and Peter K Eck[b]

3.1 ABSTRACT

Health benefits of regular consumption of whole-wheat products are attributed to the existence of secondary bioactive metabolites including phenolic acids (PAs), flavonoids, and phytosterols. The production of above-mentioned secondary wheat metabolites is strongly dependent on plant's genetic and environmental variations; however, information on how these factors influence secondary wheat metabolites including flavonoids, plant sterols, and fatty acids is lacking. Thus, the objective of this study was to explore the effects of genotype, growth year and environment on the production of secondary metabolites in Canadian wheat varieties. Eight Canadian wheat varieties (*Triticum spp*), including AC Corrine, AC Barrie, AC Crystal & Carberry, Snowbird, AC Andrew, AC Navigator & Strongfield durum wheat grains were grown at three locations over two consecutive crop years. Selected wheat varieties represent different commercial classes with different qualities. Four categories of bioactives, including phenolic acids, flavonoids, fatty acids and plant sterols were extracted separately and analyzed. Significant differences in total, free and bound phenolic acids (PA) based on wheat varieties were observed. Growing environment (i.e. location) had a larger influence on total and bound PA than genotype variabilities. A strong negative correlation was observed between the total precipitation and levels of total PA. Wheat plants responded differently to temperature, total precipitation, and altitude in regard to flavonoid production. Temperature had the largest effect on total flavonoid content (TFC) compared to total precipitation and altitude variation. The increased growing temperature resulted in a significantly higher amount of TFC in each crop year. In regard to fatty acids, linoleic acid (C18:2n6) was identified as the main fatty acid in all wheat varieties grown at different locations over two crop years. Genotype variations had the largest influence on levels of linoleic acid. Significant variation was observed in total plant sterol

contents among wheat varieties. Overall, red spring wheat varieties contained the higher amount of plant sterols than white spring wheat. The temperature had the largest effect on plant sterol levels compared to total precipitation and altitude variation. In the present study, AC Navigator, AC Crystal, and Carberry contained higher amounts of flavonoids and plant sterols within the Canadian wheat genotypes. Taken together, this knowledge creates a good foundation for breeding and provides a better understanding of how levels of health-promoting wheat bioactives are influenced by environment and genetic changes.

3.2 INTRODUCTION

Wheat is globally a critically important crop, grown on a greater land area than any other commercial cereal crop [1]. Regular consumption of whole-grain food products has been shown to be beneficial to humans, reducing the incidence of chronic diseases including cancers [2], diabetes [3], and coronary heart disease [4]. These health benefits are attributed to the existence of secondary bioactive metabolites in whole grains including phenolic acids (PAs), flavonoids, and phytosterols [5,6]. Phenolic acids in whole wheat exist in soluble free, soluble conjugated, and insoluble bound forms [2]. Ferulic, *p*-coumaric, vanillic and sinapic acids are the most dominant phenolic acids found in whole wheat [7]. Another important group of secondary compounds in whole wheat are phytosterols including fatty acids and plant sterols, which have accepted benefits in reducing blood cholesterol and therefore the risk of cardiovascular disease [8]. Wheat secondary metabolites are mostly concentrated in the bran fraction. These compounds greatly influence the quality and health properties of wheat products even though they exist at low concentrations [8].

The production of above-mentioned secondary plant products is strongly dependent on growing conditions such as light, temperature, and geographical locations as well as the plant species' physiological, genetic, and biochemical attributes [1,9].

The relative impact of genotype (G), environment (E) and genotype by environment interaction (G × E) on the production of wheat phenolic acids have been reported previously. Verna *et al*. determined the free, bound and total phenolic acids of 51 Canadian wheat grains grown at a single site in Saskatoon, Canada and observed significant variations in total phenolic acids among different wheat genotypes [10]. Mpofu *et al.* found both that genotype and growing location manifest significant effects on total phenolic content, antioxidant activity, and phenolic acid composition of six wheat varieties grown at four locations in Canada in the 2003 crop year [11]. However, information on how genetic and environmental variations influence secondary wheat metabolites including flavonoids, plant sterols, and fatty acids is lacking. This knowledge would create a good foundation for breeding and will result in better understanding of how levels of health-promoting wheat bioactives are influenced by environment and genetic changes. Thus, the objective of this study was to explore the effects of genotype, growth year and environment on the production of secondary metabolites in Canadian wheat varieties.

3.3 MATERIALS AND METHODS

3.3.1 WHEAT GENOTYPES

The samples were obtained from eight western Canadian wheat varieties (*Triticum spp*), representing different commercial classes with different qualities. Varieties included AC Corrine,

AC Barrie, AC Crystal & Carberry, Snowbird, AC Andrew (*Triticum aestivum L.*), AC Navigator & Strongfield durum wheat grains (*Triticum turgidum L. var. durum*). Detailed characteristics of selected wheat varieties and their related Canadian categories are listed in Table 3.1. These eight genotypes were grown at three locations (details on section 3.3.2) over two consecutive crop years (2010, 2011).

Table 3.1. Characteristics of the selected wheat genotypes

Class	**Characteristics**	**Genotypes**
Canada Western Red Spring (CWRS)	Hard red spring wheat	AC Barrie, Carberry
Canada Prairie Spring (CPS)	Red spring wheat Medium hard kernel	AC Crystal
Canada Western Soft White Spring (CWSWS)	Soft white spring wheat Low protein content	AC Andrew
Canada Western Amber Durum, (CWAD)	Durum wheat	AC Navigator, Strongfiled
Canada Western Extra Strong (CWES)	Hard red spring wheat Extra strong gluten	AC Corrine
Canada Western Hard White Spring (CWHWS)	Hard white spring wheat	Snowbird

3.3.2 LOCATIONS

Three locations were selected to represent the wheat growing conditions of the Canadian Prairies. These were the Cereal Grain Research Centers of the Lethbridge (Alberta (AB)), Indian Head (Saskatchewan (SK)) and Portage La Prairie (Manitoba (MB)).

Environmental data conditions such as average growing temperature and rainfall for each growing location, as recorded by Environment Canada, were obtained from Statistics Canada [12]. Location characteristics and soil composition data were obtained from Agriculture and Agri-Food Canada [13]. A brief summary of the location characteristics, soil composition, and heading and maturity dates of the wheat genotypes are presented in Table 3.2. The mean temperature was lower in 2010 compared to 2011 across all locations, whereas total precipitation was higher in 2010 compared to 2011 across all locations.

Table 3.2: Characteristics of the locations and heading and maturity data of the wheat genotypes

	Portage la Prairie (MB)		Indian Head (SK)		Lethbridge (AB)	
Year	2010	2011	2010	2011	2010	2011
Longitude	98° 17' 31" W	98° 17' 31" W	103° 40' W	103° 40' W	112° 49' 58" W	112° 49' 58" W
Latitude	49° 58' 22" N	49° 58' 22" N	50° 32' N	50° 32' N	49° 41' 39" N	49° 41' 39" N
Altitude	261	261	579	579	910	910
Soil type	Chernozemic 78% Gleysolic 3% Vertisolic 19%	Chernozemic 78% Gleysolic 3% Vertisolic 19%	Chernozemic 98% Regosolic 2%	Chernozemic 98% Regosolic 2%	Chernozemic 95% Gleysolic 5%	Chernozemic 95% Gleysolic 5%
Soil pH	7.2±0.1	7.2±0.1	6.8±0.1	6.8±0.1	8.15±0.05	8.15±0.05
Heading dates	August 10-15	August 10-15	August 5-10	August 5-10	August 10-20	August 10-20
Maturity dates	September 25-30	September 25-30	September 20-25	September 20-25	September 25-October 5	September 25-October 5
Mean temperature, heading to maturity	15.0°C	17.9°C	13.2°C	15.8°C	14.3°C	17.5°C
Total precipitation, heading to maturity (mm)	178.9	89.8	160.5	60.1	144.8	47.8

3.3.3 MATERIALS

Phenolic acids, flavonoids, and fatty acid standards were purchased from Sigma-Aldrich (Milwaukee, Wisconsin, USA). All acids and organic solvents were obtained from Fisher Scientific (Whitby, Ontario, Canada). All chemicals used were of analytical grade.

3.3.4 SAMPLE PREPARATION

The whole wheat samples were milled using an ultracentrifugal mill (Model ZM 200, Retsch, Haan, Germany) at14,000 rpm and passed through a 0.5mm sieve screen. The fine flour from each sample was individually vacuum-packed in moisture-proof packaging and stored at -20°C in the dark until analysis. Four categories of bioactives, including phenolic acids, flavonoids, fatty acids and plant sterols were extracted separately then analyzed.

3.3.5 PREPARATION OF FREE AND BOUND PHENOLIC ACIDS EXTRACTS

Free and bound phenolic acid extraction processes were performed using liquid-liquid extraction and an alkaline hydrolysis steps [14]. Briefly, for the free fraction, wheat flour (0.6 g) was extracted twice with ethyl acetate at a ratio of 1:20 (w/v). After centrifuging at 3750*g* for 10 min, the supernatants were obtained and concentrated to dryness using a rotary evaporator at 30°C. The dried extract was re-suspended in 50% dimethyl sulfoxide (DMSO)-ethanol. For the bound fraction, the dried residue obtained from the free fraction was hydrolyzed with 4 M NaOH for 4 h and adjusted to a pH of 1.5–2.0 with 6 M ice-cold HCl, then extracted with ethyl acetate three times. After centrifuging at 3750*g* for 10 min, the combined ethyl acetate fractions were

evaporated to dryness and reconstituted in 50% DMSO. Both fractions were filtered and then directly subjected to HPLC analysis.

3.3.6 HPLC-PDA ANALYSIS OF FREE AND BOUND EXTRACTS

Phenolic acids (PA) of free and bound fractions were identified using a reverse phased performance liquid chromatography (Waters 2695, Milford, MA, USA) equipped with a photodiode array detector (PDA) (Waters 996), and auto-sampler (717 plus, Waters, Milford, MA, USA) as described previously [15].

3.3.7 TOTAL FLAVONOID CONTENT (TFC)

TFC was determined by a colorimetric method [16]. Briefly, extracts were mixed with 95% ethanol, 10% aluminum chloride, 1 M potassium acetate and distilled water, then incubated at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a UV/Visible spectrophotometer.

3.3.8 FATTY ACID PROFILE ANALYSIS

Fatty acids were extracted as described by Tsen *et al.* [17], adapted from the classic Folch method [18]. Fatty acid methyl esters were then analyzed using an Agilent 6890 N (Agilent Technologies, Mississauga, ON, Canada) gas chromatograph equipped with a flame ionization detector as explained previously by Shamloo *et al.* [15]. The level of each fatty acid was calculated according

to the corresponding peak area relative to the total area of all characterized fatty acids and considered as a percentage of total fatty acids. All samples were analyzed in triplicates.

3.3.9 PLANT STEROL EXTRACTION AND DERIVATIZATION

The procedure used for plant sterol extraction included acid and alkaline hydrolyses and was done based on the method of Piironen *et al.* [19] as explained previously [15]. Prior to gas chromatographic analysis, plant sterols were derivatized to trimethylsilyl (TMS) ethers using N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA, Fisher Scientific, Grand Island, NY, USA) and trimethylchlorosilane (TMCS, Sigma Aldrich, Oakville, ON, Canada) in a ratio of 99:1 (v/v) as the reagents in anhydrous pyridine (Sigma Aldrich, Oakville, ON, Canada). Each sample was analyzed in duplicate.

3.3.10 PLANT STEROL GAS CHROMATOGRAPHIC ANALYSIS

Plant sterols were analyzed using an Agilent 6890 N gas chromatograph (Agilent Technologies, Mississauga, ON, Canada) with flame ionization detection (FID) and an on-column injector as described previously by Shamloo *et al.* [15]. Quantification of plant sterols was performed using dihydrocholesterol as the internal standard.

3.3.11 STATISTICAL ANALYSIS

All data were reported as means ± SD of triplicate independent experiments. The main effects of genotype and environment and their interaction were investigated by one way or general linear

model ANOVA with Minitab 14 Statistical software (Minitab Inc., State College, PA, USA). Significant differences were considered when ($P < 0.05$).

3.4 RESULTS

3.4.1 TOTAL, BOUND,AND FREE PHENOLIC ACIDS

Total PA. Total PA, in each of the eight wheat varieties grown at three locations in 2010 and 2011 crop years is listed in Table 3.3. The mean amounts of PA across all genotypes grown over two successive years (2010 and 2011) ranged from 487 ± 62 to 505 ± 44 (μg/g of dry matter (dm)) for MB, 500 ± 55 to 510 ± 41 (μg/g of dm) for SK, and 540 ± 62 to 582 ± 85 (μg/g of dm) for AB, and were different across all locations, however changes were not statistically significant. Concentrations of total PA were generally lower in 2010 than 2011 for MB and SK, whereas for AB levels were higher in 2010 than 2011, with the greatest variation (CV=15%) across the genotypes being evident in the samples grown in 2010, AB. Total PA of some individual genotypes grown at the same location varied across different growing years; AC Navigator grown at MB, had significantly higher total phenolic acid content in 2011 (540 μg/g of dm) than 2010 (377 μg/g of dm), while AC Barrie grown at SK, had a high total phenolic acid content in 2011 (566 μg/g of dm) but significantly lower contents in 2010 (428 μg/g of dm). In addition, a different response to the growing location was observed for some individual genotypes grown across two consecutive years. For instance, the mean value of total PA for AC Crystal, across 2010 and 2011 crop years, was AB > SK > MB. Conversely, some other genotypes (AC Corrine and Snowbird) showed consistently higher values for total PA across the 3 locations and low year-to-year variation.

Table 3.3. Total phenolic acids (microgram per gram of dry matter) in the whole grain of 8 wheat varieties grown in three locations in 2010 and 2011 crop years.

	Growth location and year						Statistics for 2 years at MB Site			Statistics for 2 years at SK Site			Statistics for 2 years at AB Site			Statistics for total data across six environments		
	MB,2010	MB,2011	SK,2010	SK,2011	AB,2010	AB,2011	av	SD	CV%	av	SD	CV%	av	SD	CV%	av	SD	CV%
AC Corrine	551e	544d	567d	510c	618d	593c	548	5	1	539	40	7	606	18	3	564	38	7
AC Navigator	377a	540d	474b	448a	652e	485a	459	115	25	461	18	4	569	118	21	496	93	19
Snowbird	495c	512c	466b	507c	539b	506b	504	12	2	487	29	6	523	23	4	504	24	5
AC Andrew	507d	511c	539c	476b	502b	470a	509	3	1	508	45	9	486	23	5	501	25	5
Carberry	440b	561d	564d	484b	617d	499b	501	86	17	524	57	11	558	83	15	528	65	12
AC Crystal	487c	439a	520c	558d	701f	650d	463	34	7	539	27	5	676	36	5	559	100	18
AC Barrie	572e	452a	428a	566d	438a	571c	512	85	17	497	98	20	505	94	19	505	72	14
Strongfiled	463b	479b	441a	531c	588c	549c	471	11	2	486	64	13	569	28	5	509	57	11
av	487	505	500	510	582	540												
SD	62	44	55	41	85	62												
CV (%)	13	9	11	8	15	11												
min	377	439	441	448	438	470												
max	572	561	574	566	701	650												

Values are means from duplicate determinations.

Means in the same column followed by different letters are significantly different ($p < 0.05$).

Statistical comparisons have been made to show the effects of annual variation, variation due to location, and variation across the six environmental conditions. In each case, the average values have been calculated, together with the standard deviations (SD) and the coefficients of variation (CV).

MB= Manitoba, SK=Saskatchewan, AB=Alberta

The highest and lowest amounts of total PA of all genotypes across the six environmental conditions were observed for AC Crystal grown at AB, 2010 (701 μg/g of dm) and AC Navigator grown at MB, 2010 (377 μg/g of dm), respectively. For individual genotypes, the variance in total phenolic acid content ranged from 5 to 19%. The genotypes exhibiting the lowest variation in total PA concentration across the six conditions included Snowbird (CV=5%), AC Andrew (CV=5%) and AC Corrine (CV=7%). Genotypes showing large differences in concentration range across the six conditions included AC Navigator (CV=19%), AC Crystal (CV=18%) and AC Barrie (CV=12%).

Free PA. Amounts of individual identifiable PA in the free fraction of different wheat genotypes grown at six environmental conditions are shown in **Figure 3.1**. Free PA profile of wheat varieties was different across six environmental conditions ($P < 0.05$). Free PA values ranged from 4.56 μg/g dm (Strongfield, MB, 2010) to 40.98 μg/g dm (AC Barrie, AB, 2011). The main individual PA identified in the free fraction were sinapic acid, caffeic acid, ferulic acid, protocatechuic acid, vanillic acid and syringic acid.

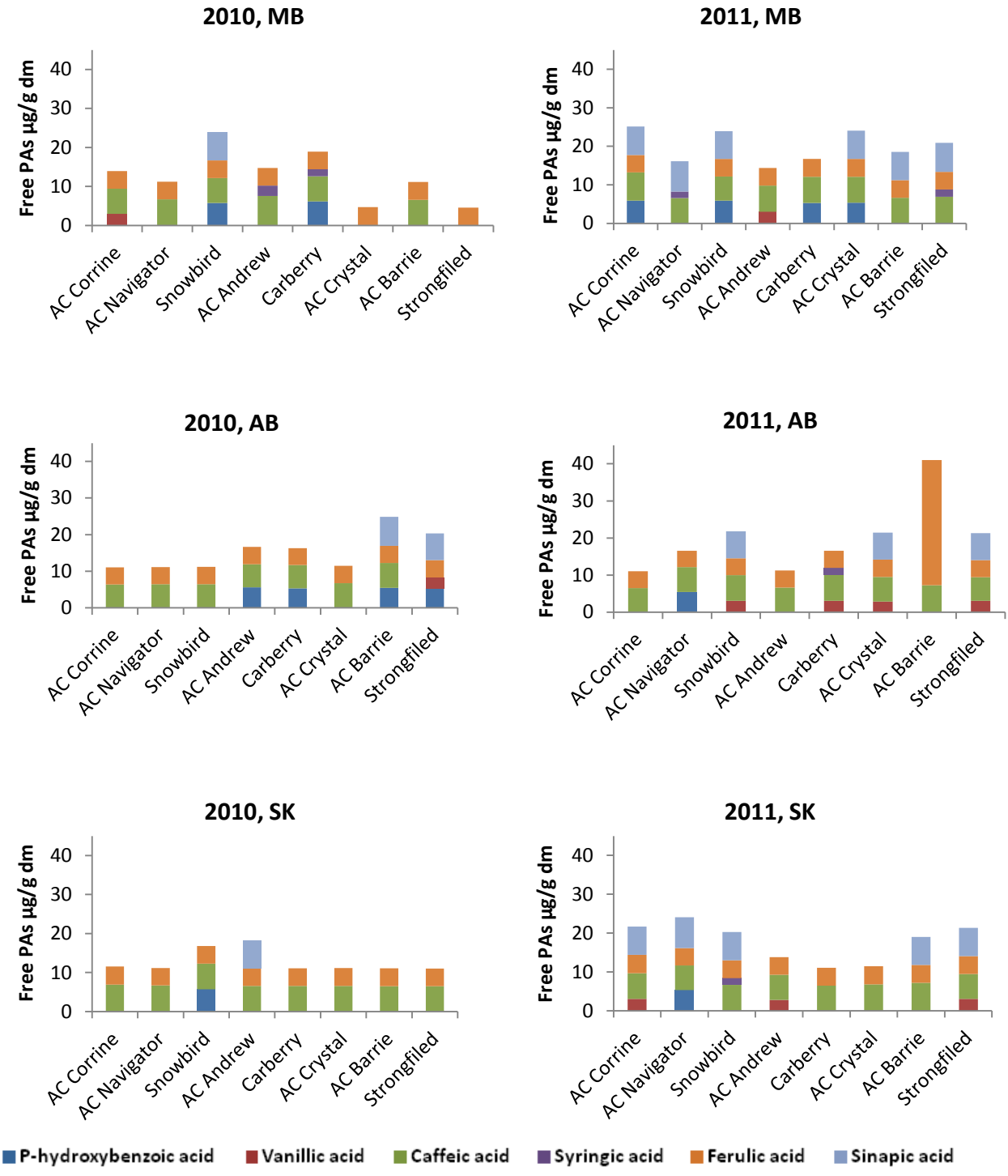


Figure 3.1. Relative distribution (microgram per gram) of individual phenolic acids across free fractions of eight wheat varieties grown in three locations in 2010 and 2011 crop years. SK=Saskatchewan, MB= Manitoba, AB= Alberta, PAs= phenolic acids, dm=dry matter

Bound PA. Amounts of individual identifiable PA in the bound fraction of different wheat genotypes grown at six environmental conditions are shown in **Figure 3.2**. The relative distribution of bound PA significantly differed between wheat varieties. Bound PA values ranged from 366.1 µg/g dm (AC Navigator, MB, 2010) to 690.12 µg/g dm (AC Crystal, AB, 2010). In the bound PA fraction, ferulic acid contents were highest across all varieties, averaging 80.1%, followed by sinapic acid (11.4%), p-coumaric acid (2.6%), vanillic acid (2.1%), syringic acid (1.9%), caffeic acid (1.32 %), p-hydroxybenzoic acid (0.3%), and lastly by o-coumaric acid (0.09%).

Among the two fractions, bound PA was the more abundant, accounting for 94-98.5% of total PA content. The free fraction contributed to the lower proportion of total phenolic acids, accounting for 1.5 to 6.0 % of the total phenolic content.

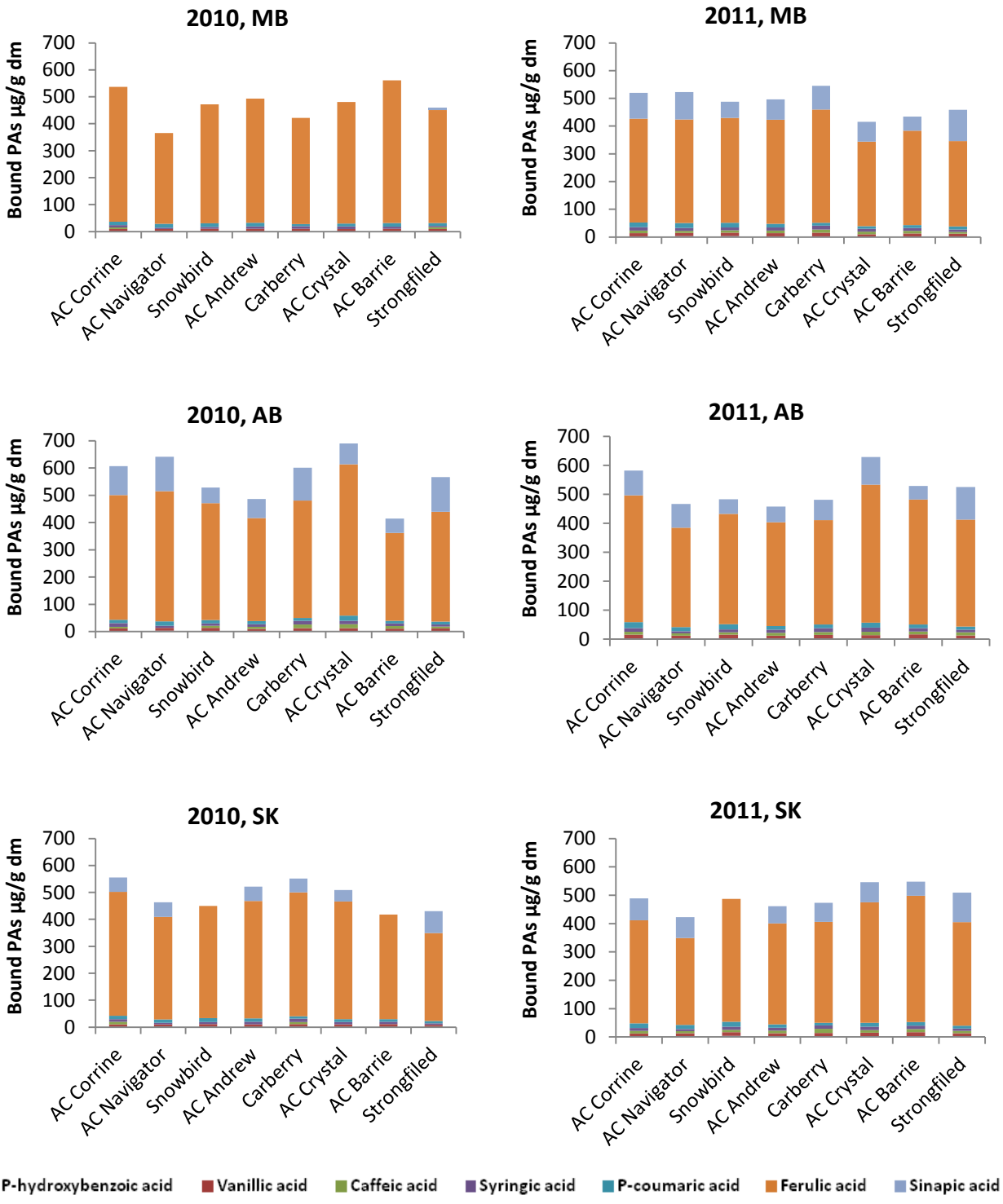


Figure 3.2. Relative distribution (microgram per gram) of individual phenolic acids across bound fractions of eight wheat varieties grown in three locations in 2010 and 2011 crop years. SK=Saskatchewan, MB= Manitoba, AB= Alberta, PAs= phenolic acids, dm=dry matter

Effects of genotype and environment on PA. As shown in Table 3.4, genotype (G), crop year (Y), and environment (E) had highly significant effects on free and bound PA levels ($P < 0.001$). Bound PA appeared most influenced by environment, whereas free PA was most influenced by crop year. Total PA, however, was strongly affected by the environment and less influenced by genotype. The crop year had the lowest significant influence on total PA.

Genotype × environment interaction (G×E), genotype × year interaction (G×Y), year × environment interaction (Y×E) and genotype × year × environment interaction (G×Y×E) had significant influences on free, bound and total PA. The later one had the largest effect on bound and total PA.

Table 3.4. Analysis of variance: influence of genotype, crop year, environment and their interactions on each compound class and each individual metabolite of eight wheat genotypes grown in three locations in 2010 and 2011 crop years.

Classes/metabolites	ANOVA significance (Mean square values)							
	Genotype (G) (*df* 7)	Crop Year (Y) (*df* 1)	Environment (E) (*df* 2)	G×E (*df* 14)	G×Y (*df* 7)	Y×E (*df* 2)	G×Y×E (*df* 14)	Error (*df* 96)
Phenolic acids								
Free PA	1.14*	**11.35***	0.77*	1.44*	1.18*	0.17*	0.55*	0.008
Bound PA	140.35*	36.41*	**569.16***	97.97*	31.28*	120.01*	146.11*	0.60
Total PA	133.01*	6.45*	**570.89***	92.36*	41.04*	130.59*	151.32*	4.50
Flavonoids								
TFC	11443.50*	20011.70*	**57790.10***	201.30*	1435.10*	443.20*	417.50*	49.70
Fatty acids								
C16:0	3.19*	1.91*	**13.43***	0.18*	0.13*	3.76*	ns	0.05
C18:0	**0.25***	0.14*	0.036*	0.012*	0.017*	0.071*	ns	0.00
C18:1n9	5.61*	13.96*	**25.47***	0.37*	0.19*	22.4*	ns	0.31
C18:2n6	**6.43***	ns	1.05*	0.58*	ns	6.37	ns	0.26
C18:3n3	1.65*	**6.84***	2.60*	0.26*	ns	0.51*	0.07*	0.02
Others	ns	**3.98***	ns	ns	ns	ns	ns	0.38
SFA	3.37*	ns	**11.22***	ns	ns	4.23*	ns	0.08
MUFA	7.32*	16.16*	**30.99***	0.30*	ns	27.42*	ns	0.13
PUFA	5.60*	ns	1.38*	0.36*	ns	**7.6***	ns	0.18
UFA	3.38*	ns	**11.22***	ns	ns	4.23*	ns	0.08
UFA/SFA	0.20*	ns	**0.64***	ns	ns	0.24*	ns	0.00
PUFA/MUFA	0.76*	2.23*	**3.39***	0.05*	ns	3.19*	0.03*	0.01
Plant sterols								
Sitosterol	14292.90	**37252.00**	7526.40	78.80	692.90	675.50	ns	34.60

Campesterol	2234.47	**2913.84**	735.39	ns	135.55	ns	ns	11.64
Sitostanol	4018.56	**8063.29**	1548.28	ns	572.23	ns	ns	23.30
Campestanol	2517.88	**4220.23**	789.4	ns	419.55	ns	ns	13.52
Stigmasterol	46.56	**105.47**	27.99	ns	30.36	ns	ns	2.23
Other sterols	35.73	**68.37**	27.18	ns	26.52	4.08	ns	1.05
Total plant sterols	84709.00	**176640.00**	36724.00	337.00	7955.00	878.00	ns	109.00

An asterisk (*) indicates significant at $P < 0.001$; a bold number is a factor or interaction most influencing the variable (highest mean square). Df=Degrees of freedom, ns=not significant

3.4.2 TOTAL FLAVONOID CONTENT

Figure 3.3 shows the TFC in the whole grains of eight wheat varieties grown in three locations in 2010 and 2011 crop years. For 2010, TFC across all wheat genotypes ranged from 224.51 μg rutin equivalent/g dm (AC Andrew, SK) to 372.97 μg rutin equivalent/g dm (AC Crystal, MB). Genotypes grown during 2010 responded differently across growing locations; the TFC for all the genotypes was significantly different across different locations as shown in **Figure 3.3**. A clear example is TFC of AC Corrine which was 328.43 μg/g, 263.5 μg/g and 228.5 μg/g in MB, AB, and SK, respectively.

For 2011, TFC across all wheat genotypes ranged from 245.04 μg rutin equivalent/g dm (AC Corrine, SK) to 396.9 μg rutin equivalent/g dm (AC Crystal, MB). Genotypes grown during 2011 also responded differently to the growing locations; the TFC for all the genotypes was significantly different across different locations. A clear example is AC Navigator which had TFC of 378.73 μg/g (MB), 339.9 μg/g (AB) and 260.2 μg/g (SK).

Comparing the two crop years, TFC of different wheat varieties were generally lower in 2010 than 2011 for all locations. All wheat varieties grown at the same location had lower TFC in 2010 compared to 2011. For instance, TFC for AC Navigator grown at MB was significantly lower in 2010 (328.43 μg/g) than 2011 (378.7 μg/g).

Location-wise the total TFC for all the wheat genotype were as follows: MB > AB > SK and was consistent over the two crop years.

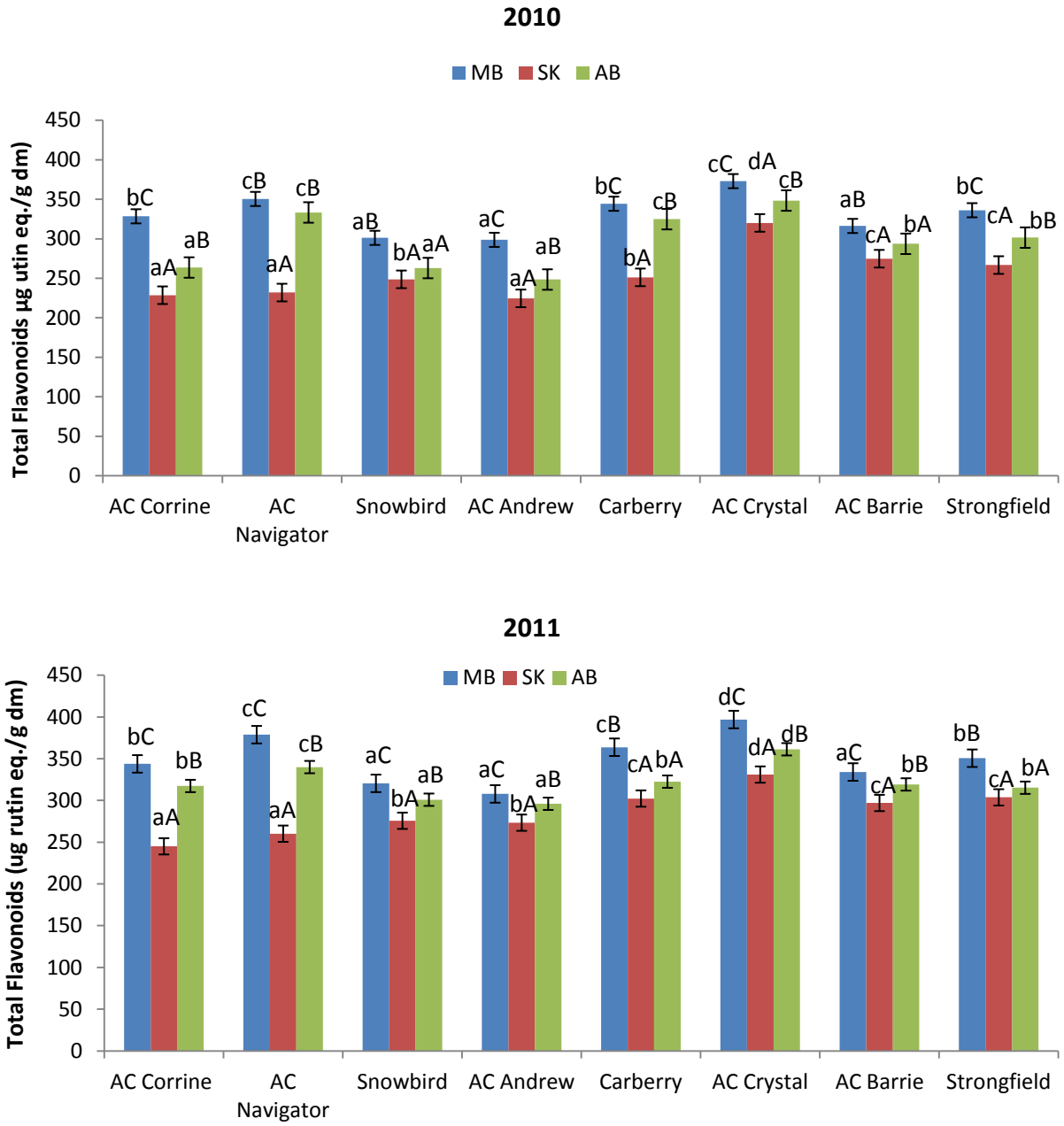


Figure 3.3. Total flavonoid contents (microgram rutin equivalent per gram of dry matter) of eight wheat varieties grown in three locations in 2010 and 2011 crop years. [a,b,c,d,e] Different small letter superscripts indicate significant differences ($P < 0.05$) between different genotypes grown at the same location. [A,B,C] Different capital letters superscripts indicate significant differences ($P < 0.05$) within the same genotype grown in different locations. (SK=Saskatchewan, MB= Manitoba, AB= Alberta)

Based on wheat genotypes, in all locations over the 2010 and 2011 crop years, the mean value of TFC was as follows: AC Crystal > Carberry > AC Navigator > Strongfield > AC Barrie > AC Corrine > Snowbird > AC Andrew.

Effects of genotype and environment on flavonoid content: As shown in Table 3.4, genotype (G), crop year (Y), and environment (E) had highly significant influences on TFC, however, TFC appeared most influenced by environment and crop year, and less influenced by genotype variation. G×E, G×Y, Y×E, G×Y×E interactions had significant influences on TFC and genotype × crop year interaction had the largest effect on TFC.

3.4.3 FATTY ACID PROFILE

Fatty acid profiles of the eight wheat genotypes grown under the different environments are summarized in Table 3.5. All fatty acid concentrations were calculated as percentage values of total identified fatty acids measured. In all wheat genotypes, regardless of their growing environments, linoleic acid (C18:2n6) was measured as the main fatty acid ranging in concentration from 58.34 % (AC Navigator, 2010, AB) to 62.14 % (AC Corrine, 2010, SK). The second highest fatty acid was palmitic acid (C16:0), accounted for 16.3 to 20.06 % of total fatty acids with the highest amount for AC Barrie, AB, 2010 and the lowest amount for AC Navigator, MB, 2010. And finally, the third dominant fatty acid was oleic acid (C18:1n9) which accounted for 10.22 % (AC Corrine, AB, 2010) to 15.99 % (AC Navigator, SK, 2011). In 2010, a significant increase in palmitic acid (C16:0) was observed for all genotypes grown at AB compared to those grown at SK and MB. For all other individual fatty acids, there were no

Table 3.5. Contents (%) of fatty acid profile of 8 wheat varieties grown in three locations in 2010 and 2011 crop years.

Year	Growing Location	Fatty acids	Genotypes							
			AC Corrine	AC Navigator	Snowbird	AC Andrew	Carberry	AC Crystal	AC Barrie	Strongfiled
2010	MB	C16:0	17.24^{A}	16.93^{A}	17.60^{A}	16.29^{A}	17.75^{A}	17.63^{A}	17.81^{A}	17.24^{A}
		C18:0	0.770	1.150	0.940	1.010	1.090	0.830	0.820	1.320
		C18:1n9	14.51^{a}	15.98^{b}	14.85^{b}	14.19^{a}	14.66^{ab}	14.36^{a}	13.97^{a}	14.58^{a}
		C18:2n6	59.79^{b}	58.38^{a}	59.49^{ab}	60.64^{b}	59.50^{ab}	59.23^{ab}	58.80^{a}	58.58^{a}
		C18:3n3	4.220	3.790	4.170	4.360	3.970	4.490	4.710	4.340
		Others	2.640	2.930	2.250	2.740	2.410	2.710	3.150	3.250
		SFA	19.00	19.10	19.41	18.31	19.80	19.59	20.08	20.08
		MUFA	15.74	17.44	15.48	15.43	15.70	15.52	15.24	15.87
		PUFA	60.89^{ab}	59.50^{a}	60.44^{a}	61.73^{b}	60.37^{a}	60.25^{a}	59.83^{a}	59.54^{a}
		UFA	81.00	80.90	80.59	81.69	80.20	80.41	79.92	79.92
		UFA/SFA	4.260	4.24	4.150	4.460	4.050	4.110	3.980	3.980
2010	SK	C16:0	17.74^{A}	17.31^{A}	18.07^{A}	16.38^{A}	17.85^{A}	17.84^{A}	18.25^{A}	18.27^{A}
		C18:0	0.930	1.020	0.880	0.890	0.950	0.680	0.740	1.210
		C18:1n9	12.14^{a}	15.04^{b}	12.87^{a}	12.63^{a}	12.58^{a}	12.58^{a}	12.32^{a}	12.34^{a}
		C18:2n6	62.14^{c}	58.81^{a}	60.78^{b}	61.37^{bc}	60.78^{b}	60.38^{b}	60.49^{b}	59.64^{ab}
		C18:3n3	4.090	4.080	4.360	5.020	4.670	4.890	4.620	4.790
		Others	2.300	2.910	2.320	2.970	2.580	2.840	2.840	2.970
		SFA	19.59	19.59	19.93	18.66	19.90	19.85	20.27	20.86
		MUFA	13.10	16.26	13.82	13.74	13.60	13.66	13.47	13.48
		PUFA	63.07^{c}	59.92^{a}	61.76^{a}	62.43^{bc}	61.66^{b}	61.47^{b}	61.50^{b}	60.70^{a}
		UFA	80.41	80.41	80.07	81.34	80.10	80.15	79.73	79.14
		UFA/SFA	4.100	4.10	4.020	4.360	4.020	4.040	3.930	3.790
2010	AB	C16:0	19.01^{B}	18.90^{B}	19.77^{B}	17.84^{B}	19.56^{B}	18.76^{B}	20.06^{B}	19.76^{B}
		C18:0	0.83	0.840	0.880	0.720	0.930	0.660	0.650	1.050
		C18:1n9	10.22^{a}	13.48^{b}	10.95^{a}	10.74^{a}	11.22^{a}	10.36^{a}	10.44^{a}	11.02^{a}
		C18:2n6	61.88^{b}	58.34^{a}	60.78^{a}	61.68^{ab}	60.43^{a}	60.09^{a}	60.04^{a}	59.93^{a}
		C18:3n3	4.7	4.570	4.640^{a}	5.470^{a}	4.730^{a}	6.640^{a}	5.370^{a}	4.820^{a}
		Others	2.78	3.040	2.400	2.920	2.510	2.940	2.800	2.750
		SFA	21.29	21.16	21.67	19.68	21.55	20.67	21.71	21.98
		MUFA	11.1	14.68	11.84	11.86	12.22	11.55	11.71	12.13
		PUFA	62.77^{b}	59.46^{a}	61.72^{ab}	62.68^{b}	61.36^{ab}	60.99^{a}	60.99^{a}	60.92^{a}
		UFA	78.71	78.84	78.33	80.14	78.45	79.33	78.29	78.02

		UFA/SFA	3.700	3.730	3.610	4.030	3.640	3.850	3.610	3.550
2011	MB	C16:0	18.18	17.70	18.56	17.17	18.61	18.56	18.60	18.48
		C18:0	1.050	1.080	0.870	0.910	1.000	0.720	0.720	1.350
		C18:1n9	12.96 ^{a}	15.0 ^{b}	13.57^{a}	13.63^{a}	13.73^{a}	13.13^{a}	12.99^{a}	13.40 ^{a}
		C18:2n6	61.02 ^{b}	59.05 ^{a}	60.23^{ab}	60.90^{ab}	60.01^{a}	60.06^{a}	59.99^{a}	59.44^{a}
		C18:3n3	3.890	3.640	3.890	3.940	3.700	4.420	4.450	3.950
		Others	2.420	2.700	2.240	2.740	2.430	2.560	2.520	2.670
		SFA	20.12	19.85	20.30	19.22	20.54	20.16	20.20	20.78
		MUFA	13.98	16.17	14.51	14.74	14.59	14.22	14.00	14.41
		PUFA	61.84 ^{b}	60.18 ^{a}	61.16 ^{b}	61.96^{b}	60.99^{ab}	61.05^{b}	61.18^{b}	60.66^{a}
		UFA	79.88	80.15	79.70	80.78	79.46	79.84	79.80	79.22
		UFA/SFA	3.970	4.040	3.930	4.200	3.870	3.960	3.950	3.810
2011	SK	C16:0	17.53	17.71	18.26	17.19	17.95	18.16	18.59	18.63
		C18:0	1.090	1.110	0.97	0.97	1.13	0.79	0.78	1.33
		C18:1n9	13.71^{a}	15.99 ^{b}	14.53 ^{a}	13.75 ^{a}	14.37^{a}	13.75^{a}	14.17^{a}	14.39^{a}
		C18:2n6	60.6 ^{b}	58.25 ^{a}	59.54^{a}	60.89^{b}	59.7^{ab}	59.91^{ab}	59.17^{a}	58.75^{a}
		C18:3n3	3.960	3.560	3.92	4.02	3.94	4.37	4.18	3.9
		Others	2.600	2.700	2.16	2.6	2.38	2.42	2.47	2.4
		SFA	19.64	19.74	19.99	19.04	20.13	19.83	20.24	20.8
		MUFA	14.60	17.07	15.31	14.78	15.25	14.85	15.33	15.47
		PUFA	61.59 ^{b}	59.43 ^{a}	60.61^{ab}	61.98 ^{b}	60.53^{a}	60.81^{ab}	60.11^{a}	59.66^{a}
		UFA	80.36	80.26	80.01	80.96	79.87	80.17	79.76	79.2
		UFA/SFA	4.090	4.070	4.00	4.25	3.97	4.04	3.94	3.81
2011	AB	C16:0	18.39	18.61	18.98	17.6	18.88	18.9	19.08	19.69
		C18:0	1.200	1.070	1.01	0.89	1.12	0.65	0.8	1.36
		C18:1n9	13.35 ^{b}	15.50 ^{c}	13.74^{b}	12.7^{b}	13.53^{b}	11.6^{a}	13.14^{b}	13.34 ^{b}
		C18:2n6	60.39^{bc}	57.86 ^{a}	59.53^{b}	60.98^{c}	59.59^{b}	60.23^{b}	59.41^{b}	58.5^{a}
		C18:3n3	3.820	3.670	3.95	4.68	3.98	5.48	4.49	4.09
		Others	2.330	2.580	2.16	2.53	2.28	2.51	2.44	2.49
		SFA	20.45	20.45	20.74	19.3	20.87	20.2	20.76	21.85
		MUFA	14.33	16.73	14.62	13.9	14.43	12.94	14.26	14.52
		PUFA	61.21^{bc}	59.00 ^{a}	60.52^{b}	61.95^{c}	60.54^{b}	61.16^{bc}	60.34^{b}	59.35^{a}
		UFA	79.55	79.55	79.26	80.7	79.13	79.8	79.24	78.15
		UFA/SFA	3.890	3.890	3.82	4.18	3.79	3.95	3.82	3.58

^{a,b,c} Different superscript small letters in the same row indicate significant difference ($P < 0.05$).

^{A,B,C} Different superscript capital letters in the same column for the same dependent variable indicate significant difference ($P < 0.05$).

significant differences observed among different growing locations. In 2011, there were no differences observed among different growing locations for individual fatty acids.

The amount of linolenic acid (C18:3n3) across all the genotypes ranged from 3-5 % of total fatty acids and did not differ significantly. Polyunsaturated fatty acids (PUFA) ranged from 59.0 to 63 % and were three to four times greater than monounsaturated fatty acids (MUFA). The wheat varieties displayed different PUFA compositions regardless of growing conditions. Generally, unsaturated fatty acid levels were three to four times higher than saturated fatty acid levels.

Effects of genotype and environment on fatty acids: As shown in Table 3.4, C16:0, C18:1n9, SFA, MUFA, UFA, UFA/SFA, and PUFA/MUFA were most influenced by environment (E). For C18:0 and C18:2n6 levels across all samples, genotype (G) was the main influencing factor. C18:3n3 and other minor fatty acids were most influenced by crop year (Y). And finally for PUFA, the Y×E interaction made the greatest contribution.

3.4.4 PLANT STEROLS

Tables 3.6 and 3.7 show the plant sterol composition and total plant sterol contents of eight wheat varieties grown in three locations in the 2010 and 2011 crop years. Sitosterol was the most abundant plant sterol in all wheat genotypes accounting for 39.1 to 41.2 % of the total sterols, regardless of their growing conditions. Next most abundant were campesterol (16-18 %), sitostanol (18-20 %), campestanol (16-18 %) and stigmasterol (2 %).

Table 3.6. Individual plant sterol contents (microgram per gram of dry matter) of 8 wheat varieties grown in three locations in 2010 and 2011 crop years.

Year	Growing Location	Plant sterols	Genotypes							
			AC Corrine	AC Navigator	Snowbird	AC Andrew	Carberry	AC Crystal	AC Barrie	Strongfiled
2010	MB	Sitosterol	347.23^{cC}	373.46^{dB}	299.66^{aB}	313.22^{abC}	361.02^{cdB}	358.41^{cdC}	323.54^{bB}	318.94^{bC}
		Campesterol	146.85^{cB}	158.32^{dAB}	129.94^{aAB}	137.45^{abAB}	149.19^{cdB}	146.24^{cB}	141.73^{bcAB}	139.21^{bcBC}
		Sitostanol	164.97^{bcB}	178.67^{dB}	147.15^{aB}	156.84^{bBC}	173.89^{cdB}	172.12^{cdB}	161.63^{bB}	159.29^{bB}
		Campestanol	156.10^{c}	168.93^{dB}	142.59^{a}	148.28^{abC}	160.70^{cdB}	159.24^{cB}	152.09^{bcB}	149.35^{bcB}
		Stigmasterol	17.300	19.390^{AB}	17.600^{B}	17.630	19.020^{AB}	17.840^{AB}	18.230	17.810
		Others	17.080	18.410	16.060	16.370	17.380	17.280	17.040	16.800
2010	SK	Sitosterol	311.72^{cA}	333.28^{dA}	287.97^{abA}	284.88^{aA}	323.65^{cdA}	313.59^{cA}	299.30^{bA}	293.67^{abA}
		Campesterol	136.73^{bcA}	152.51^{dA}	124.88^{aA}	130.77^{abA}	140.01^{cA}	138.59^{bcA}	136.88^{bcA}	128.89^{abA}
		Sitostanol	149.96^{bcA}	170.25^{dA}	140.39^{aA}	144.72^{abA}	154.27^{bcA}	158.82^{cA}	148.93^{bA}	148.74^{bA}
		Campestanol	155.08^{d}	157.31^{dA}	139.88^{ab}	134.78^{aA}	148.91^{cdA}	147.22^{cA}	141.50^{bcA}	141.85^{bcA}
		Stigmasterol	16.560	17.820^{A}	17.260^{B}	16.550	17.260^{A}	16.120^{A}	16.890	16.940
		Others	16.240	16.980	15.720	15.990	16.200	15.180	16.230	16.080
2010	AB	Sitosterol	332.24^{cB}	364.90^{dB}	300.07^{aB}	302.44^{aB}	352.40^{dB}	335.70^{cB}	321.98^{bcB}	307.49^{abB}
		Campesterol	142.46^{bcAB}	155.56^{dA}	125.60^{aA}	134.35^{bA}	145.83^{cAB}	146.61^{cdB}	143.38^{cB}	133.71^{abAB}
		Sitostanol	162.44^{bcB}	174.96^{dAB}	139.43^{aA}	151.02^{abB}	165.59^{cdB}	164.90^{cdA}	158.78^{bcB}	153.06^{bA}
		Campestanol	156.10^{c}	163.85^{dAB}	140.39^{a}	142.57^{abB}	156.30^{cB}	151.80^{bcA}	148.47^{bAB}	145.18^{abA}
		Stigmasterol	17.040^{ab}	18.510^{bA}	17.290^{aB}	17.150^{ab}	17.750^{abA}	15.850^{aA}	17.420^{ab}	17.380^{ab}
		Others	16.720	18.220	16.800	16.700	16.810	15.470	16.780	16.470
2011	MB	Sitosterol	365.03^{dD}	419.04^{fD}	316.99^{aC}	333.35^{bD}	405.06^{fD}	387.11^{eE}	357.86^{dD}	341.68^{cD}
		Campesterol	152.84^{cC}	184.09^{eC}	133.81^{aB}	140.90^{bB}	163.4^{dC}	154.9^{cdC}	149.55^{cC}	145.26^{bC}
		Sitostanol	176.31^{cC}	209.36^{eD}	149.43^{aB}	159.04^{bC}	203.08^{eD}	187.13^{dC}	172.44^{cC}	170.91^{cC}
		Campestanol	161.22^{bc}	194.23^{eD}	146.26^{a}	147.01^{aC}	185.11^{dD}	171.95^{cC}	158.57^{bC}	155.71^{bB}
		Stigmasterol	19.000^{b}	25.370^{dC}	16.900^{aB}	18.170^{b}	23.840^{cdB}	21.530^{cB}	18.310^{b}	17.480^{a}
		Others	19.410	21.780	16.390	16.380	21.780	21.760	17.580	17.240
2011	SK	Sitosterol	352.19^{cC}	392.52^{eC}	302.32^{aB}	310.98^{abBC}	387.26^{eC}	364.33^{dC}	341.36^{cC}	323.17^{bC}
		Campesterol	146.37^{bcB}	167.93^{dB}	127.43^{aAB}	132.63^{aA}	153.34^{cB}	152.50^{cC}	143.23^{bB}	136.63^{abB}
		Sitostanol	170.97^{cBC}	193.23^{dC}	144.50^{aAB}	148.62^{aAB}	190.24^{dC}	171.87^{cB}	164.27^{bcB}	160.91^{bB}
		Campestanol	155.36^{bc}	181.56^{dC}	139.64^{a}	140.30^{aB}	176.95^{dC}	163.77^{cB}	152.20^{bB}	151.23^{bB}
		Stigmasterol	17.960^{b}	21.430^{dB}	14.600^{aA}	15.660^{a}	21.370^{dB}	20.300^{cdB}	17.660^{b}	16.650^{ab}
		Others	17.010	19.150	14.490	14.440	19.550	20.080	17.100	14.690
2011	AB	Sitosterol	361.18^{dD}	399.99^{fC}	307.99^{aBC}	318.56^{bC}	392.87^{eC}	370.91^{dD}	346.87^{cC}	334.16^{cD}

	Campesterol	150.41^{bcBC}	171.59^{dB}	132.83^{aB}	137.87^{aAB}	163.15^{cdC}	153.68^{cC}	145.80^{bBC}	141.53^{abC}
	Sitostanol	174.23^{cC}	206.30^{dD}	141.30^{aA}	151.56^{aB}	203.36^{dD}	180.60^{cC}	166.45^{bcBC}	163.93^{bBC}
	Campestanol	158.90^{bcAB}	186.23^{dCD}	143.18^{a}	142.46^{aB}	180.51^{dD}	166.98^{cC}	155.50^{bBC}	153.50^{bB}
	Stigmasterol	18.650^{b}	22.710^{cdBC}	15.290^{aA}	16.600^{a}	23.200^{dB}	21.530^{cB}	18.360^{b}	17.150^{b}
	Others	18.100	19.570	15.230	13.900	22.180	21.450	17.480	14.870

^{a,b,c} Different superscript small letters in the same row indicate significant difference ($P < 0.05$).

^{A,B,C} Different superscript capital letters in the same column for the same dependent variable indicate significant difference ($P < 0.05$)

Table 3.7. Total plant sterol contents (microgram per gram of dry matter) of 8 wheat varieties grown in three locations in 2010 and 2011 crop years.

		AC Navigator	Carberry	AC Crystal	AC Corrine	AC Barrie	Strongfield	AC Andrew	Snowbird
2011	MB	1053.9 ± 10.9^{fD}	1002.3 ± 10.7^{eE}	944.4 ± 11.4^{dE}	893.8 ± 8.30^{cE}	874.6 ± 11.9^{cE}	848.3 ± 9.00^{bD}	815.3 ± 15.1^{aC}	779.8 ± 17.7^{aB}
	AB	1006.4 ± 12.1^{eC}	985.20 ± 11.7^{eE}	915.2 ± 14.5^{dD}	881.5 ± 3.60^{cD}	850.5 ± 11.1^{bDE}	825.1 ± 14.6^{bD}	780.9 ± 6.80^{aC}	755.8 ± 15.5^{aB}
	SK	975.80 ± 18.5^{fC}	948.00 ± 8.20^{fD}	892.8 ± 13.6^{eD}	859.8 ± 6.80^{dC}	835.8 ± 13.5^{dCD}	803.2 ± 6.10^{cC}	762.6 ± 4.01^{bB}	742.9 ± 2.80^{aA}
2010	MB	917.20 ± 16.5^{fB}	881.20 ± 6.20^{eC}	871.1 ± 3.20^{dC}	849.5 ± 3.80^{cC}	814.3 ± 12.0^{bBC}	801.2 ± 12.3^{bC}	798.8 ± 16.2^{bC}	753.0 ± 3.30^{aB}
	AB	896.00 ± 16.8^{fB}	854.60 ± 2.70^{eB}	830.3 ± 16.6^{dcB}	827.0 ± 4.80^{dB}	806.8 ± 8.20^{cB}	773.3 ± 5.50^{bB}	764.2 ± 3.30^{bB}	739.6 ± 6.90^{aA}
	SK	848.10 ± 9.30^{eA}	800.30 ± 3.50^{dA}	789.5 ± 5.40^{cA}	786.3 ± 6.90^{cA}	759.7 ± 6.60^{bA}	746.1 ± 8.90^{bA}	727.7 ± 6.90^{aA}	726.1 ± 6.40^{aA}

^{a,b,c,d,e,f} Different superscripts small letters in the same row indicate significant difference ($P < 0.05$).

^{A,B,C,D,E} Different superscripts capital letters in the same column indicate significant difference ($P < 0.05$).

The total plant sterol contents ranged from 726.1 µg/g dm (Snowbird, SK, 2010) to 1053.9 µg/g dm (AC Navigator, MB, 2011). Total plant sterols for wheat varieties grown at all locations across two crop years 2010 and 2011 follows: AC Navigator > Carberry > AC Crystal > AC Corrine > AC Barrie > Strongfiled > AC Andrew > Snowbird. Total plant sterol contents for wheats grown at 2011 were significantly higher than 2010 for all locations across all genotypes.

Effects of genotype and environment on plant sterols: As shown in Table 3.4, genotype (G), crop year (Y), environment (E) and G×Y had significant effects on all individual and total plant sterol contents. Crop year had the largest influence on all individual and total plant sterol contents.

3.5 DISCUSSION

This research investigated the relative contribution of genotypes and environmental differences, including year and location variations, on the four main classes of Canadian wheat secondary metabolites. Accumulation of these metabolites depends on environmental factors such as temperature, precipitation, altitude, soil type [20,21] and plant genetic background [1,10]. Presently, we observed significant shifts in the concentration and composition of secondary metabolites of Canadian wheat grains based on genotypes and growth environments during 2010 and 2011 crop years.

Phenolic acids: Significant differences in total, free and bound phenolic acids (PA) based on wheat varieties were observed. AC Corrine and AC Crystal had higher total and bound PA, while AC Navigator and AC Andrew had lower total and bound PA than other genotypes. Mpofu *et al.*

discovered significant differences in total PA across different western Canadian wheat cultivars [11]. Presently, individual PA profiles were significantly different across genotypes. However, in line with previous reports [22-24], ferulic acid identified as the main individual PA in free and bound fractions followed by sinapic acid, p-coumaric acid, vanillic acid, syringic acid, caffeic acid, and p-hydroxybenzoic acid.

Similar to previous findings [1,23,25], in the present study growing environment (i.e. location) had a larger influence on total and bound PA than genotype in wheat grains grown at each crop year. For the 2010 crop year, mean values of total PA in different locations was AB > SK > MB and was consistent for 2011 crop year. Regardless of genotype and crop year, wheat grains grown at Alberta (AB) contained highest total PA while those grown at Manitoba (MB) contained the lowest total PA. Considering the amount of total precipitation of each year heading to maturity for each location (shown in Table 3.2), this result revealed that there is a strong negative correlation (correlation coefficient= -0.497 for 2010 and = -0.26 for 2011) between the total precipitation and levels of total PA. This finding was consistent with recent results reported for other plants. For instance, Liu *et al.* found that annual average precipitation conversely affects the total PA content of *potentilla fruticosa L.* [26].

On the other hand, a significant positive correlation was shown to exist presently between total PA and altitude for 2010 crop year (correlation coefficient= 0.51). For 2011 crop year, this positive correlation remained, however it did not reach statistically significant (correlation coefficient =0.29). Wheat grains grown at higher altitudes contained higher total PA. Altitude is an overall reflection of several environmental factors such as temperature, humidity, soil

composition and solar radiation [26]. Landscape matrix has been reported as having a significant effect on the response of insect species [27,28]. Significant positive correlation between total PA and altitude could be the response of the wheat grown at a higher altitude to the strong ultraviolet radiation, and/or it could be the response to the changing herbivorous insect's population to protect the plant against harmful UV radiation and/or insects. However, those responses require further investigations.

Flavonoids: TFC of different wheat genotypes varied significantly. Red spring wheat cultivars, AC Crystal and Carberry, contained higher TFC where white spring wheat, Snowbird, and AC Andrew, contained lower TFC than other genotypes. These results are in line with previous findings where higher TFC was reported for colored wheat varieties [29].

The environment had the largest influence on TFC. Wheat plants responded differently to temperature, total precipitation, and altitude in regard to flavonoid production. Unlike phenolic acids, the temperature had the largest effect on TFC compared to total precipitation and altitude variation. The increased growing temperature resulted in a higher amount of TFC in each crop year.

Higher TFC in wheat grains grown at the location with higher amounts of rainfall (correlation coefficient=0.35 for 2010 and = 0.47 for 2011) were observed in the present study. This could be the result of nitrogen (N) leaching from fields with higher amounts of rainfall. Leaching losses of N occur when soils have more incoming water (rain or irrigation) than it can hold [30,31]. Based on

the carbon/nutrient balance hypothesis [32], low nitrogen limits the growth of the plants more than photosynthesis, and plant allocates the extra carbon that cannot be used for growth to produce secondary metabolites, including flavonoids [33,34]. Mohd Hafiz *et al.* observed a significant increase in production of TFC under low nitrogen levels in different varieties of Kacip Fatimah [34].

Presently, altitude was inversely correlated to TFC (correlation coefficient= -0.31 for 2010 and = -0.32 for 2011). There are controversial results on how altitude affects TFC in different plants; altitude was not the major factor governing the total PA and flavonoids in *Sphagnum junghuhnianum* [35]*;* TFC in bryophytes exhibited no obvious relationship with altitude [36]. However, in *Leontodon* species and *Eucommia ulmoides*, which are medicinal plants, analyses of samples originating from different altitudes showed a significant positive correlation between altitudes of collection sites and TFC [37,38]. By contrast, the TFC of some medicinal plants, such as *Buxus sempervirens* L., decreased with increased altitude [39]. In the present study, other environmental factors such as rainfall and temperature had a stronger effect on TFC than altitude.

Fatty acids: Similar to previous reports [15,40], linoleic acid (C18:2n6) was identified as the main fatty acid in all wheat varieties grown at different locations over two crop years. Genotype variations had the largest influence on levels of linoleic acid (C18:2n6). Significant increase observed for palmitic acid (C16:0) levels for wheat grains grown in AB (where rainfall was lower) compare to those grown in MB and SK. However, for other fatty acids no clear link was

identified between the level of fatty acids and environmental factors such as rainfall, temperature or altitude.

Plant sterols: Sitosterol was identified as the most abundant plant sterol in all wheat varieties in the present study as reported previously [15,41,42]. Significant variation was observed in total plant sterol contents among wheat varieties. Overall, red spring wheat varieties contained the higher amount of plant sterols than white spring wheat.

The crop year had the largest influence on all individual plant sterol contents compared to other factors (Table 3.4). Total plant sterols for wheat grains grown at 2011 were significantly higher than those of 2010 for all locations across all genotypes. Also, the mean value of total plant sterols in different locations was MB > AB > SK and was consistent over the two crop years. As described in Table 3.2, the mean temperature was lower in 2010 compared to 2011 across all locations, whereas total precipitation was higher in 2010 compared to 2011 across all locations. Taken together, our result showed that by increasing the temperature and decreasing the total precipitation total plant sterol contents increased.

In conclusion, profiles and contents of secondary metabolites presently studied in different wheat varieties grown in different environments were significantly influenced by genotype, crop year, growth location and their interactions.

The present study provided comprehensive and valuable data on the diversity in wheat secondary metabolite contents and composition based on genotype and grown environment, which could be used by wheat breeders to select wheat varieties containing higher amounts of bioactives. This would be expected to lead to enhanced intakes of health-promoting compounds from natural sources by consumers. For instance, in the present study, AC Navigator, AC Crystal, and Carberry contained higher amounts of flavonoids and plant sterols within the Canadian wheat genotypes.

The present study, however, has some limitations. Data on some other environmental factors such as elevated CO2, UV light and changing herbivorous insect populations, which can induce different physiological responses in plants, are lacking and need further investigation.

3.6 ACKNOWLEDGEMENTS

This research was funded in part by Manitoba Science and Technology International Collaboration Fund (STIC) and Food Advancement through Science and Training (FAST) program.

Authors would like to thank Drs. Susan Arntfield and Nancy Ames, for their assistance with finding the wheat samples through Cereal Research Centre, Canadian Wheat Board and Agriculture and Agri-Food Canada, and Dennis Labossiere, Tracy Exley, Kim Kuzminski and Camille Rhymer for their technical assistance.

3.7 REFERENCES

1 Fernandez-Orozco, R., Li, L., Harflett, C., Shewry, P. R. & Ward, J. L. Effects of environment and genotype on phenolic acids in wheat in the HEALTHGRAIN diversity screen. *Journal of agricultural and food chemistry* **58**, 9341-9352, doi:10.1021/jf102017s (2010).

2 Liu, R. H. Whole grain phytochemicals and health. *Journal of Cereal Science* **46**, 207-219, doi:http://dx.doi.org/10.1016/j.jcs.2007.06.010 (2007).

3 Montonen, J., Knekt, P., Jarvinen, R., Aromaa, A. & Reunanen, A. Whole-grain and fiber intake and the incidence of type 2 diabetes. *The American journal of clinical nutrition* **77**, 622-629 (2003).

4 Slavin, J., Jacobs, D. & Marquart, L. Whole-grain consumption and chronic disease: Protective mechanisms. *Nutrition and Cancer* **27**, 14-21, doi:10.1080/01635589709514495 (1997).

5 Shahidi, F. Functional foods: Their role in health promotion and disease prevention. *Journal of Food Science* **69**, R146-R149 (2004).

6 Yao, L. H. *et al.* Flavonoids in food and their health benefits. *Plant foods for human nutrition (Dordrecht, Netherlands)* **59**, 113-122 (2004).

7 Laddomada, B., Caretto, S. & Mita, G. Wheat bran phenolic acids: bioavailability and stability in whole wheat-based foods. *Molecules* **20**, 15666-15685 (2015).

8 Shewry, P. R. & Hey, S. J. The contribution of wheat to human diet and health. *Food and Energy Security* **4**, 178-202, doi:10.1002/fes3.64 (2015).

9 Pek, Z., Daood, H., Nagyne, M., Nemenyi, A. & Helyes, L. Effect of environmental conditions and water status on the bioactive compounds of broccoli. *Central European Journal of Biology* **8**, 777-787, doi:10.2478/s11535-013-0172-7 (2013).

10 Verma, B., Hucl, P. & Chibbar, R. N. Phenolic content and antioxidant properties of bran in 51 wheat cultivars. *Cereal Chemistry Journal* **85**, 544-549, doi:10.1094/CCHEM-85-4-0544 (2008).

11 Mpofu, A., Sapirstein, H. D. & Beta, T. Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. *Journal of agricultural and food chemistry* **54**, 1265-1270, doi:10.1021/jf052683d (2006).

12 Environment Canada, Historical Data, available at http://climate.weather.gc.ca/ (2017), Accessed on 3rd Jan 2017.

13 Agriculture and Agri-Food Canada, The National Soil DataBase (NSDB), Available at http://sis.agr.gc.ca/cansis/nsdb/index.html, (2017), Accessed on Jan 2017.

14 Krygier, K., Sosulski, F. & Hogge, L. Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and purification procedure. *Journal of agricultural and food chemistry* **30**, 330-334, doi:10.1021/jf00110a028 (1982).

15 Shamloo, M. *et al.* Effects of genotype and temperature on accumulation of plant secondary metabolites in Canadian and Australian wheat grown under controlled environments *Scientific Reports* **7**, 9133, doi:10.1038/s41598-017-09681-5 (2017).

16 Chang, C. C., Yang, M. H., Wen, H. M. & Chern, J. C. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.* **10**, 178-182 (2002).

17 Tsen, C. C., Levi, I. & Hlynka., I. A rapid method for the extraction of lipids from wheat products. *Cereal Chemistry* **39**, 195-203 (1962).

18 Folch, J., Lees, M. & Sloane Stanley, G. H. A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of biological chemistry* **226**, 497-509 (1957).

19 Piironen, V., Toivo, J. & Lampi, A. M. Plant sterols in cereals and cereal products. *Cereal Chemistry* **79**, 148-154, doi:10.1094/cchem.2002.79.1.148 (2002).

20 Ramakrishna, A. & Ravishankar, G. A. Influence of abiotic stress signals on secondary metabolites in plants. *Plant signaling & behavior* **6**, 1720-1731, doi:10.4161/psb.6.11.17613 (2011).

21 Liu, W. *et al.* Influence of environmental factors on the active substance production and antioxidant activity in Potentilla fruticosa L. and its quality assessment. *Scientific reports* **6**, 28591, doi:10.1038/srep28591 https://www.nature.com/articles/srep28591#supplementary-information (2016).

22 Hernandez, L., Afonso, D., Rodriguez, E. M. & Diaz, C. Phenolic compounds in wheat grain cultivars. *Plant Foods Hum Nutr* **66**, 408-415, doi:10.1007/s11130-011-0261-1 (2011).

23 Martini, D. *et al.* Variation of total antioxidant activity and of phenolic acid, total phenolics and yellow coloured pigments in durum wheat (Triticum turgidum L. var. durum) as a function of genotype, crop year and growing area. *Journal of Cereal Science* **65**, 175-185, doi:http://doi.org/10.1016/j.jcs.2015.06.012 (2015).

24 Klepacka, J. & Fornal, L. Ferulic acid and its position among the phenolic compounds of wheat. *Critical reviews in food science and nutrition* **46**, 639-647, doi:10.1080/10408390500511821 (2006).

25 Mpofu, A., Sapirstein, H. D. & Beta, T. Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. *Journal of Agricultural and Food Chemistry* **54**, 1265-1270, doi:10.1021/jf052683d (2006).

26 Liu, W., Liu, J., Yin, D. & Zhao, X. Influence of Ecological Factors on the Production of Active Substances in the Anti-Cancer Plant Sinopodophyllum hexandrum (Royle) T.S. Ying. *PLoS ONE* **10**, e0122981, doi:10.1371/journal.pone.0122981 (2015).

27 Hodkinson, I. D. Terrestrial insects along elevation gradients: species and community responses to altitude. *Biological reviews of the Cambridge Philosophical Society* **80**, 489-513 (2005).

28 Damken, C., Perry, G. L. W. & Beggs., J. R. Complex habitat changes along elevational gradients interact with resource requirements of insect specialist herbivores. *Ecosphere* **3**, 123, doi:http://dx.doi.org/10.1890/ES12-00216.1 (2012).

29 Li, Y. *et al.* Total phenolic, flavonoid content, and antioxidant activity of flour, noodles, and steamed bread made from different colored wheat grains by three milling methods. *The Crop Journal* **3**, 328-334, doi:http://doi.org/10.1016/j.cj.2015.04.004 (2015).

30 Vitosh M.L. , Johnson J.W. & Mengel D.B. Nitrogen losses from soil. *Tri-state Fertilizer Recommendations for Corn, Soybeans, Wheat and Alfalfa, Extension Bulletin E-2567*, 1-3 (1995).

31 Lehmann, J. & Schroth, G. in *Trees, Crops and Soil Fertility: Concepts and Research Methods* (ed G Schroth) Ch. 7, 151-166 (Centre for Agriculture and Biosciences International, 2003).

32 Bryant, J. P., Chapin, F. S. & Klein, D. R. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* **40**, 357-368, doi:10.2307/3544308 (1983).

33 Fritz, C., Palacios-Rojas, N., Feil, R. & Stitt, M. Regulation of secondary metabolism by the carbon–nitrogen status in tobacco: nitrate inhibits large sectors of phenylpropanoid metabolism. *The plant Journal* **46**, 533-548 (2006).

34 Mohd Hafiz, I., Jaafar, H. Z. E., Rahmat, A. & Rahman, Z. A. Effects of nitrogen fertilization on synthesis of primary and secondary metabolites in three varieties of Kacip Fatimah (Labisia Pumila Blume). *International Journal of Molecular Sciences* **12**, 5238-5254, doi:10.3390/ijms12085238 (2011).

35 Majuakim, L., Ng, S. Y., Abu Bakar, M. F. & Suleiman., M. Effect of altitude on total phenolics and flavonoids in Sphagnum junghuhnianum in tropical montane forests of Borneo. *Sepilok Bulletin* **19 & 20**, 23-32 (2014).

36 Wang, X. *et al.* Total flavonoid concentrations of bryophytes from Tianmu Mountain, Zhejiang Province (China): Phylogeny and ecological factors. *PLoS One* **12**, e0173003, doi:10.1371/journal.pone.0173003 (2017).

37 Dong, J., Ma, X., Wei, Q., Peng, S. & Zhang, S. Effects of growing location on the contents of secondary metabolites in the leaves of four selected superior clones of Eucommia ulmoides. *Industrial Crops and Products* **34**, 1607-1614, doi:https://doi.org/10.1016/j.indcrop.2011.06.007 (2011).

38 Zidorn, C. & Stuppner, H. Evaluation of chemosystematic characters in the Genus Leontodon (Asteraceae). *Taxon* **50**, 115-133, doi:10.2307/1224515 (2001).

39 Bernal, M., Llorens, L., Julkunen-Tiitto, R., Badosa, J. & Verdaguer, D. Altitudinal and seasonal changes of phenolic compounds in Buxus sempervirens leaves and cuticles. *Plant physiology and biochemistry : PPB* **70**, 471-482, doi:10.1016/j.plaphy.2013.06.012 (2013).

40 Nikolic, N. *et al.* Fatty acids composition and rheology properties of wheat and wheat and white or brown rice flour mixture. *European Food Research and Technology* **227**, 1543 (2008).

41 Nurmi, T. *et al.* Distribution and composition of phytosterols and steryl ferulates in wheat grain and bran fractions. *Journal of Cereal Science* **56**, 379-388, doi:https://doi.org/10.1016/j.jcs.2012.04.010 (2012).

42 Nurmi, T., Nyström, L., Edelmann, M., Lampi, A.-M. & Piironen, V. Phytosterols in wheat genotypes in the HEALTHGRAIN diversity screen. *Journal of agricultural and food chemistry* **56**, 9710-9715, doi:10.1021/jf8010678 (2008).

TRANSITION STATEMENT 2

As one of the main objective of this thesis is to evaluate how genotype, growth year and location variations translate into physiologic relevance, we decided to evaluate if the phenolic acids in wheats with different genetic background grown in different environments would inhibit the glucose uptake in a cell model for small intestinal transport.

We established that genotype and environmental shifts greatly influence the content of wheat secondary metabolites and as such, to evaluate the effects of those changes on health benefits of whole wheat products is required. It had been shown that the polyphenols found in other plants, such as flavonoids and particularly quercetin, inhibit glucose transport of intestinal membrane. However, the effects of whole wheat phenolic acids on inhibition of glucose uptake into Caco-2 cell have not yet been studied. This mechanism is of specific interest in the treatment and prevention of type-2 diabetes.

In the following manuscript, we evaluated how the contents of phenolic acids in wheats vary by genotype and environmental shifts, and how these changes affect the cellular glucose transporter inhibitions. This is the first report on inhibition of glucose transporters by phenolic acids extracted from whole wheat.

CHAPTER 4

MANUSCRIPT 3

This manuscript is under revision by the Journal of Nutrition and Metabolism

INHIBITION OF INTESTINAL CELLULAR GLUCOSE UPTAKE BY PHENOLICS EXTRACTED FROM WHOLE WHEAT GROWN AT DIFFERENT LOCATIONS

Maryam Shamloo[a,c], Peter JH Jones[a,c] , and Peter K Eck[b]*

Affiliations: [a]Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB R3T 2N2, Canada. [b]Department of Human Nutritional Sciences, W569 Duff Roblin Building, 190 Dysart Road, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada. [c]Department of Food Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada.

*Corresponding coauthor: Peter K Eck[b]

4.1 ABSTRACT

Diets high in whole grains are linked to reduced risk of type 2 diabetes. This is associated with a variety of compounds accumulated in whole grains, mainly polyphenols. The class and quantity of polyphenols vary within and between the cereal grains based on their genetic background and the growing environments. A number of major polyphenols, such as phenolic acids and flavonoids had been shown to have a lower glycemic index through inhibition of intestinal glucose transport proteins. This study was conducted to evaluate if the phenolic acids in wheats with different genetic background grown in different environments would inhibit the glucose uptake in a cell model for small intestinal transport.

The inhibitory effects of phenolic acids, extracted from eight wheat varieties representing different commercial classes including winter, spring and durum wheat grains, grown at different locations over two crop years, on the glucose uptake in human CaCo-2E cells were determined. Free and bound phenolic acids extracts of all wheat genotypes were effective inhibitors of glucose uptake. Here, wheat genotype AC Barrie grown at AB in the 2011 crop year and AC Crystal grown at AB in the 2010 crop year showed the highest glucose uptake inhibition of 56.32 %, and 21.22 % for free and bound phenolic acids extracts, respectively.

The degree of glucose uptake inhibitions positively correlated with the content of free and bound phenolic acids; the correlation coefficient were R^2=0.91 and R^2 =0.89, respectively. Genotype and environmental conditions influenced the content of free and bound phenolic acids which linearly translated to the degree of glucose uptake inhibition in a model of intestinal absorption. ($P < 0.05$).

Results of the present study could also enable the selection of particular wheat grains to be used as a nutritious food source to control postprandial hyperglycemia in diabetic patients.

4.2 INTRODUCTION

Diabetes mellitus has become an emerging global health problem. Currently, an estimated 422 million adults are suffering from diabetes and that number will increase to 642 million by 2040 [1]. Type 2 diabetes is characterized by the body's insensitivity to insulin and is largely the result of increased sugar uptake and lack of physical activity [2]. In diabetic patients, the expression of intestinal glucose transporters has been reported to be 3 to 4-fold higher than healthy controls [3], therefore, a higher amount of glucose will be absorbed by these patients in a shorter period of time, leading to increased postprandial glycemia. Thus, an effective treatment option for diabetes and diabetes-related complications is to dampen or inhibit intestinal glucose transporters and/or glucose absorption.

Wheat is the main ingredient in a range of staple foods that form part of a healthy diet [4]. Regular intake of whole grain products is associated with a 20-30 % reduction in the risk of type 2 diabetes [5,6]. The whole wheat bread has a lower glycemic index than white bread [7], which is associated to polyphenols found in the bran fraction of whole wheat [8]. These polyphenols are known to be effictive in lowering blood glucose levels and ameliorating oxidative stress [9]. On the other hand, compelling evidence reveals that genetic and growing environments play a critical role in influencing the accumulation of secondary plant metabolites [10], including polyphenols. Winter wheat varieties contain twice as many total phenolic acids than the average level of 175

wheat genotypes [11]. Free phenolic acids of 26 wheat genotypes grown in Hungry in three consecutive crop years were largely influenced by the effect of environment, whereas bound phenolic acids were stable throughout the different growing environments and were more influenced by the genetic variation effect [12].

The mechanism of how whole grains might reduce rapid increases in blood glucose is unknown [13]. Some plant bioactives inhibit intestinal glucose transporters [14,15] via blunted glucose absorption [7,13,16].

We observed extracts of the free phenolic acids from the whole grains of wheat genotypes grown under controlled environments inhibited glucose uptake into CaCo-2 cell monolayers, however, to our best knowledge, there are no studies investigating the free and bound phenolic acids extracts of different wheat varieties grown in different environmental conditions to explore to what extent free and bound phenolic acids inhibit glucose uptake in a CaCo-2 model of intestinal absorption.

4.3 MATERIALS AND METHODS

4.3.1 WHEAT GENOTYPES

The samples were obtained from eight western Canadian wheat genotypes (*Triticum spp*), including AC Corrine, AC Barrie, AC Crystal & Carberry, Snowbird, AC Andrew (*Triticum aestivum L.*), AC Navigator & Strongfield durum wheat grains (*Triticum turgidum L. var. durum*). Characteristics of wheat varieties and their related Canadian categories are described

previously (Table 3.1). All wheat genotypes were grown at three locations over 2010 and 2011 crop years.

4.3.2 LOCATIONS

Three locations were selected to grow wheats including the Cereal Grain Research Centers of the Lethbridge (Alberta (AB)), Indian Head (Saskatchewan (SK)) and Portage La Prairie (Manitoba (MB)).

Environmental data conditions and locations characteristics and soil composition data were obtained from Statistics Canada [17] and Agriculture and Agri-Food Canada [18], respectively. A brief summary of the location characteristics, soil composition, and heading and maturity dates of the wheat genotypes was presented previously (Table 3.2). In 2010, the mean temperature was lower than 2011 in all three locations, and the total precipitation was higher.

4.3.3 MATERIALS

Phenolic acids standards were purchased from Sigma-Aldrich (Milwaukee, Wisconsin, USA). All acids and organic solvents were obtained from Fisher Scientific (Whitby, Ontario, Canada). All chemicals used were of analytical grade.

4.3.4 PREPARATION OF FREE AND BOUND PHENOLIC ACIDS EXTRACTS

The whole wheat samples were prepared as explained in a previous report [19]. Briefly, they were milled and passed through a 0.5mm sieve screen. The fine flour from each sample was stored at -20°C in the dark until analysis. Free and bound phenolic acid extraction processes were performed using liquid-liquid extraction and an alkaline hydrolysis steps [20] as described before [19].

4.3.5 HPLC-PDA ANALYSIS OF FREE AND BOUND EXTRACTS

Phenolic acids of free and bound fractions were identified by a reverse phased performance liquid chromatography system (Waters 2695, Milford, MA, USA) equipped with a photodiode array detector (PDA) (Waters 996), and auto-sampler (717 plus, Waters, Milford, MA, USA) as described by Shamloo *et al* [19].

4.3.6 GLUCOSE UPTAKE INHIBITION ACTIVITY ASSAYS

4.3.6.1 INHIBITION OF GLUCOSE UPTAKE INTO CACO-2E ENTEROCYTE MONOLAYERS

The ability of wheat phenolic acids extracts to reduce cellular glucose uptake was investigated through the modified method described by Kwon *et al*., [14]. Confluent caco-2 cells grown on 96-well plates were rinsed 3 times with PBS and incubated in pre-incubation buffer (HEPES buffer with 5 mM glucose) for 30 minutes at 37°C. Transport experiments were initiated by replacing the pre-incubation buffer with 100 µL transport buffer (HEPES buffer, pH 7.4, glucose free)

supplemented with [^{3}H] 2-deoxyglucose (5 mM) and wheat extracts. Transport buffer with no wheat extracts used as the positive control. The cells were incubated at room temperature for 15 minutes in the dark and transport experiment was stopped by adding 100 µL of ice cold pre-incubation buffer immediately after removal of transport buffer. Cells were washed with 100 µL of pre-incubation buffer and then lysed with 60 µL lysis buffer (20 mg SDS in 1 mL 0.2M NaOH) and incubated at room temperature for 1 hour. 45 µL aliquot of cell lysates was added to 5ml scintillation cocktail and the [^{3}H] 2-deoxyglucose concentration was quantified by scintillation spectrometry. The protein content of the remaining cell lysates was determined using DC Protein Assay Kit (Bio-Rad, USA). The glucose uptake into Caco-2E cells was expressed as counts per minute beta (cpma) per mg protein. The viability of cells and validity of assay was demonstrated by the linearity of the uptake rates of glucose in the absence of wheat extracts.

4.3.7 STATISTICAL ANALYSIS

All cell culture data represent means of three experimental sets. Each of the experimental sets consisted of three parallel transport experiments (n = 6). All data were calculated on a one way analysis of variance (ANOVA) on a Minitab 14 Statistical software (Minitab Inc., State College, PA, USA). Sample means were compared using Tukey HSD method and significant differences were considered when $P < 0.05$. Correlations between wheat extract phenolic acid and flavonoid contents and inhibition capacity were done by Pearson's correlation test.

4.4 RESULTS AND DISCUSSION

4.4.1 GENOTYPE AND ENVIRONMENTAL VARIATION INFLUENCE ON PHENOLIC ACIDS LEVELS

Phenolic acid (PA) contents in the free and bound fractions of the eight wheat varieties grown at different locations are listed in Table 4.1 and Table 4.2, respectively. Free PA contents across all wheat genotypes grown during 2010 and 2011, ranged from 4.56 microgram per gram of dry matter (μg/g dm) in Strongfield grown at Manitoba (MB) over the crop year 2010 to 40.98 μg/g dm in AC Barrie, grown at Alberta (AB) over the crop year 2011. In all locations, contents of free PA in wheat varieties of AC Navigator and AC Barrie were significantly lower in 2010 than 2011. Same trend was observed for AC Corrine grown at MB and SK, Snowbird grown in AB, AC Crystal grown at MB and AB, and Strongfield grown at MB and SK. Conversely, AC Andrew grown at SK and AB contained significantly higher levels of free PA in 2010 than 2011. For all other genotypes grown at MB, SK and AB, no significant changes in the levels of free PA was identified over the 2010 and 2011 crop years. Bound PA contents across all wheat genotypes grown during 2010 and 2011, ranged from 366.1 μg/g dm (AC Navigator, MB, 2010) to 690.12 μg/g dm (AC Crystal, AB, 2010). AC Corrine grown at all locations, AC Navigator grown at SK and AB, Snowbird grown in AB, AC Andrew grown at SK and AB, AC Crystal grown at MB and AB, AC Barrie grown in MB and Strongfield grown in AB had significantly higher levels of bound PA in 2010 compared to 2011. AC Navigator grown in MB, Snowbird grown in SK, Carberry grown in MB, AC Crystal grown in SK, AC Barrie grown at SK and AB, and Strongfield grown in SK had significantly lower amounts of bound PA in 2010 compared to 2011.

In accordance with the current results, the genotype had previously been shown to influence the levels of polyphenols of wheat cultivars [21]. Overall, crop year, genotypes and growing locations influenced how the wheat plant responses to biotic and abiotic conditions and hence the production of free and bound phenolic acids.

Table 4.1. Free phenolic acids content (microgram per gram of dry matter) in the whole grain of 8 wheat varieties grown in three locations in 2010 and 2011 crop years.

Genotype	Year	Growing Locations		
		MB	**SK**	**AB**
AC Corrine	2010	13.93±0.98[a]	11.56±1.27[a]	11.08±1.09[a]
	2011	25.11±2.92[b]	21.68±2.65[b]	11.16±1.82[a]
AC Navigator	2010	11.19±1.07[a]	11.14±1.67[a]	11.10±2.93[a]
	2011	16.13±1.81[b]	24.07±2.94[b]	16.53±2.87[b]
Snowbird	2010	23.91±1.73[a]	16.79±2.90[a]	11.17±2.43[a]
	2011	23.98±1.09[a]	20.28±2.31[a]	21.79±0.95[b]
AC Andrew	2010	14.75±1.60[a]	18.34±1.01[a]	16.62±0.09[a]
	2011	14.34±0.62[a]	13.81±1.21[b]	11.24±1.01[b]
Carberry	2010	18.93±3.20[a]	11.10±0.29[a]	16.31±2.07[a]
	2011	16.72±1.90[a]	11.15±2.35[a]	16.55±1.98[a]
AC Crystal	2010	4.680±0.63[a]	11.14±2.85[a]	11.49±0.93[a]
	2011	24.03±1.01[b]	11.42±2.39[a]	21.43±1.02[b]
AC Barrie	2010	11.12±1.29[a]	11.06±0.93[a]	24.83±2.87[a]
	2011	18.51±2.76[b]	18.92±2.01[b]	40.98±2.81[b]
Strongfiled	2010	4.560±2.90[a]	11.03±1.76[a]	20.34±2.09[a]
	2011	20.91±1.87[b]	21.33±0.98[b]	21.29±2.76[a]

Values are means ± SD from duplicate determinations.
[a,b] Different superscripts capital letters in the same column in the same dependent variable indicate significant difference ($P < 0.05$).
MB= Manitoba, SK=Saskatchewan, AB=Alberta.

Table 4.2. Bound phenolic acids content (microgram per gram of dry matter) in the whole grain of 8 wheat varieties grown in three locations in 2010 and 2011 crop years.

Genotype	Year	Growing Locations		
		MB	SK	AB
AC Corrine	2010	537.41±5.23[a]	555.51±5.44[a]	607.17±9.19[a]
	2011	519.54±6.31[b]	489.23±3.33[b]	582.34±8.32[b]
AC Navigator	2010	366.11±5.03[a]	463.47±4.65[a]	641.60±9.03[a]
	2011	522.78±7.42[b]	422.61±3.98[b]	467.28±6.17[b]
Snowbird	2010	471.71±6.54[a]	450.01±5.87[a]	528.61±5.54[a]
	2011	488.32±7.30[a]	487.24±2.63[b]	483.01±4.59[b]
AC Andrew	2010	492.97±5.20[a]	521.54±4.93[a]	486.34±8.98[a]
	2011	495.96±4.82[a]	461.28±5.75[b]	457.65±6.46[b]
Carberry	2010	421.35±7.17[a]	551.45±5.43[a]	600.87±7.34[a]
	2011	544.86±8.43[b]	472.96±4.54[b]	481.57±8.22[b]
AC Crystal	2010	481.24±9.32[a]	509.15±6.38[a]	690.12±7.56[a]
	2011	415.12±6.82[b]	545.49±10.43[b]	629.27±6.41[b]
AC Barrie	2010	560.98±8.38[a]	417.86±7.77[a]	414.11±5.89[a]
	2011	433.89±5.16[b]	547.56±6.09[b]	528.81±7.94[b]
Strongfiled	2010	459.11±6.32[a]	430.32±5.98[a]	566.96±6.89[a]
	2011	458.68±5.22[a]	509.01±8.89[b]	525.81±3.37[b]

Values are means ± SD from duplicate determinations.
[a,b] Different superscripts capital letters in the same column in the same dependent variable indicate significant difference ($P < 0.05$).
MB= Manitoba, SK=Saskatchewan, AB=Alberta.

4.4.2 EFFECT OF FREE AND BOUND PHENOLIC ACIDS ON GLUCOSE UPTAKE

Free phenolic acid extracts of all whole wheat varieties grown at MB, SK and AB over 2010 and 2011inhibited glucose uptake in confluent CaCo-2E monolayers as shown in **Figure 4.1** and **Figure 4.2** (Panels A-H), respectively. For 2010 crop year, extracts from AC Barrie, grown in AB showed the highest inhibitory potency (46.18 %) (Figure 1 (G)), followed by Snowbird grown in MB (45.12 %) (Figure 1 (C)). For 2011 crop year, again extracts from AC Barrie, grown in AB showed the highest inhibitory potency (56.32 %) (Figure 2 (G)), followed by AC Corrine grown at MB (48.62 %) (Figure 2 (A)). The free phenolic acids content of all wheat genotypes correlated positively the degree of inhibition of glucose uptake, as shown in **Figure 4.3A** (R^2=0.903; $P < 0.05$).

Bound phenolic acid extracts of all wheat genotypes grown at MB, SK and AB over 2010 and 2011 also inhibited the uptake of glucose in confluent CaCo-2E monolayers, as shown in **Figure 4.4** and **Figure 4.5** (Panels A-H), respectively.

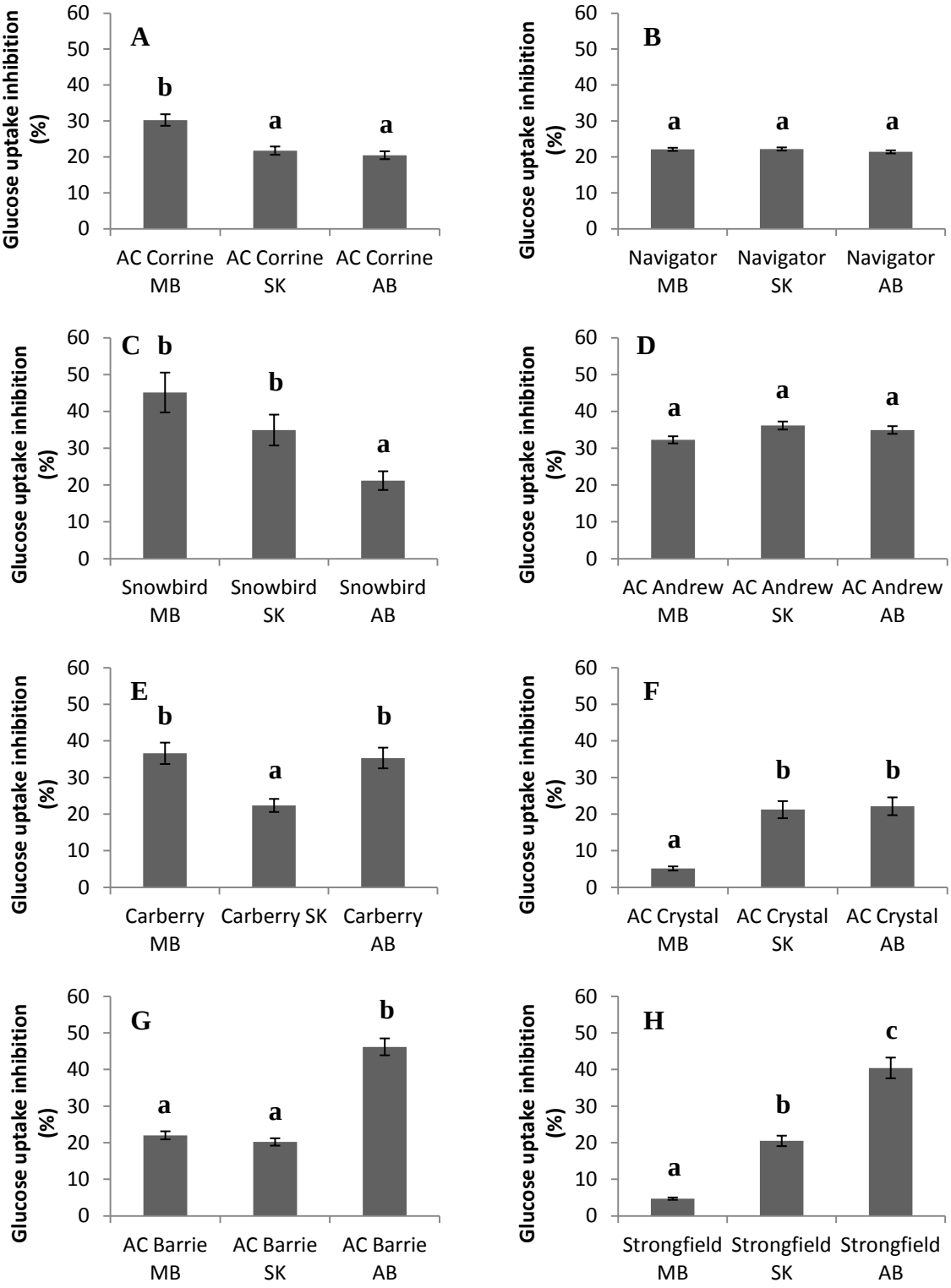


Figure 4.1. Relative inhibition of the uptake of [3H] 2-Deoxyglucose into CaCo-2 monolayers caused by extracts of free phenolic acids obtained from eight wheat genotypes (panels A-H) grown in MB, SK, and AB over 2010 crop year. [a,b,c] Different small letter superscripts indicate significant differences ($P < 0.05$).

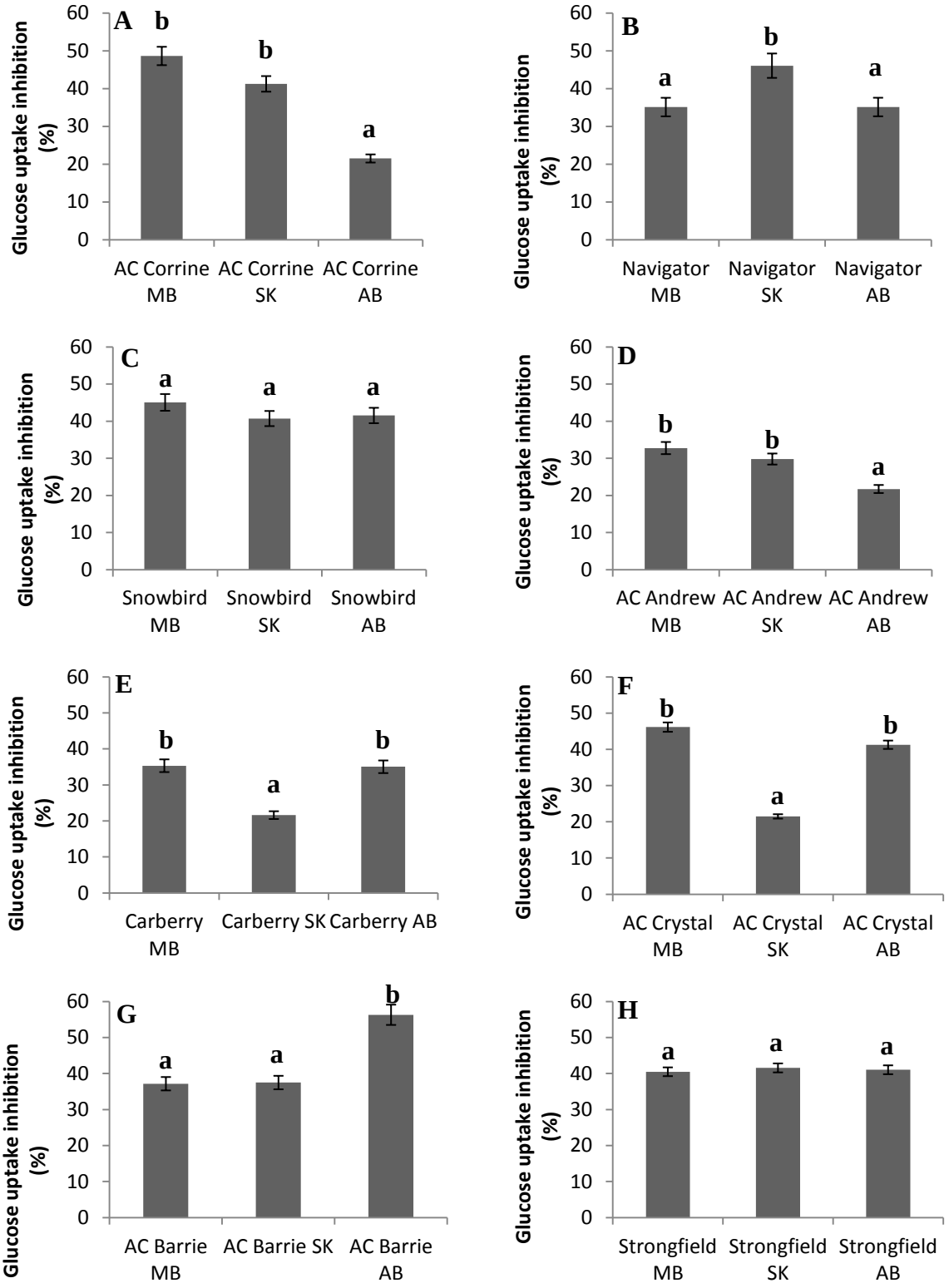


Figure 4.2. Relative inhibition of the uptake of [3H] 2-Deoxyglucose into CaCo-2 monolayers caused by extracts of free phenolic acids obtained from eight wheat genotypes (panels A-H) grown in MB, SK, and AB over 2011 crop year. [a,b,c] Different small letter superscripts indicate significant differences ($P < 0.05$).

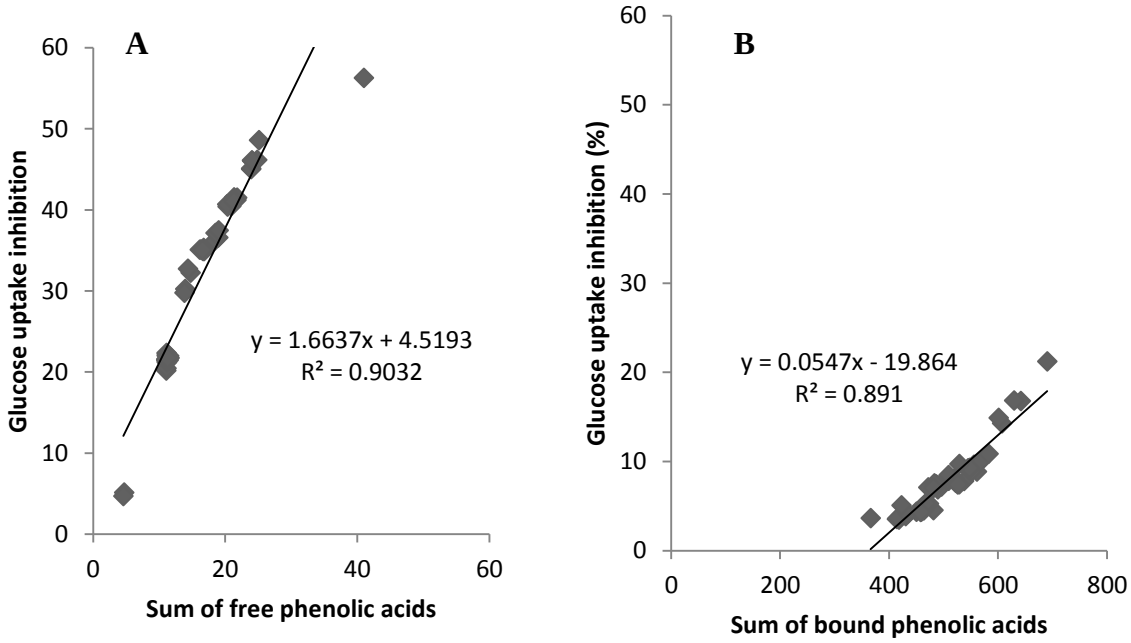


Figure 4.3. Correlation between the relative inhibition of glucose uptake into CaCo-2 monolayers and free phenolic acid (A), and bound phenolic acid (B) contents in extracts of eight wheat genotypes grown in MB, SK, and AB over 2011 crop year.

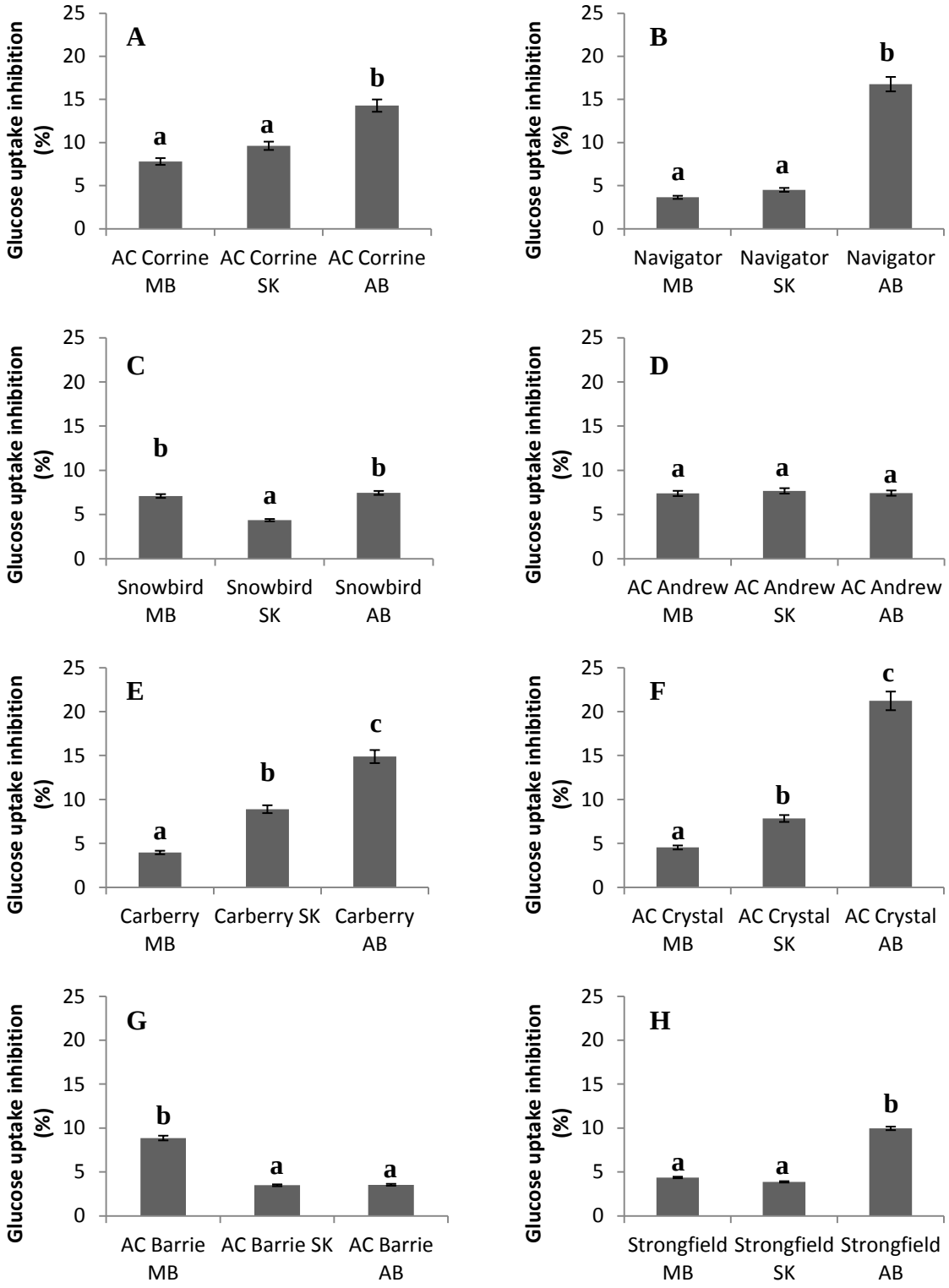


Figure 4.4 Relative inhibition of the uptake of [3H] 2-Deoxyglucose into CaCo-2 monolayers caused by extracts of bound phenolic acids obtained from eight wheat genotypes (panels A-H) grown in MB, SK, and AB over 2010 crop year. [a,b,c] Different small letter superscripts indicate significant differences ($P < 0.05$).

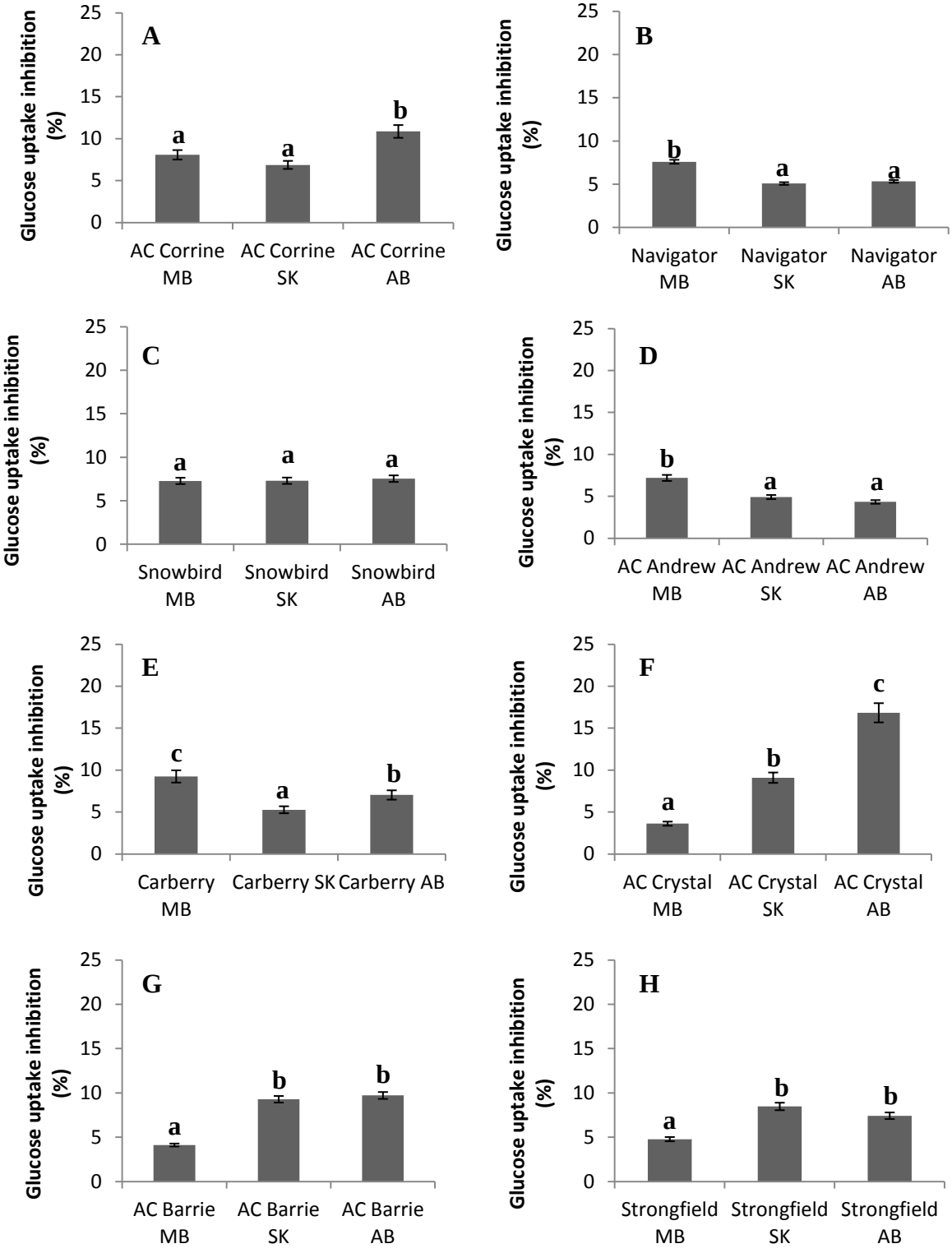


Figure 4.5. Relative inhibition of the uptake of [3H] 2-Deoxyglucose into CaCo-2 monolayers caused by extracts of bound phenolic acids obtained from eight wheat genotypes (panels A-H) grown in MB, SK, and AB over 2011 crop year. [a,b,c] Different small letter superscripts indicate significant differences ($P < 0.05$).

The highest inhibitory potencies of bound phenolic extracts were observed in AC Crystal (21.22 %) and AC Navigator (16.78 %) grown in AB in the 2010 crop year (**Figure 4.4 (F and B)**), respectively. In comparison to the free phenolic acids, the bound extracts showed a lower degree of glucose uptake inhibition for all wheat genotypes.

The degree of glucose uptake inhibitions positively correlated with the content of bound phenolic acids, as depicted in **Figure 4.3B** (R^2=0.891; $P < 0.05$). Our results, therefore, suggest that phenolic acids inhibit glucose uptake in free or bound form and the effect of free phenolic acids is higher than that of bound phenolic acids.

Free phenolic acids are bioavailable in the small intestine [22]. However, both free and bound forms of phenolic acids inhibit glucose transporters located on the apical pole of the enterocyte, as shown by our Caco-2model, indicating that phenolic acids as a component found in the whole wheat products could be useful in dampening postprandial hyperglycemia and by adding the whole wheat products to the diet the glycemic response can be improved. As reported previously [23], the most abundant compound in both free and bound phenolic acids extracts was identified as ferulic acid and it has prior been reported that daily dietary uptake of 77 mg of ferulic acid may effectively suppress hyperglycemia [22]. Wheat bran is one of the main food sources of ferulic acid (5mg/g) [24]. However, the daily intake of (free or bound) ferulic acid largely depends on the selection of cereal products. It had been estimated that whole wheat and refined wheat bread contain 330.1 μg/g and 25.3 μg/g ferulic acid, respectively [25], and therefore choosing the whole wheat products over the refined products clearly influence the physiologic efficacy.

4.5 CONCLUSIONS

Free and bound phenolic acids of wheat grains inhibited glucose uptake in a model of intestinal absorption, indicating efficacy in the control of postprandial blood glucose spikes; however, wheat genotype background and growing environmental conditions significantly influenced the content of these free and bound phenolic acids and hence shift the ability of wheat phenolic acids extracts to reduce cellular glucose uptake.

Daily consumption of whole wheat products may increase the public health through reduction of incidence of type 2 diabetes.

4.6 ACKNOWLEDGEMENTS

This research was funded in part by Manitoba Science and Technology International Collaboration Fund (STIC) and Food Advancement through Science and Training (FAST) program.

Authors would like to thank Drs. Susan Arntfield and Nancy Ames, for their assistance with finding the wheat samples through Cereal Research Centre, Canadian Wheat Board and Agriculture and Agri-Food Canada, and Dennis Labossiere, Tracy Exley, Kim Kuzminski and Camille Rhymer for their technical assistance.

4.7 REFERENCES

1 World Health Organization, Global report on diabetes. (2016) Available at: http://apps.who.int/iris/bitstream/10665/204871/1/9789241565257_eng.pdf. (Accessed on 3rd Jan 2017).

2 American Diabetes, A. Diagnosis and classification of diabetes mellitus. *Diabetes Care* **32**, S62-S67, doi:10.2337/dc09-S062 (2009).

3 Dyer, J., Wood, I. S., Palejwala, A., Ellis, A. & Shirazi-Beechey, S. P. Expression of monosaccharide transporters in intestine of diabetic humans. *American journal of physiology. Gastrointestinal and liver physiology* **282**, G241-248, doi:10.1152/ajpgi.00310.2001 (2002).

4 Lafiandra, D., Riccardi, G. & Shewry, P. R. Improving cereal grain carbohydrates for diet and health. *Journal of cereal science* **59**, 312-326, doi:10.1016/j.jcs.2014.01.001 (2014).

5 Gil, A., Ortega, R. M. & Maldonado, J. Wholegrain cereals and bread: a duet of the Mediterranean diet for the prevention of chronic diseases. *Public health nutrition* **14**, 2316-2322, doi:10.1017/s1368980011002576 (2011).

6 Belobrajdic, D. P. & Bird, A. R. The potential role of phytochemicals in wholegrain cereals for the prevention of type-2 diabetes. *Nutr J* **12**, 62, doi:10.1186/1475-2891-12-62 (2013).

7 Ludwig, D. S. & Eckel, R. H. The glycemic index at 20 y? *American Journal of Clinical Nutrition* **76**, 264S-265S. (2002).

8 Scalbert, A. & Williamson, G. Dietary intake and bioavailability of polyphenols. *The Journal of nutrition* **130**, 2073s-2085s (2000).

9 Pandey, K. B. & Rizvi, S. I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity* **2**, 270-278 (2009).

10 Ramakrishna, A. & Ravishankar, G. A. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior* **6**, 1720-1731, doi:10.4161/psb.6.11.17613 (2011).

11 Li, L., Shewry, P. R. & Ward, J. L. Phenolic acids in wheat varieties in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry* **56**, 9732-9739, doi:10.1021/jf801069s (2008).

12 Fernandez-Orozco, R., Li, L., Harflett, C., Shewry, P. R. & Ward, J. L. Effects of environment and genotype on phenolic acids in wheat in the HEALTHGRAIN diversity screen. *J Agric Food Chem* **58**, 9341-9352, doi:10.1021/jf102017s (2010).

13 Belobrajdic, D. P. & Bird, A. R. The potential role of phytochemicals in wholegrain cereals for the prevention of type-2 diabetes. *Nutrition Journal* **12**, 62, doi:10.1186/1475-2891-12-62 (2013).

14 Kwon, O. *et al.* Inhibition of the intestinal glucose transporter GLUT2 by flavonoids. *Faseb Journal* **21**, 366-377, doi:10.1096/fj.06-6620com (2007).

15 Song, J. *et al.* Flavonoid inhibition of sodium-dependent vitamin C transporter 1 (SVCT1) and glucose transporter isoform 2 (GLUT2), intestinal transporters for vitamin C and Glucose. *The Journal of biological chemistry* **277**, 15252-15260, doi:10.1074/jbc.M110496200 (2002).

16 Scalbert, A., Morand, C., Manach, C. & Remesy, C. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **56**, 276-282 (2002).

17 Environment Canada, Historical Data, available at http://climate.weather.gc.ca/ (2017), Accessed on 3rd Jan 2017.

18 Agriculture and Agri-Food Canada, The National Soil DataBase (NSDB), Available at http://sis.agr.gc.ca/cansis/nsdb/index.html, (2017), Accessed on Jan 2017.

19 Shamloo, M. *et al.* Effects of genotype and temperature on accumulation of plant secondary metabolites in Canadian and Australian wheat grown under controlled environments. *Scientific Reports* **7**, 9133, doi:10.1038/s41598-017-09681-5 (2017).

20 Krygier, K., Sosulski, F. & Hogge, L. Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and purification procedure. *Journal of Agricultural and Food Chemistry* **30**, 330-334, doi:10.1021/jf00110a028 (1982).

21 Hernandez, L., Afonso, D., Rodriguez, E. M. & Diaz, C. Phenolic compounds in wheat grain cultivars. *Plant Foods for Human Nutrition* **66**, 408-415, doi:10.1007/s11130-011-0261-1 (2011).

22 Malunga, L. N., Eck, P. & Beta, T. Inhibition of Intestinal alpha-Glucosidase and Glucose Absorption by Feruloylated Arabinoxylan Mono- and Oligosaccharides from Corn Bran and Wheat Aleurone. *Journal of Nutrition and Metabolism*, doi:10.1155/2016/1932532 (2016).

23 Shamloo, M., Eck, P. K. & Jones, P. J. Effects of Genotype and Environment on Secondary Metabolites of Canadian Wheat Grains. *Journal of Agriculture and Food Chemistry* **Submitted**, x (2017).

24 Boz, H. Ferulic acid in cereals – a Review. *Czech Journal of Food Sciences* **33**, 1-7 (2015).

25 Yu, L., Nanguet, A.-L. & Beta, T. Comparison of antioxidant properties of refined and whole wheat flour and bread. *Antioxidants* **2**, 370-383, doi:10.3390/antiox2040370 (2013).

TRANSITION STATEMENT 3

We investigated the changes in secondary wheat metabolites for whole wheat grains grown at different field conditions and observed that the environmental variations had the largest impact on the levels of secondary compounds in wheat. Among the environmental factors, the temperature had the largest influence on secondary metabolites levels.

Considering the global climate change, it is important to investigate effects of increased temperature on secondary metabolites of wheat grains and related impacts on nutritional values as wheat include the large proportion of individual's diet around the world. Previous studies reported that raising the growth temperature will increase the levels of plants secondary metabolites and particularly polyphenols.

In the following manuscript, we investigated if different growing temperatures could alter the accumulation of secondary metabolites in wheat grains differing in their genetic background. This is the first study to evaluate the temperature effect on whole wheat secondary metabolites grown under the controlled environment.

CHAPTER 5

MANUSCRIPT4

This manuscript was published in the Scientific Reports

(Scientific Reports 2017; 7: 9133 Copyright © 2017)

EFFECTS OF GENOTYPE AND TEMPERATURE ON ACCUMULATION OF PLANT SECONDARY METABOLITES IN CANADIAN AND AUSTRALIAN WHEAT GROWN UNDER CONTROLLED ENVIRONMENTS

Maryam Shamloo[a,c], Elizabeth A. Babawale[a], Agnelo Furtado[d], Robert J. Henry[d], Peter K. Eck[b] and Peter J. H. Jones[a,c]**

Affiliations: [a]Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB R3T 2N2, Canada. [b]Department of Human Nutritional Sciences, W569 Duff Roblin Building, 190 Dysart Road, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada. [c]Department of Food Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada. [d]Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia QLD 4072, Australia.

*Corresponding author: Peter JH Jones[a,c]

5.1 ABSTRACT

Predictions of global increased temperature are for 1.8-4ºC by 2100. Increased temperature as an abiotic stress may exert a considerable influence on the levels of secondary metabolites in plants. These secondary metabolites may possibly exert biological activities beneficial in prevention or treatment of disorders linked to oxidative stress in the human. Wheat secondary compounds in three Canadian and three Australian genotypes were grown under controlled environments, in which the only changing parameter was temperature, were investigated. Kennedy and AC Navigator contained the highest amount of total phenolic acids among Australian and Canadian wheat genotypes, respectively. The total phenolic acids and total flavonoid contents of wheat genotypes increased following the increase of the growing temperature. In all the wheat genotypes, regardless of their growing temperatures, linoleic acid (C18:2n6) was measured as the main fatty acid. Significant increases in palmitic acid (C16:0) and oleic acid (C18:1n9) and significant decreases in linoleic acid (C18:2n6) and linolenic acid (C18:3n3) were observed at increased of growing temperature for all wheat genotypes. Growing temperature decreased campesterol content of wheat genotypes. Genotype and growing temperature significantly shifted the production of wheat secondary metabolites. This information might be used as a guide for breeding wheat varieties with higher antioxidant properties.

5.2 INTRODUCTION

Wheat (*Triticum* spp.) is a major food cereal in the world grain market. The total global wheat production was around 711.2 million tons in 2013 [1]. Some wheat genotypes have been reported to have high levels of secondary bioactive metabolites including phenolic acids (PAs), flavonoids, and phytosterols [2]. The phenolic group in polyphenols and flavonoids can accept an

electron to form relatively stable phenoxyl radicals, thereby disrupting chain oxidation reactions in cellular components and therefore, limit the risk of various degenerative diseases associated with oxidative stress such as diabetes, chronic cardiovascular diseases and cancer [3-5]. Genotype and growing factors such as temperature and light affect the levels of phenolics and phytosterols in wheat grains as previously reported [6,7]. However, most studies only evaluated the polyphenols and phytosterols of wheat grains grown in different geographical farm fields, while information on how genotypes and temperature impact the secondary metabolites levels of wheat grown under controlled conditions is limited.

As defined by the Intergovernmental Panel on Climate Change (IPCC, 2007), global warming relates to the increase in mean temperature that has been observed since the mid-twentieth century resulting from anthropogenic emissions of greenhouse gases into the atmosphere [8]. The IPCC's fourth assessment predicted that the global temperature will increase by 1.8 to 4.0°C by 2100. Increased temperature as an abiotic stress may exert a considerable influence on the levels of secondary metabolites in plants including wheat grains [9]. Therefore, considering the rapidly accelerating environmental changes on local and global scales, it is important to understand how secondary metabolites levels and composition change by increased temperature as such knowledge may have significant implications on nutritional values of wheat grains.

In order to adapt to temperature stress, plants use different protective mechanisms ranging from structural to biochemical [10]. One of the important biochemical defense mechanism is through enhanced production of secondary compounds. Phenolic acids and flavonoids are two important groups of plant secondary compounds, which are suggested to protect plants against abiotic

stresses such as drought or increased temperature through their antioxidant properties to remove reactive oxygen species (ROS) before they oxidize cell walls and membranes [11,12]. Elevated temperatures could increase the production of phenolics and flavonoids in plants, probably through the activation of their catalytic enzymes [13]. For instance, strawberry grown at increased temperature regimes had higher values of phenolic acid, flavonols, anthocyanins, and antioxidant capacities [14]. Jeong *et al*., [15] reported a synergistic effect between high night-time temperatures and cultivation duration which produced lettuce rich in polyphenols compared to that at low temperature. Similarly, more soluble phenolics were measured in sugarcane (*Saccharum officinarum*) sprouts grown at 40/35˚C than those grown at 28/23˚C (day/night) temperatures [16]. However, to our knowledge, information concerning the influence of increased temperature on secondary compounds within cereal grains, including wheat, remains unknown. The purpose of the current study, therefore, was to investigate whether different wheat genotypes and/or growing temperatures could alter the accumulation of secondary metabolites in wheat grains differing in their genetic background.

5.3 METHODS

5.3.1 EXPERIMENTAL DESIGN.

Three separate growth chambers (GR192) were used to control all environmental factors and provide three temperature regimes, at the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, Canada, 2015, where the temperature was altered and other environmental factors including photoperiod, carbon-dioxide levels, humidity and wind velocity were fixed. The chambers shared a common, re-circulating nutrient solution

developed for wheat. Photosynthetic photon flux (PPF) was provided with cool-white, VII0 fluorescent lamps. The photoperiod was set to be 20-h. The temperature was maintained at 20±0.2°C, 25±0.2°C and 30±0.2°C in growth chambers 1, 2, and 3, respectively. Chamber CO_2 concentration was controlled by mixing pure CO_2 with outside air. Air flow into each plant growth chamber was maintained at 30 L min^{-1} to provide a rapid air turnover rate (once per minute).

The experiment was a completely randomized block design (using 6 wheat genotypes) within three temperature treatments and three replications (individual plants). Each variety was grown as three replicates and each replicate consisted of a minimum of five potted plants, i.e., a total of 15 plants per variety (as described in Appendix 1). The seeds of each genotype were harvested at maturity, manually cleaned and air-dried until a moisture content of 10% ± 0.5 was reached. The dried samples from each replicate were individually vacuum-packed in moisture-proof packaging and stored at -20°C in the dark until analysis.

5.3.2 WHEAT GENOTYPES

Six Wheat Genotypes (*Triticum Spp*) Were Used In This Study As Follows:

-Three Canadian wheat genotypes: 1. AC Crystal, red spring wheat (*Triticum aestivum L.*), 2. AC Navigator durum wheat (*Triticum turgidum L. var. durum*), 3. Carberry, a hard red spring wheat (*Triticum aestivum L.*), were kindly donated by Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg, MB, Canada.

-Three Australian wheat genotypes: 1. Kennedy, quick maturing spring wheat (*Triticum aestivum L.*), 2. Fango60, drought tolerant wheat (*Triticum aestivum L.*), 3. EGA Gregory, hard spring wheat (*Triticum aestivum L.*), were kindly donated from Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, QLD, Australia.

5.3.3 SAMPLE PREPARATION

The whole wheat samples were milled using an ultracentrifugal mill (Model ZM 200, Retsch, Haan, Germany) using rpm of 14,000 and passed through a 0.5mm sieve screen. The fine flour from each sample was individually vacuum-packed in moisture-proof packaging and stored at -20°C in the dark until analysis. Four categories of bioactives, including phenolic acids, flavonoids, fatty acids and plant sterols, extracted separately and analyzed from these growth chamber grown wheat grains.

5.3.4 FREE AND BOUND PHENOLIC ACID EXTRACTION

The extraction was performed using liquid-liquid extraction and alkaline hydrolysis steps [17] with slight modification. Briefly, Wheat flour (0.6 g) was extracted twice with ethyl acetate at a ratio of 1:20 (w/v). Each time, the mixture was kept on a mechanical shaker (Thermo/Lab-Line/Barnstead MAX Q 5000, Artisian Scientific, Champaign, IL, USA) for 1 h at room temperature. After centrifuging (Model Sorvall Legend RT, Thermo Electron Corporation, Osterode, Germany) at 3750*g* for 10 min, the supernatants obtained from each time were combined and concentrated to dryness by using an analytical nitrogen rotary evaporator (Model N-EVAP 112, Organomation Assocuates, Inc, Berlin, MA, USA) at 30°C. The dried extract was

re-suspended in 1.2 mL of 50% dimethyl sulfoxide (DMSO)-ethanol as crude extracts and kept in a sealed amber vial container at 4°C. This extract was referred to as free fraction. The dried residue obtained from crude extraction was hydrolyzed with 18 mL of 4 M NaOH on a shaker (Thermo/Lab-Line/Barnstead MAX Q 5000, Artisian Scientific, Champaign, IL, USA) under nitrogen gas for 4 h. After digestion, the solution was adjusted to a pH 1.5–2.0 with 6 M ice-cold HCl and then extracted with 12 mL of ethyl acetate three times. After centrifuging (Model Sorvall Legend RT, Thermo Electron Corporation, Osterode, Germany) at 3750*g* for 10 min, the combined ethyl acetate fractions were evaporated to dryness and reconstituted in 1.2 mL of 50% dimethyl sulfoxide (DMSO)-ethanol and kept in a sealed amber vial container at 4°C. This extract obtained from residues was referred as bound fraction. Both fractions were directly subjected to HPLC analysis. Prior to HPLC analysis, they were filtered through a 0.45 μm syringe filter.

5.3.5 HPLC-PDA ANALYSIS

The HPLC (Waters 2695, Milford, MA, USA) equipped with a photodiode array detector (PDA) (Waters 996), and auto-sampler (717 plus, Waters, Milford, MA, USA) used to analyze phenolic acids. A 250 × 4.6 mm, 5 μm RP 18 column (Shim-pack HRC-ODS, SHIMADZU Corp., Tokyo, Japan) was used for separation. Each sample (20 μl) was injected via an auto-sampler and eluted through the column with a gradient mobile phase consisting of A (0.1% acetic acid in water) and B (0.1% acetic acid in methanol) with a flow rate of 0.5 ml/min.

A 75 min linear gradient was programmed as follows: 0-11min, 9-14% B; 11-14min, 14-15% B; 14-17min, 15% B;17-24 min, 15-16.5% B; 24-28 min, 16.5-19% B; 28-30 min, 19-25% B; 30-36 min, 25-26% B; 36-38 min, 26-28% B; 38-41 min, 28-35% B; 41-46 min, 35-40% B; 46-48 min, 40-48% B; 48-53 min, 48-53% B; 53-70 min, 53-70% B; 70-72 min, 70-9% B; 72-75 min; 9% B. The peaks of phenolic acids were detected at a wavelength of 280 nm. The quantification of phenolic acid content were calculated using external calibration curves of gallic acid (0.001 to 0.01 mg/ml, equation: y=0.0021x+1.0216), protocatechiuic acid (0.001 to 0.01 mg/ml, equation: y=0.0036x+0.3842), p-hydroxybenzoic acid (0.00099 to 0.0099 mg/ml, equation: y=0.0022x+0.2571), vanillic (0.001 to 0.01 mg/ml, equation: y=0.0017x+0.6503), caffeic (0.001 to 0.01 mg/ml, equation: y=0.0022x+0.2152), syringic (0.001 to 0.01 mg/ml, equation: y=0.001x+0.2548), p-coumaric (0.001 to 0.01 mg/ml, equation: y=0.0007x+0.3395), ferulic (0.001 to 0.01 mg/ml, equation: y=0.0011x+0.2972), sinapic (0.001 to 0.01 mg/ml, equation: y=0.0025x+0.2613), isoferulic (0.001 to 0.01 mg/ml, equation: y=0.0009 x +0.2747), o-coumaric (0.001 to 0.01 mg/ml, equation: y=0.0006+ 0.1171).

5.3.6 TOTAL FLAVONOID CONTENT

Flavonoid contents of wheat fractions were assayed using the aluminum chloride colorimetric method of Chang *et al* [18]. The appropriate dilution of extracts (0.5 ml) were mixed with1.5 ml of 95% ethanol, followed by 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a UV/Visible spectrophotometer (Model DU 800, Beckman Coulter, Inc., CA, USA). The flavonoid content was calculated using a standard

calibration of rutin solution and expressed as micrograms of rutin equivalent (RE) per gram of sample.

5.3.7 FATTY ACID PROFILE ANALYSIS

Fatty acids were extracted as described by Tsen *et al.* [19], adapted from the classic Folch method [20], using chloroform-methanol (2:1, volume to volume (v/v)) containing 0.01% butylated hydroxytoluene (Sigma-Aldrich, Oakville, Ontario, Canada), followed by methylation with methanolic HCl. Fatty acid methyl esters were then analyzed using an Agilent 6890 N (Agilent Technologies, Mississauga, ON, Canada) gas chromatograph (GC) equipped with a flame ionization detector. During the extraction and methylation, heptadecanoic acid (C17:0) was used as an internal standard (Sigma-Aldrich, Oakville, Ontario, Canada). Known fatty acid standards (Sigma-Aldrich, Oakville, Ontario, Canada) were used to identify the individual fatty acids in wheat samples. The level of each fatty acid was then calculated according to the corresponding peak area relative to the total area of total interested fatty acids and considered as a percentage of the total fatty acids.

5.3.8 PHYTOSTEROL EXTRACTION AND DERIVATIZATION

The procedure used for phytosterol extraction included acid and alkaline hydrolyses and was based on the method of Piironen *et al* [21]. The internal standard dihydrocholesterol (DHC, 40 μg) was first added into a 0.5 gram cereal sample. The sample was then subjected to acid hydrolysis with hydrochloric acid (HCl) to liberate sterols from their glycosidic conjugates. After acid hydrolysis and the extraction of lipids, alkaline hydrolysis with potassium hydroxide (KOH)

saponified the lipids and hydrolysed the esterified sterols into free sterols. The unsaponifiable lipids (containing free sterols) were extracted into cyclohexane and purified by solid-phase extraction (SPE) using silica cartridges (Strata SI-1, 500 mg, Phenomenex, Torrance, CA, USA). Prior to the gas chromatographic analysis, phytosterols were derivatized to trimethylsilyl (TMS) ethers using N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA, Fisher Scientific, Grand Island, NY, USA) and trimethylchlorosilane (TMCS, Sigma Aldrich, Oakville, ON, Canada) in a ratio of 99:1 (v/v) as the reagents in anhydrous pyridine (Sigma Aldrich, Oakville, ON, Canada). Each sample was analyzed in duplicate.

5.3.9 PHYTOSTEROL GAS CHROMATOGRAPHIC ANALYSIS

Phytosterols were analyzed using an Agilent 6890 N gas chromatograph (Agilent Technologies, Mississauga, ON, Canada) with flame ionization detection (FID) and an on-column injector. The GC was equipped with a SAC-5 silica capillary column (30 m × 0.25 mm × 0.25 μm, Supelco Inc., Bellefonte, PA, USA). Peak identification was accomplished by comparing the retention times with those of a standard mixture of pure sterols. Quantification of phytosterols was performed using dihydrocholesterol as the internal standard.

5.3.10 STATISTICAL ANALYSIS

All data were reported as means ± SD of triplicate independent experiments. The main effects of genotype and environment and their interaction were investigated by one way or general linear model ANOVA with Minitab 14 Statistical software (Minitab Inc., State College, PA, USA). Significant differences were considered when ($P < 0.05$) unless stated otherwise.

5.4 RESULTS

5.4.1 YIELD

The durum wheat variety AC Navigator, which was developed for the Canadian prairies, when grown at 30^{o}C did not develop grains. All other varieties prevalent in Canada (AC Crystal and Carberry), or Australia (Kennedy, Fango60, and EGA Gregory) did develop grains at 20^{o}C, 25^{o}C and 30^{o}C.

Table 5.1, summarizes the grain yield in each temperature regime. Increased temperatures resulted in decreased wheat grain yields for all genotypes and for AC Navigator, there was no grain produced at 30^{o}C. Adverse impact of higher temperatures on grain yields are in line with previous studies [22-24].

Table 5.1. Thousand kernel weight (g) of six wheat varieties grown in controlled temperatures.

Growing Temperature	Genotypes					
	AC Crystal	AC Navigator	Carberry	Kennedy	Fango60	EGA Gregory
20^{o}C	46.50^{Ac}	43.35^{Ab}	51.82^{Bb}	44.93^{Ac}	57.16^{Cc}	46.00^{Ab}
25^{o}C	38.89^{Bb}	32.09^{Aa}	33.33^{Aa}	36.68^{Ab}	48.34^{Cb}	42.21^{Bb}
30^{o}C	22.45^{Aa}	na	29.32^{Ba}	28.56^{Ba}	35.67^{Ca}	26.76^{Aa}

^{A,B,C,D} Different superscripts capital letters in the same row indicate significant difference ($P < 0.05$).
^{a,b,c} Different superscripts small letters in the same column indicate significant difference ($P < 0.05$).
na = data not available, no grain produced.

5.4.2 PHENOLIC ACIDS

Phenolic acids (PA), in each of the six wheat varieties grown at three temperatures are listed in Table 5.2. The mean value of total PAs, ranged from 389.54±32.36 (Fango60, 20^{o}C) to 1007.61±87.32 mg/kg dm (Kennedy, 30^{o}C). The highest content of total PAs, was observed for the genotype of Kennedy grown at 30^{o}C (1007.61±87.32 mg/kg dm), followed by Kennedy grown at 25^{o}C (906.56±46.17 mg/kg dm), and AC Navigator grown at 20^{o}C (857.65±50.90 mg/kg dm). The lowest value was determined for Fango60 grown at 20^{o}C (389.54±32.36 mg/kg dm). Based on the wheat genotypes, the mean value of total PAs was as follows: Kennedy > AC Navigator > AC Crystal > Carberry > EGA Gregory > Fango60.

Table 5.2. Free, bound and total phenolic acids content (microgram per gram of dry matter (dm)) in the whole grain of six wheat varieties grown in controlled environments.

Growing Environment	Phenolic acids	Genotypes					
		AC Crystal	AC Navigator	Carberry	Kennedy	Fango60	EGA Gregory
20°C	Free	6.520±0.02 ^{Ea}	7.040±0.02 ^{Fa}	5.820±0.03 ^{Ca}	6.270±0.01 ^{Da}	5.020±0.03 ^{Aa}	5.480±0.03 ^{Ba}
	Bound	701.3±36.6 ^{Ca}	850.6±50.9 ^{Da}	546.2±16.2 ^{Ba}	674.3±55.6 ^{Ca}	384.5±32.3 ^{Aa}	493.8±41.91 ^{ABa}
	Total	707.8±36.6 ^{Ca}	857.6±50.9 ^{Da}	552.0±16.2 ^{Ba}	680.6±55.6 ^{Ca}	389.5±32.3 ^{Aa}	499.3±41.88 ^{ABa}
25°C	Free	9.900±0.01 ^{Eb}	9.350±0.02 ^{Db}	9.190±0.03 ^{Cb}	10.99±0.04 ^{Fb}	8.040±0.02 ^{Ab}	8.450±0.03 ^{Bb}
	Bound	723.6±84.3 ^{Ca}	820.0±95.1 ^{CDa}	614.1±62.6 ^{BCab}	895.6±46.1 ^{CDb}	465.4±39.3 ^{ABab}	526.4±58.3 ^{ABa}
	Total	733.5±84.3 ^{Ca}	829.4±95.1 ^{CDa}	623.3±62.5 ^{BCab}	906.5±46.1 ^{CDb}	473.4±39.3 ^{ABab}	534.9±58.3 ^{ABa}
30°C	Free	14.48±0.04 ^{Dc}	na	13.70±0.04 ^{Cc}	16.61±0.03 ^{Ec}	12.31±0.01 ^{Ac}	12.69±0.01 ^{Bc}
	Bound	743.6±47.3 ^{Ca}	na	698.2±31.9 ^{BCb}	991.0±87.3 ^{Db}	557.6±37.9 ^{Ab}	600.8±55.0 ^{ABa}
	Total	758.1±47.3 ^{Ca}	na	711.9±32.0 ^{BCb}	1007 ±87.3 ^{Db}	569.9±37.9 ^{Ab}	613.5±55.0 ^{ABa}

^{A,B,C,D,E,F} Different superscripts capital letters in the same row indicate significant difference ($P < 0.05$).

^{a,b,c} Different superscripts small letters in the same column in the same dependent variable indicate significant difference ($P < 0.05$).

na = data not available, no grain produced.

Irrespective of genotype, wheat grains grown at 30^{o}C produced higher amount of PAs followed by those grown at 25^{o}C, and 20^{o}C, respectively, except for the genotype of AC Navigator, in which, the total PAs of wheat grown at 20^{o}C was higher than 25^{o}C, and where no grain was produced by the plant at 30^{o}C. Increased growing temperature, resulted in a significant (P < 0.05) increase in free phenolics for all genotypes, as the free phenolics of all genotypes grown at 30^{o}C was twice to three times more than that for those grown at 20^{o}C. In addition, in all wheat samples grown, without considering the genotypes and the growing temperature, the contents of bound phenolics were significantly higher than free ones (P < 0.001).

The relative distribution of individual identifiable PAs across free and bound phenolic acid fractions for different wheat genotypes grown at 20^{o}C, 25^{o}C, and 30^{o}C are shown in **Figures** 5.**1**, 5.**2**, and 5.**3**, respectively. In the bound PA fraction, ferulic acid contents were highest across all varieties, averaging 81.7%, followed by sinapic acid (7.8%), p-coumaric acid (4.3%), vanillic acid (2.4%), p-hydroxybenzoic acid (2.0%), and lastly by syringic acid (1.6%). The relative distribution of bound PA did not significantly differ between wheat varieties. For free PA, ferulic acid was most abundant, averaging 51.4%, followed by vanillic acid (28.4%), p-coumaric acid (6.7%), sinapic acid (5.3%), p-hydroxybenzoic acid (4.2%), syringic acid (3.7%) and gallic acid (0.5%). The relative distribution of free PA did not significantly differ between wheat varieties.

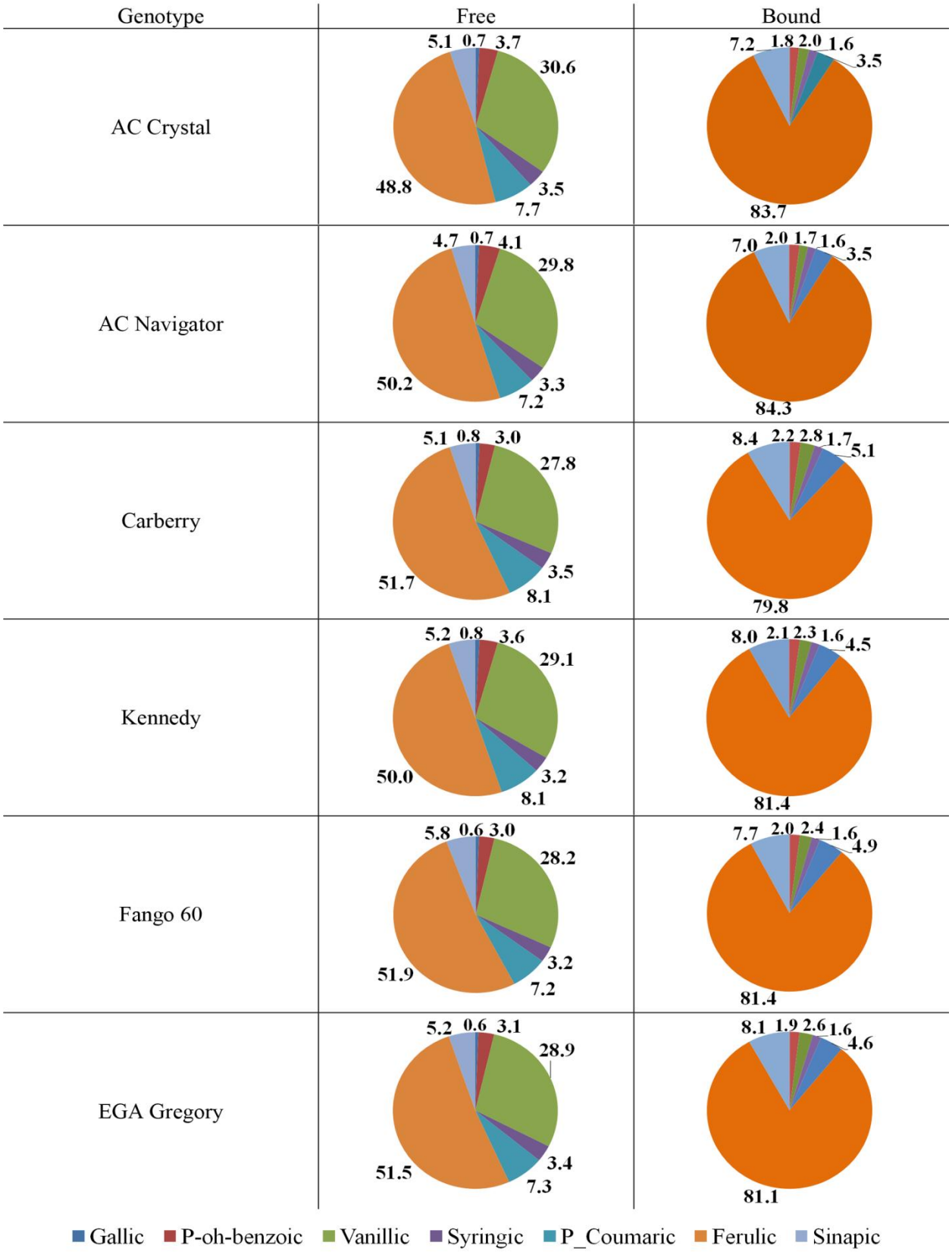


Figure 5.1. Relative distribution (%) of individual phenolic acids across free and bound fractions of six wheat varieties grown at 20°C.

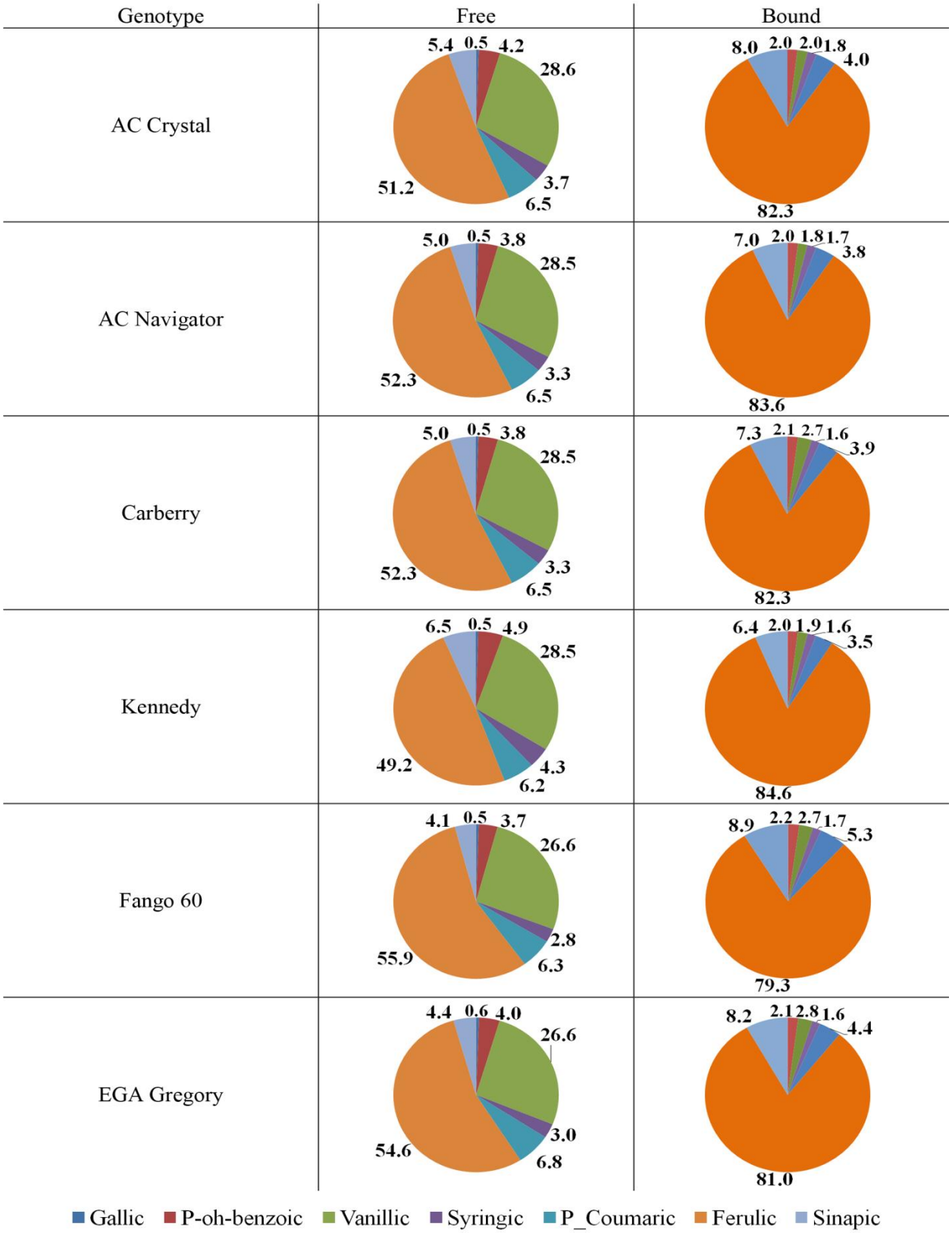
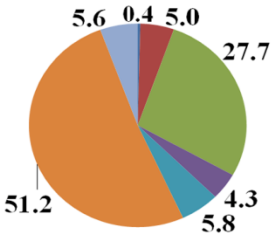
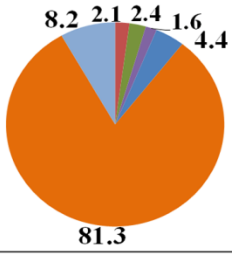
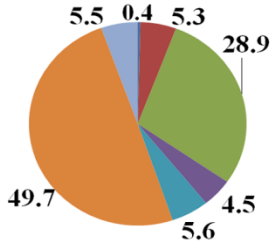
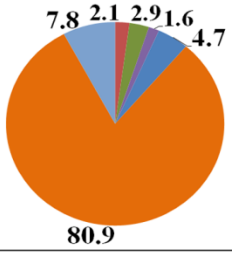
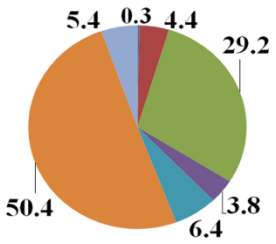
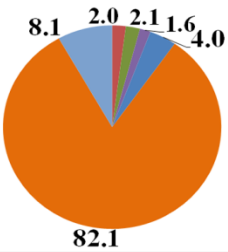
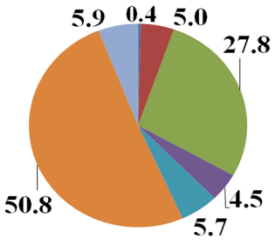
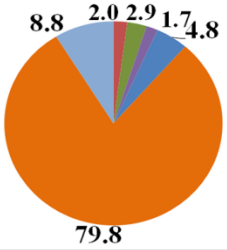
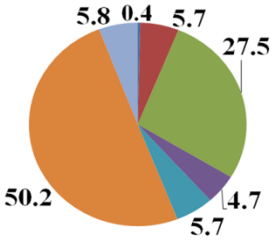
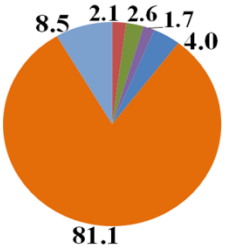


Figure 5.2. Relative distribution (%) of individual phenolic acids across free and bound fractions of six wheat varieties grown at 25°C.

Genotype	Free	Bound
AC Crystal		
AC Navigator	No Sample	No Sample
Carberry		
Kennedy		
Fango 60		
EGA Gregory		

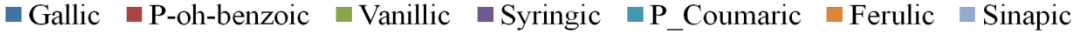


Figure 5.3. Relative distribution (%) of individual phenolic acids across free and bound fractions of six wheat varieties grown at 30°C.

5.4.3 FLAVONOIDS

Figure 5.4, shows the total flavonoid content in the grains of six wheat varieties grown in controlled environments. All genotypes showed significantly ($P < 0.05$) increased total flavonoids at increased growing temperatures. The lowest amounts of total flavonoid contents were observed in Fango60 and EGA Gregory grown at 20^{o}C, with 170.05 ± 2.24 and 187.59 ± 2.48 μg rutin equivalent/g dm, respectively. The highest amounts of total flavonoids were observed in Kennedy and AC Crystal grown at 30^{o}C, with 343.23 ± 3.03 and 328.6 ± 8.67 μg rutin equivalent/g dm, respectively.

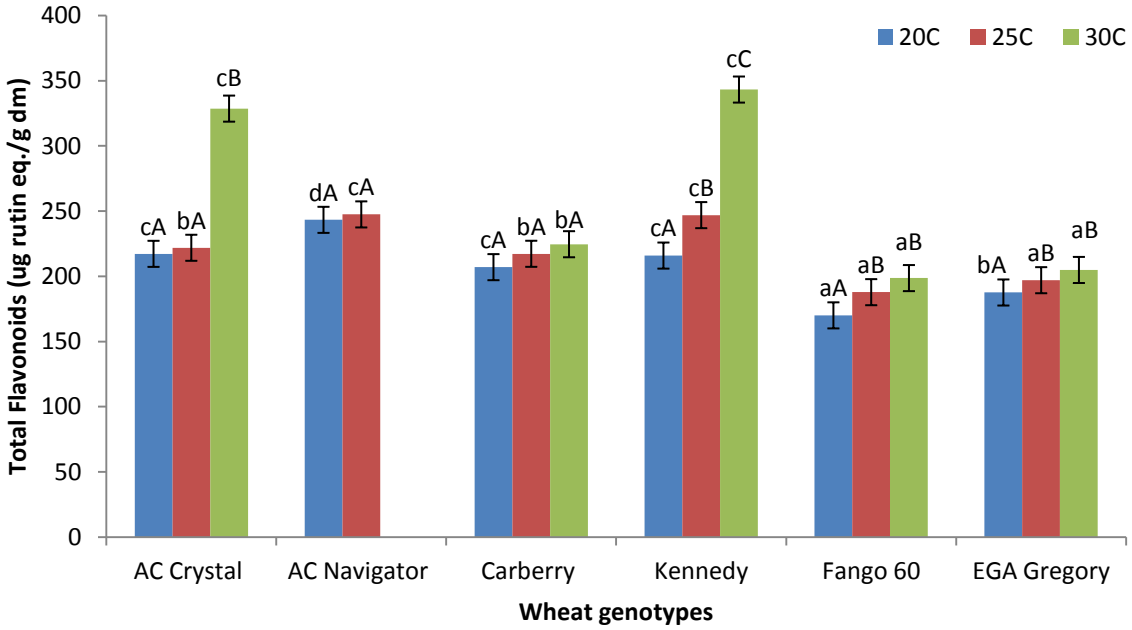


Figure 5.4. Total flavonoid contents (microgram rutin equivalent per gram of dry matter) of six wheat genotypes grown in controlled environments. [a,b,c] Different small letter superscripts indicate significant differences ($P < 0.05$) between genotypes grown at the same temperature. [A,B,C] Different capital letters superscripts indicate significant differences ($P < 0.05$) within the same genotype grown under different temperatures. (Data not available for AC Navigator grown at 30 ^{o}C).

5.4.4 FATTY ACIDS

Total fatty acid profiles of the six wheat genotypes grown under the different controlled environments are summarized in Table 5.3. All fatty acid concentrations were calculated as percentage values of total identified fatty acids measured. In all wheat genotypes, regardless of their growing temperatures, linoleic acid (C18:2n6) was measured as the main fatty acid ranging from 49.47 % (EGA Gregory grown at 30^{o}C) to 57.73 % (Carberry grown at 20^{o}C). Significant increases in palmitic acid (C16:0) and oleic acid (C18:1n9) and significant decreases in linoleic acid (C18:2n6) and linolenic acid (C18:3n3) were observed at increased growing temperatures for all wheat genotypes. Polyunsaturated fatty acids (PUFA) ranged from 50.4 to 59 % of fatty acids profile and were two to three times greater than that of monounsaturated fatty acids (MUFA). The wheat varieties displayed different PUFA compositions regardless of growing conditions (Table 5.3). Generally, unsaturated fatty acid levels were three to four times higher than saturated fatty acid levels.

5.4.5 PHYTOSTEROL PROFILES

Table 5.4 shows the phytosterol compositions of 6 wheat genotypes grown under controlled environments. The total phytosterol content ranged from 604.5 µg/g dm (AC Crystal, 30^{o}C) to 1120.2 µg/g dm (AC Navigator, 25^{o}C). Sitosterol was the most abundant phytosterol in all wheat genotypes, regardless of their growing temperatures, accounting for 38-45 % of the total sterols, followed by campesterol (14-18 %), sitostanol (15-21 %), campestanol (12-18 %) and stigmasterol (1-3 %). Growing temperature significantly decreased ($P < 0.05$) campesterol content of wheat genotypes growing at 30^{o}C compared to 25^{o}C, while other phytosterols did not

Table 5.3. Contents (%) of fatty acid profile of six wheat genotypes grown under controlled environments.

Growing Temperature	Fatty acids	Genotypes					
		AC Crystal	AC Navigator	Carberry	Kennedy	Fango60	EGA Gregory
20°C	C16:0	20.26[a]	17.73[a]	18.35[a]	18.16[a]	19.03[a]	19.36[a]
	C18:0	0.920	1.040	1.310	1.200	0.950	1.110
	C18:1n9	16.74[a]	18.88[a]	16.96[a]	19.05[a]	16.76[a]	19.09[a]
	C18:2n6	55.74[a]	56.82[a]	57.73[a]	55.81[a]	57.55[a]	54.98[a]
	C18:3n3	4.420[a]	3.400[a]	3.560[a]	3.290[a]	3.990[a]	4.080[a]
	Others	1.910	21.12	2.100	2.490	1.700	1.370
	SFA	21.59	19.72	20.87	20.86	21.12	21.87
	MUFA	17.08	19.43	19.47	16.95	18.16	24.24
	PUFA	56.86[A]	57.48[A]	55.53[AB]	59.05[A]	57.19[AB]	51.75[B]
	UFA	78.41	80.28	79.13	79.14	78.88	78.13
	UFA/SFA	3.630	4.070	3.790	3.790	3.740	3.570
25°C	C16:0	20.18[ab]	17.90[a]	19.49[ab]	19.07[ab]	21.00[ab]	19.56[ab]
	C18:0	0.91	1.27	0.980	1.030	1.440	1.360
	C18:1n9	16.01[a]	20.18[a]	16.79[a]	19.86[a]	16.62[a]	17.80[a]
	C18:2n6	57.40[a]	55.86[a]	57.33[a]	55.71[a]	56.03[a]	56.06[a]

	C18:3n3	3.81^{ab}	2.850^{ab}	3.070^{ab}	2.97^{ab}	3.290^{ab}	3.510^{ab}
	Others	1.700	1.940	2.340	1.370	1.610	1.700
	SFA	19.13	20.18	21.29	20.29	21.95	22.18
	MUFA	19.45	17.10	16.33	20.46	18.35	21.85
	PUFA	57.96^{A}	58.70^{A}	58.54^{AB}	56.27^{A}	56.21^{AB}	53.10^{B}
	UFA	80.87	79.82	78.71	79.71	78.05	77.82
	UFA/SFA	4.230	3.960	3.700	3.930	3.560	3.510
30^{o}C	C16:0	20.37^{b}	na	20.94^{b}	20.32^{b}	20.46^{b}	20.84^{b}
	C18:0	1.170	na	1.100	1.150	1.300	1.340
	C18:1n9	17.99^{b}	na	23.66^{b}	23.76^{b}	21.42^{b}	23.97^{b}
	C18:2n6	55.11^{b}	na	49.51^{b}	50.74^{b}	52.06^{b}	49.47^{b}
	C18:3n3	3.420^{b}	na	2.330^{b}	2.590^{b}	2.810^{b}	2.670^{b}
	Others	1.930	na	2.450	1.440	1.940	1.340
	SFA	20.02	na	19.52	23.07	22.47	22.60
	MUFA	17.46	na	20.58	16.95	24.13	24.45
	PUFA	58.89^{A}	na	56.98^{AB}	56.59^{A}	50.50^{AB}	50.46^{B}
	UFA	79.98	na	78.71	76.93	77.53	77.40
	UFA/SFA	4.000	na	3.70	3.330	3.450	3.430

[a,b] Different small letters superscripts indicate significant differences in the same column in the same dependent variable ($P < 0.05$).

[A,B] Different capital letters superscripts in the same row indicate significant differences ($P < 0.05$). na = data not available.

Table 5.4. Phytosterol contents (microgram per gram of dry matter) of six wheat genotypes grown under controlled environments.

Growing Temperature	Plant sterols	Genotypes					
		AC Crystal	AC Navigator	Carberry	Kennedy	Fango60	EGA Gregory
20°C	Sitosterol	358.3^{B}	395.9^{BC}	316.1^{AB}	423.7^{C}	400.2^{BC}	287.7^{A}
	Campesterol	148.1^{abB}	188.7^{abC}	127.1^{abAB}	169.4^{abBC}	164.4^{abB}	119.6^{abA}
	Sitostanol	127.3^{AB}	195.9^{D}	128.9^{AB}	158.4^{C}	145.4^{BC}	102.2^{A}
	Campestanol	106.2^{ABC}	188.8^{D}	99.30^{AB}	124.2^{C}	118.0^{BC}	87.50^{A}
	Stigmasterol	12.27^{AB}	22.9^{D}	11.40^{A}	15.00^{C}	14.00^{BC}	15.10^{C}
	Others	37.60^{B}	50.5^{C}	30.80^{A}	38.10^{B}	38.30^{B}	27.20^{A}
	Total	790.1^{BC}	1042.9^{E}	713.8^{AB}	929.1^{DE}	880.5^{CD}	639.6^{A}
25°C	Sitosterol	352.0^{B}	428.7^{D}	346.1^{B}	449.7^{D}	397.8^{C}	299.4^{A}
	Campesterol	140.2^{aA}	192.0^{aC}	136.7^{aA}	182.7^{aBC}	167.5^{aB}	121.8^{aA}
	Sitostanol	151.2^{AB}	222.7^{D}	162.6^{BC}	209.6^{D}	181.8^{C}	131.4^{A}
	Campestanol	110.2^{A}	197.5^{D}	114.8^{AB}	150.0^{C}	130.9^{B}	103.0^{A}
	Stigmasterol	12.40^{A}	23.70^{C}	11.50^{A}	15.60^{B}	12.90^{AB}	13.30^{AB}
	Others	41.60^{AB}	55.40^{C}	36.90^{AB}	54.50^{C}	47.80^{BC}	30.90^{A}
	Total	807.9^{B}	1120^{D}	809.0^{B}	1062^{D}	938.9^{C}	700.0^{A}

30^{o}C	Sitosterol	259.1^{A}	na	360.2^{BC}	349.3^{B}	386.4^{C}	370.0^{BC}
	Campesterol	90.50^{bA}	na	110.8^{bB}	110.2^{bB}	133.9^{bC}	126.7^{bC}
	Sitostanol	126.3^{A}	na	156.3^{B}	169.5^{B}	175.0^{B}	168.2^{B}
	Campestanol	87.30^{A}	na	97.80^{AB}	100.6^{BC}	109.7^{C}	106.1^{BC}
	Stigmasterol	11.10^{A}	na	19.60^{C}	15.20^{B}	15.60^{B}	19.40^{C}
	Others	29.90^{A}	na	41.00^{B}	31.80^{A}	48.00^{B}	47.00^{B}
	Total	604.5^{A}	na	785.5^{B}	776.9^{B}	868.8^{C}	837.6^{BC}

^{a,b} Values with different superscripts in the same column in the same dependent variable are significantly different ($p \leq 0.05$)

^{A,B,C,D,E} Values with different superscripts in the same row are significantly different ($p \leq 0.05$). Abbreviations; na = data not available.

show significant variation due to the growing environment. Overall, the wheat grown at 25^{o}C contained higher amounts of phytosterols compared to other temperatures although the values were not statistically significant, except for EGA Gregory in which the total phytosterol value was significantly higher at 30 ^{o}C than other temperatures. Among the wheat genotypes, the mean value of total phytosterols follows AC Navigator (1081.5 µg/g dm) > Kennedy (922.7 µg/g dm) > Fango60 (896.0 µg/g dm) > Carberry (769.4 µg/g dm) > AC Crystal (734.1 µg/g dm) > EGA Gregory (725.7 µg/g dm). The variation was much higher when it came to wheat genotypes for each growing environment, as wheat genotypes significantly ($P < 0.05$) altered all the individual phytosterols regardless of growing conditions as presented in Table 5.4.

5.4.6 EFFECTS OF GENOTYPE AND TEMPERATURE ON WHEAT PHENOLIC ACIDS

As presented in Table 5.5 genotype, environment and their interactions significantly ($P < 0.01$) influenced phenolic acid levels including free, bound and total phenolic acids.

5.4.7 EFFECTS OF GENOTYPE AND TEMPERATURE ON WHEAT FLAVONOIDS

Total flavonoids influenced significantly by genotype, environment and genotype-by-environment interaction. Genotype-by-environment interaction effect was larger than genotype or environment effect individually (Table 5.5).

5.4.8 EFFECTS OF GENOTYPE AND TEMPERATURE ON WHEAT FATTY ACIDS

Genotype variations had higher influence on C16:0, C18:0, and SFA concentrations, where C18:2n6, C18:3n9, PUFA, UFA, UFA/SFA, PUFA/MUFA levels were mostly influenced by environmental changes. Genotype-by-environment interactions had higher influence only on C18:1n9 and MUFA as indicated in Table 5.5. In general, the greater effect of environment compared to wheat genotypes was observed for fatty acid profiles.

Table 5.5. Analysis of variance: Influence of genotype, environment and genotype environment interaction, on each compound class and each individual metabolite of six wheat genotypes grown under controlled environments.

Classes/metabolites	**ANOVA significance (Mean square values)**		
	Genotype (*df* 5)	**Environment (*df* 2)**	**G×E (*df* 10)**
Phenolic acids			
Free phenolics	36.05*	**142.9***	36.58*
Bound phenolics	**176272***	30419*	159503*
Total phenolics	**179838***	30483*	164322*
Free phenolics			
Gallic acid	0.0000003*	0.0000002**	**0.0000004***
P-oh-benzoic	0.0000514*	**0.0004060***	0.0000631*
Vanillic	0.0019348*	**0.0066585***	0.0019014*
Syringic	0.0000425*	**0.0002598***	0.0000448*
P-coumaric	0.0000925*	**0.0001454***	0.0000808*
Ferulic	0.0053631*	**0.0231092***	0.0058324*
Sinapic	0.0000922*	**0.0003282***	0.0000000*
Bound phenolics			
P-oh-benzoic	**0.0000167***	0.0000031*	0.0000166*
Vanillic	**0.0000215***	0.0000037*	0.0000148*
Syringic	**0.0000117***	0.0000032*	0.0000107*
P-coumaric	0.0000536*	0.0000029*	**0.0000580***
Ferulic	**0.0319640***	0.0062590*	0.0278880*
Sinapic	0.0002175*	0.0000159**	**0.0002247***
Flavonoids			

Total flavonoids content	15138*	815*	**17127***
Fatty acids			
C16:0	**94.3490***	26.267*	62.917*
C18:0	**0.20440***	ns	0.18133*
C18:1n9	73.53*	1.97**	**100.637***
C18:2n6	450.44*	**1110.41***	443.98*
C18:3n3	3.3321*	**10.1768***	0.9317*
Others	0.58909*	0.54828*	**0.69966***
SFA	**106.797***	29.554*	72.615*
MUFA	76.350*	1.992**	**104.922***
PUFA	468.21*	**1152.94***	462.44*
UFA	935.5*	**1267.9***	1049.1*
UFA/SFA	1.3828*	**5.4187***	2.5248*
PUFA/MUFA	2.1351	**8.7418***	0.8051*
Plant sterols			
Sitosterol	22446*	**43208***	30529*
Campesterol	2109.1*	**21312.0***	4786.1*
Sitostanol	3139.1*	**9520.0***	8696.3*
Campestanol	1538.2*	**12442.1***	5626.4*
Stigmasterol	19.263*	15.476*	**124.931***
Other sterols	141.31*	618.75*	**645.14***
Total plant sterols	74095*	**325481***	198126*

An asterisk (*) indicates significant at $P < 0.001$; Two asterisks (**) indicate significant at $P < 0.01$; ns indicates not significant at $P < 0.05$. A bold number is the factor or interaction most influencing the variable (highest mean square for each variable); *df*: degrees of freedom. G×E: Genotypes, environment interactions.

5.4.9 EFFECTS OF GENOTYPE AND TEMPERATURE ON WHEAT PHYTOSTEROLS

Environment contributed to a higher extent on the total plant sterol level and on all individual sterols except for the stigmasterol, which was mostly influenced by genotype-by-environment interactions (Table 5.5).

5.5 DISCUSSION

This research provides new information on how genotype and growing temperature impact the production of wheat secondary metabolites, which can be used to produce the wheat products that contain higher amounts of targeted bioactives.

Increased growth temperature from 18/12^{o}C to 30/22^{o}C (day/night) for strawberry, yielded fruit with the most phenolic contents [14]. Similarly, in the present study, the total phenolic acids of wheat genotypes increased following the increase of the growing temperature. The increased levels of total phenolic acids could be related to plant's defense mechanism against temperature stress as also reported in other studies [10,25] and could be a response to the generation of ROS. Therefore, the wheat grains produced at higher temperatures possess higher antioxidant properties which is a positive nutritional enhancement. These possible health benefits of phenolic acids depend on their absorption and metabolism, which in turn are determined by their structure including their conjugation with other phenolics, degree of glycosylation/acylation, molecular size and solubility. For example, it was reported that phenolic acids, when ingested in the free form, are rapidly absorbed by the small intestine and therefore may have health benefits of protection against cardiovascular disease and certain types of cancer due to their antioxidant

properties which are the lowering of the levels of free radicals present in the body [5,26]. However, bound phenolic acids are naturally esterified in plant products and esterification impairs their absorption because intestinal mucosa, liver, and plasma do not possess esterases, and therefore hydrolysis can be performed only by microflora present in the colon [5]. As these compounds reach the colon, they will be degraded by the colon microflora and may exert antioxidant activities, as several studies have linked microbial metabolism of bound phenolic acids to colon cancer prevention [5,27,28].

The individual phenolic acids identified in the present study were similar to those observed in other research [29,30], including levels of five main phenolic acids: ferulic acid, vanillic acid, *p*-coumaric acid, sinapic acid, and syringic acid. As reported in a previous study [29], the results of this work also suggest that bound phenolic acids in wheat, which included the major proportion of the total phenolic acids, were strongly affected by the genotype variation and less influenced by the environment. However, presently the environmental effect was larger than genotypic differences for free phenolic acids [29]. Other investigators observed different trends for environmental changes compared to genotypic variation; for example, the results of Mpofu *et al*., showed that environmental effects on the content of phenolic compounds were considerably larger than genotypic effects [31]. Although these investigators used different wheat genotypes in their study, the main possible reason could have been that these investigators collected their samples from different fields across Western Canada, where all the environmental factors including soil pH, temperature and rainfall were different. In contrast, presently we used controlled environments in which the only changing parameter was temperature. The profile and levels of phenolic acids determined in the wheat genotypes in the present study were similar to

those observed in previous work [7,32-35] , confirming that biosynthesis of phenolic compounds in plants including wheat grains is under genetic control and is strongly influenced by biotic and abiotic factors such as temperature.

Total flavonoid contents of wheat measured in the present study were in agreement with those reported in previous work [36,37]. Within the Canadian wheat genotypes, AC Crystal contained higher amounts of flavonoids as expected: red spring wheat and colored wheat varieties have been reported to contain higher flavonoid content previously[36]. The increased growing temperature resulting in higher amount of total flavonoids can be explained as the effect of temperature forcing the plant to produce extra flavonoids as a defense strategy against the environmental changes. Enhanced levels of flavonoids suggest that the grains grown at higher temperatures possess not only higher total phenolic acids but they also have higher amounts of flavonoids which again increases the overall antioxidant properties of such grains. Such information could be used as useful strategies to produce wheat products with higher nutritional value considering the genotype and the temperatures during the growing season in different geographical environments such as Australia and Canada.

The main fatty acids identified in the wheat in the present study were similar to those reported previously [38-40]. Linoleic acid (C18:2n6) and linolenic acid (C18:3n3), two essential fatty acids, were both decreased with increased growing temperature for all wheat genotypes. Also, palmitic acid (C16:0), a saturated fatty acid, increased at higher temperatures. Increased levels of palmitic acid in the diet has been linked to elevated LDL level and thereby increased cardiovascular

diseases [41]. Therefore, unlike the phenolic acids and flavonoids contents, the fatty acid profile of wheat grains produced under higher temperatures was negatively affected by temperature.

Although the growing environment, genotype, and genotype-by-environment interactions all significantly affected the SFA and UFA compositions of wheat, the genotypes variation had a higher influence on saturated fatty acids, while the unsaturated fatty acids were more affected by the environment changes. These findings are in agreement with results of Bleggia *et al* [42] who also observed a large effect of genotype-by-environment interactions on fatty acid profiles of wheat cultivars harvested across three cultivation years and two cultivation systems (conventional and organic), however, these investigators observed a non-significant effect for the genotype variation on SFA and UFA compositions. This finding could be related to differences in genotypes or growing conditions.

The most abundant phytosterol in all wheat genotypes was sitosterol which is in line with previous findings [43,44]. The total phytosterol contents were also in the range as previously found [6,44]. The genotype and environment and their interaction resulted in significant differences in the proportions of the individual plant sterols and total plant sterols which confirmed the previous report [6]. Environment effects were greater than the genotype variation for all individual plant sterols, except the stigmasterol which was highly influenced by genotype-by-environment interaction.

Overall, the effect of global warming on wheat secondary bioactive metabolites appears to be genotype-specific as well as dependent on the category of metabolite. However, some trends seem to be related to the type of environmental stressor as well. For example, in the present study the increase of phenolic compounds, including phenolic acids and flavonoids, agrees with data in the literature indicating that they are usually positively enhanced by an elevated temperature.

In conclusion, the profiles and contents of the secondary metabolites presently studied in wheat grains were significantly influenced by the genotype, growth environment and genotype by environment interactions. The comprehensive data set produced in this study constitutes a valuable basis to further our understanding of the variations of wheat bioactives grown under different temperatures and enables the selection of particular wheat grains to be used as a nutritious food source depending on growth environment and genotype. For example, of all six genotypes used in the present study, Kennedy and AC Navigator contained higher amounts of phenolic acids and phytosterols within the Australian and Canadian wheat genotypes, respectively. Knowledge of such genotypic differences in phenolic acids and phytosterols can be used for breeding wheat varieties with higher antioxidant properties. Another example is that based on the present study, the Canadian genotype of AC Navigator will not yield at the increased temperature above 25^{o}C, which can be a useful information for wheat breeders.

The present study, however, has some limitations. The only variable parameter in the present work was the temperature and it can be expected that other environmental factors such as water deprivation, elevated CO2, and UV light can induce a different physiological response in plants.

Therefore, it is difficult to predict the outcome of present and future climatic changes based on the evaluation of only one or two parameters at a time. Thus our present results are lacking information regarding the combined effect of these abiotic factors on wheat secondary metabolites. Future studies should, therefore, focus on simultaneously testing the effects of multiple environmental factors to gain a more realistic perspective of how global climatic changes may impact the production of secondary bioactive metabolites of wheat grains.

5.6 ACKNOWLEDGEMENTS

This research was funded in part by Mitacs Accelerate Graduate Research Internship Program, Haplotech Inc. and Richardson Centre for Functional Foods and Nutraceuticals. Authors would like to thank Dr. Rale Gjuric, Dr. Debbie Jones, Jill Lockery for their assistance with growing and harvesting the wheat grains, Dr. Parimalan Rangan and Dennis Labossiere, for their technical assistance.

5.7 REFERENCES

1 World Health Organization, Global report on diabetes. (2016) Available at: http://apps.who.int/iris/bitstream/10665/204871/1/9789241565257_eng.pdf. (Accessed on 3rd Jan 2017).

2 Giambanelli, E. *et al.* A comparative study of bioactive compounds in primitive wheat populations from Italy, Turkey, Georgia, Bulgaria and Armenia. *Journal of the science of food and agriculture* **93**, 3490-3501, doi:10.1002/jsfa.6326 (2013).

3 Parr, A. J. & Bolwell, G. P. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *Journal of the science of food and agriculture* **80**, 985-1012, doi:10.1002/(SICI)1097-0010(20000515)80:7<985::AID-JSFA572>3.0.CO;2-7 (2000).

4 Slavin, J. Whole grains and human health. *Nutrition research reviews* **17**, 99-110, doi:10.1079/nrr200374 (2004).

5 Pandey, K. B. & Rizvi, S. I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity* **2**, 270-278 (2009).

6 Nurmi, T., Lampi, A.-M., Nystrom, L. & Piironen, V. Effects of environment and genotype on phytosterols in wheat in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry* **58**, 9314-9323, doi:10.1021/jf100192t (2010).

7 Martini, D. *et al.* Variation of total antioxidant activity and of phenolic acid, total phenolics and yellow coloured pigments in durum wheat (Triticum turgidum L. var. durum) as a function of genotype, crop year and growing area. *Journal of cereal science* **65**, 175-185, doi:http://doi.org/10.1016/j.jcs.2015.06.012 (2015).

8 IPCC (2007). *Contribution of Working Group I To The Fourth Assessment Report of The Intergovernmental Panel On Climate Change.* Cambridge University Press, Cambridge, United Kingdom.

9 Dixon, R. A. & Paiva, N. L. Stress-induced phenylpropanoid metabolism. *The Plant cell* **7**, 1085-1097, doi:10.1105/tpc.7.7.1085 (1995).

10 Meseret C. Egigu *et al.* Effects of rising temperature on secondary compounds of Yeheb (Cordeauxia edulis Hemsley). *American Journal of Plant Sciences* **5**, doi:DOI: 10.4236/ajps.2014.5506 (2014).

11 Vickers, C. E., Gershenzon, J., Lerdau, M. T. & Loreto, F. A unified mechanism of action for volatile isoprenoids in plant abiotic stress. *Nature Chemical Biology* **5**, 283-291, doi:10.1038/nchembio.158 (2009).

12 Fares, S., Oksanen, E., Lannenpaa, M., Julkunen-Tiitto, R. & Loreto, F. Volatile emissions and phenolic compound concentrations along a vertical profile of Populus nigra leaves exposed to realistic ozone concentrations. *Photosynthesis Research* **104**, 61-74, doi:10.1007/s11120-010-9549-5 (2010).

13 Wu, G. *et al.* Growth temperature and genotype both play important roles in sorghum grain phenolic composition. *Scientific Reports* **6**, doi:10.1038/srep21835 (2016).

14 Wang, S. Y. & Zheng, W. Effect of plant growth temperature on antioxidant capacity in strawberry. *Journal of Agricultural and Food Chemistry* **49**, 4977-4982, doi:10.1021/jf0106244 (2001).

15 Jeong, S. W. *et al.* The effects of different night-time temperatures and cultivation durations on the polyphenolic contents of lettuce: Application of principal component

analysis. *Journal of Advanced Research* **6**, 493-499, doi:10.1016/j.jare.2015.01.004 (2015).

16 Wahid, A. Physiological implications of metabolite biosynthesis for net assimilation and heat-stress tolerance of sugarcane (Saccharum officinarum) sprouts. *Journal of Plant Research* **120**, 219-228, doi:10.1007/s10265-006-0040-5 (2007).

17 Krygier, K., Sosulski, F. & Hogge, L. Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and purification procedure. *Journal of Agricultural and Food Chemistry* **30**, 330-334, doi:10.1021/jf00110a028 (1982).

18 Chang, C. C., Yang, M. H., Wen, H. M. & Chern, J. C. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.* **10**, 178-182 (2002).

19 Tsen, C. C., Levi, I. & Hlynka., I. A Rapid method for the extraction of lipids from wheat products. *Cereal Chemistry* **39**, 195-203 (1962).

20 Folch, J., Lees, M. & Sloane Stanley, G. H. A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of biological chemistry* **226**, 497-509 (1957).

21 Piironen, V., Toivo, J. & Lampi, A. M. Plant sterols in cereals and cereal products. *Cereal Chemistry* **79**, 148-154, doi:10.1094/cchem.2002.79.1.148 (2002).

22 Abeledo, L. G., Savin, R. & Slafer, G. A. Carpel size and temperature in pre-anthesis modify potential grain weight in wheat. *The Journal of Agricultural Science* **132**, 453-459, doi:10.1017/S0021859699006504 (1999).

23 Gambín, B. L. & Borrás, L. Resource distribution and the tradeoff between grain number and grain weight: a comparison across crop species. *Anna. Appl. Biol.* **156**, 91-102, doi:10.1111/j.1744-7348.2009 (2010).

24 Sadras, V. O. & Slafer, G. A. Environmental modulation of yield components in cereals: heritabilities reveal a hierarchy of phenotypic plasticities. *Field Crops Res.* **127**, doi:10.1016/j.fcr.2011.11.014 (2012).

25 De Abreu, I. N. & Mazzafera, P. Effect of water and temperature stress on the content of active constituents of Hypericum brasiliense choisy. *Plant Physiology and Biochemistry* **43**, 241-248, doi:10.1016/j.plaphy.2005.01.020 (2005).

26 Bravo, L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition reviews* **56**, 317-333, doi:10.1111/j.1753-4887.1998.tb01670.x (1998).

27 Adom, K. K. & Liu, R. H. Antioxidant activity of grains. *Journal of Agricultural and Food Chemistry* **50**, 6182-6187, doi:10.1021/jf0205099 (2002).

28 Cardona, F., Andres-Lacueva, C., Tulipani, S., Tinahones, F. J. & Isabel Queipo-Ortuno, M. Benefits of polyphenols on gut microbiota and implications in human health. *Journal of Nutritional Biochemistry* **24**, 1415-1422, doi:10.1016/j.jnutbio.2013.05.001 (2013).

29 Fernandez-Orozco, R., Li, L., Harflett, C., Shewry, P. R. & Ward, J. L. Effects of environment and genotype on phenolic acids in wheat in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry* **58**, 9341-9352, doi:10.1021/jf102017s (2010).

30 Ma, D. *et al.* Accumulation of phenolic compounds and expression profiles of phenolic acid biosynthesis-related genes in developing grains of white, purple, and red wheat. *Frontiers in Plant Science* **7**, doi:10.3389/fpls.2016.00528 (2016).

31 Mpofu, A., Sapirstein, H. D. & Beta, T. Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. *Journal of Agricultural and Food Chemistry* **54**, 1265-1270, doi:10.1021/jf052683d (2006).

32 Martini, D. *et al.* Effects of genotype and environment on phenolic acids content and total antioxidant capacity in durum wheat. *Cereal Chemistry* **91**, 310-317, doi:10.1094/cchem-09-13-0201-cesi (2014).

33 Wang, L. *et al.* Determination of phenolic acid concentrations in wheat flours produced at different extraction rates. *Journal of Cereal Science* **57**, 67-72, doi:10.1016/j.jcs.2012.09.013 (2013).

34 Li, L., Shewry, P. R. & Ward, J. L. Phenolic acids in wheat varieties in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry* **56**, 9732-9739, doi:10.1021/jf801069s (2008).

35 Adom, K. K., Sorrells, M. E. & Liu, R. H. Phytochemical profiles and antioxidant activity of wheat varieties. *Journal of Agricultural and Food Chemistry* **51**, 7825-7834, doi:10.1021/jf030404l (2003).

36 Li, Y. *et al.* Total phenolic, flavonoid content, and antioxidant activity of flour, noodles, and steamed bread made from different colored wheat grains by three milling methods. *The Crop Journal* **3**, 328-334, doi:10.1016/j.cj.201504.004 (2015).

37 Abozed, S. S., El-kalyoubi, M., Abdelrashid, A. & Salama, M. F. Total phenolic contents and antioxidant activities of various solvent extracts from whole wheat and bran. *Annals of Agricultural Sciences* **59**, 63-67, doi:10.1016/j.aoas.201406.009 (2014).

38 Valcárcel-Yamani, B. & Caetano da Silva Lannes, S. Applications of quinoa (Chenopodium Quinoa Willd.) and amaranth (Amaranthus Spp.) and their influence in the nutritional value of cereal based foods. *Food and Public Health* **6**, 265-275, doi:10.5923/j.fph.20120206.12 (2012).

39 Liu, K. S. Comparison of lipid content and fatty acid composition and their distribution within seeds of 5 small grain species. *Journal of Food Science* **76**, C334-C342, doi:10.1111/j.1750-3841.2010.02038.x (2011).

40 Nikolic, N. *et al.* Fatty acids composition and rheology properties of wheat and wheat and white or brown rice flour mixture. *European Food Research and Technology* **227**, 1543-1548, doi:10.1007/s00217-008-0877-z (2008).

41 Siri-Tarino, P. W., Sun, Q., Hu, F. B. & Krauss, R. M. Saturated fatty acids and risk of coronary heart disease: modulation by replacement nutrients. *Current Atherosclerosis Reports* **12**, 384-390, doi:10.1007/s11883-010-0131-6 (2010).

42 Beleggia, R. *et al.* Effect of genotype, environment and genotype-by-environment interaction on metabolite profiling in durum wheat (Triticum durum Desf.) grain. *Journal of Cereal Science* **57**, 183-192, doi:10.1016/j.jcs.2012.09.004 (2013).

43 Nurmi, T., Nystrom, L., Edelmann, M., Lampi, A. M. & Piironen, V. Phytosterols in wheat genotypes in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry* **56**, 9710-9715, doi:10.1021/jf8010678 (2008).

44 Nurmi, T. *et al.* Distribution and composition of phytosterols and steryl ferulates in wheat grain and bran fractions. *Journal of Cereal Science* **56**, 379-388, doi:10.1016/j.jcs.2012.04.010 (2012).

TRANSITION STATEMENT 4

Increased levels of phenolic compounds had been reported in elevated growth temperatures for several plant species, and we observed, from our controlled environment experiment, that levels of polyphenols increased in wheat grains grown at higher temperature. However, the research to correlate changes in growth temperatures with changes in wheat phenolic acids and the mechanisms of actions impacting health outcomes is lacking.

With rising the air temperature globally, it is important to investigate how major crops respond to the heat stress and what are the related impacts on their nutritional values. In the following manuscript, we determined how the increased growing temperature influences the ability of wheat phenolic acid extracts to reduce cellular glucose uptake. In addition, we proposed the mechanisms involved on how phenolics inhibit intestinal glucose transporters.

CHAPTER 6

MANUSCRIPT 5

This manuscript is under review by the Nature Communications

ELEVATED GROWTH TEMPERATURES INCREASE WHEAT PHENOLICS WHICH INHIBIT CELLULAR GLUCOSE TRANSPORT

Maryam Shamloo[a,c], Peter JH Jones[a,c] , and Peter K Eck[b]*

Affiliations: [a]Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB R3T 2N2, Canada. [b]Department of Human Nutritional Sciences, W569 Duff Roblin Building, 190 Dysart Road, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada. [c]Department of Food Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada.

*Corresponding coauthor: Peter K Eck[b]

6.1 ABSTRACT

In spite of their importance to optimal nutrient supplies, the impacts of elevated growth temperatures on crop nutritional values had not been investigated. Health benefits in wheat, including the control of elevated blood sugar levels, are attributed to phenolic compounds, which are influenced by environmental factors.

The concentrations of phenolics in whole wheat were genotype dependent and increased with the growth temperature under controlled conditions. Extracts of these phenolics inhibited glucose accumulation in the CaCo-2 model of intestinal uptake, and this effect positively correlated with the phenolic contents and growth temperatures.

Based on these observations it can be speculated that rising global temperatures could increase the nutritional value of whole wheat, considering that elevated phenolics will lead to a better control of postprandial hyperglycemia. Since postprandial hyperglycemia is associated with obesity and type 2 diabetes, this effect might contribute to the mitigation of these major non-communicable diseases.

6.2 INTRODUCTION

It is predicted that climate change negatively impacts on crop yields and therefore food availability [1], and it is often assumed that effects on food security and nutrient density will be adverse [2]. In regard to declining nutrient density, it could be speculated that this would increase

malnutrition and therefore decrease the diet's function in preventing some of the most detrimental non-communicable diseases, such as type 2 diabetes.

Wheat is one of the most important commodities worldwide, grown on the largest land area of all commercial crops, making it the most important grain source in the human diet [3]. This makes wheat a prime target to investigate how its composition of health-beneficial phytochemicals changes as a result of climate change.

Whole wheat contains health-beneficial phytochemicals, which consumption is associated with reduced total mortality [4,5] as well as reduced risk of coronary heart disease (CHD)[6], ischemic stroke [6], and type 2 diabetes [7,8]. The mechanisms of disease prevention have not been fully determined, but phenolic compounds has been suggested to have the greatest potential of being beneficial to health [3]. It has been proposed, that wheat phenols exhibit their beneficial effect through antioxidant mechanisms [3]; however, due to limited bioavailability this might be an effect *in vitro* rather than *in vivo* [9]. Some plant derived polyphenols inhibit intestinal glucose transporters [10-12], resulting in improved postprandial glycemic responses through blunted glucose absorption [13-15]. This offers an alternative mechanism for disease prevention mediated by polyphenols [16], but experimental data are not available.

Increased levels of phenolic compounds had been reported in elevated growth temperatures for several plant species [17-21], suggesting that this phenomenon might also apply to wheat species. Moreover, several studies examined the impact of elevated CO_2 levels on wheat composition [22-

[24], while reports on the impact of the growth temperatures are sparse [3], and studies examining the changes in nutrients contents of crops grown at different temperatures in controlled environments are rare [31].

Significantly, to our best knowledge, there are no experimental attempts to correlate changes in growth temperatures with changes in bioactive plant compounds and the mechanisms of actions impacting health outcomes.

This study addresses these shortfalls by determining levels of the main phenols in different wheat genotypes grown at three temperatures in controlled environments. Moreover, soluble and bound phenolics were extracted to test if they inhibit glucose uptake in a CaCo-2 model of intestinal absorption, in order to extrapolate on their impact on the glycemic index.

6.3 MATERIALS AND METHODS

6.3.1 MATERIALS

Six wheat genotypes (*Triticum spp*), including AC Crystal, red spring wheat (*Triticum aestivum L.*), AC Navigator durum wheat (*Triticum turgidum L. var. durum*), Carberry, a hard red spring wheat (*Triticum aestivum L.*), Kennedy, quick maturing spring wheat (*Triticum aestivum L.*), Fango60, drought tolerant wheat (*Triticum aestivum L.*), EGA Gregory, hard spring wheat (*Triticum aestivum L.*), were grown in three separate growth chambers (GR192), where the temperature was altered and all other environmental factors including photoperiod, carbon-dioxide levels, humidity and wind velocity were fixed as described before [25].

[^{3}H] 2-Deoxyglucose (25.5 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, USA). Dulbecco's modified Eagle medium (DMEM) (25 mM glucose) was obtained from Biofluids (Rockville, MD, USA) and all other media supplements were from GIBCO Life Technologies (Gaithersburg, MD, USA). Caco-2 cells were a gift from Dr. David Fitzgerald (National Cancer Institute, Bethesda, MD, USA). Phenolic acids standards were purchased from Sigma-Aldrich (Milwaukee, Wisconsin, USA). All acids and organic solvents were obtained from Fisher Scientific (Whitby, Ontario, Canada). All chemicals used were of analytical grade.

6.3.2 PREPARATION OF FREE AND BOUND PHENOLIC ACIDS EXTRACTS

The whole wheat samples were prepared as reported previously [25]. Briefly, they were milled and passed through a 0.5mm sieve screen. The fine flour from each sample was stored at -20°C in the dark until further use. Free and bound phenolic acid extraction was performed using liquid-liquid extraction as described previously [25]. Briefly, for the free fraction, wheat flour (0.6 g) was extracted twice with ethyl acetate at a ratio of 1:20 (w/v). After centrifugation at 3,750*g* for 10 min, the supernatants were obtained and concentrated to dryness using a rotary evaporator at 30°C. The dried extract was re-suspended in 50% dimethyl sulfoxide (DMSO)-ethanol. For the bound fraction, the dried residue obtained from the free fraction was hydrolyzed with 18 mL of 4 M NaOH for 4 h and adjusted to a pH 1.5–2.0 with 6 M ice-cold HCl and then extracted with 12 mL of ethyl acetate three times. After centrifugation at 3,750*g* for 10 min, the combined ethyl acetate fractions were evaporated to dryness and reconstituted in 50% dimethyl sulfoxide (DMSO)-ethanol. Both fractions were filtered and then directly subjected to HPLC analysis.

6.3.3 HPLC-PDA ANALYSIS OF FREE AND BOUND EXTRACTS

Phenolic acids of free and bound fractions were identified by a reverse phased performance liquid chromatography system (Waters 2695, Milford, MA, USA) equipped with a photodiode array detector (PDA) (Waters 996), and auto-sampler (717 plus, Waters, Milford, MA, USA) as described by Shamloo *et al* [25].

6.3.4 GLUCOSE UPTAKE INHIBITION ASSAYS

6.3.4.1 CELL CULTURE

Caco-2 stock cell cultures were maintained in 75-cm^2 plastic flasks and cultured at 37 °C in a 95% air, 5% CO_2 atmosphere in Dulbecco's modified Eagle's minimal essential medium containing 15 mM glucose supplemented with 10% heat-inactivated FBS, 0.1 mM non-essential amino acids, and 0.1 mM glutamine. All experiments were carried out on cells of passage number 13 and 31. For experiments, cells were seeded into 96 well plates and grown to confluency. Media was refreshed 4 hours prior to experiments.

6.3.4.2 INHIBITION OF GLUCOSE UPTAKE INTO CACO-2 ENTEROCYTE MONOLAYERS

The ability of wheat phenolic acids extracts to reduce cellular glucose uptake was investigated through the modified method described by Kwon *et al.* [11]. Confluent CaCo-2 cells grown on 96-well plates were rinsed 3 times with PBS and incubated in pre-incubation buffer (HEPES buffer with 5 mM glucose) for 30 minutes at 37°C. Transport experiments were initiated by replacing

the pre-incubation buffer with 100 µL transport buffer (HEPES buffer, pH 7.4, glucose free) supplemented with [^{3}H] 2-deoxyglucose (5 mM) and wheat extracts. Transport buffer with no wheat extracts used as the positive control. The cells were incubated at room temperature for 15 minutes in the dark and transport experiments were stopped by adding 100 µL of ice cold pre-incubation buffer immediately after removal of the transport buffer. Cells were washed three times with 100 µL of pre-incubation buffer and then lysed with 60 µL lysis buffer (20 mg SDS in 1 mL 0.2M NaOH) and incubated at room temperature for 1 hour. 45 µL aliquot of cell lysates was added to 5ml scintillation cocktail and the [^{3}H] 2-deoxyglucose concentration was quantified by scintillation spectrometry. The protein content of the remaining cell lysates was determined using DC Protein Assay Kit (Bio-Rad, USA). The glucose uptake into CaCo-2 cells was expressed as counts per minute (cpm) per mg protein. The viability of cells and validity of assay was demonstrated by the linearity of the uptake rates in the absence of wheat extracts.

6.3.5 STATISTICAL ANALYSIS

All cell culture data represent means of three experimental sets in which the results were consistent. Each of the experimental sets consisted of three parallel transport experiments (n = 6). All data were calculated using one way analysis of variance (ANOVA) on a Minitab 14 Statistical software (Minitab Inc., State College, PA, USA). Sample means were compared using the Tukey HSD method and significant differences were considered when $P < 0.05$. Correlations between wheat extract phenolic acid contents and inhibition capacity were assessed by the Pearson's correlation test.

6.4 RESULTS

6.4.1 GENOTYPE AND GROWTH TEMPERATURE DETERMINE THE PHENOLIC ACIDS CONTENTS AND COMPOSITION

When grown in environmental chambers at 20^{o}C, 25^{o}C and 30^{o}C, all wheat varieties developed grains, except the durum wheat AC Navigator failed at 30^{o}C.

Phenolic acid (PA) contents in the free and bound fractions and the concentrations of individual compounds differed between the six wheat varieties, with ferulic acid being the most abundant, averaging 51.4 % and 81.7 % in the free and bound fractions, respectively (Table 6.1 and Table 6.2).

All individual free phenolic acids increased ($P < 0.05$) with the growth temperature except for gallic acid (Table 6.1 and Table 6.2). Kennedy grown at 30^{o}C produced the highest amounts of free phenolic acids, followed by AC Crystal.

Table 6.1. Individual free phenolic acids (µg/g dry matter) extracted from six wheat varieties grown in controlled environments at 20°C, 25°C and 30°C.

Growth Temp.	Free phenolic acid	Wheat Genotypes					
		AC Crystal	AC Navigator	Carberry	Kennedy	Fango60	EGA Gregory
20°C	Galic acid	0.045±0.006^{ABa}	0.052±0.007^{Ba}	0.047±0.009^{ABa}	0.051±0.010^{ABa}	0.032±0.004^{Aa}	0.032±0.004^{Aa}
	P-oh-benzoic acid	0.238±0.006^{CDa}	0.290±0.006^{Da}	0.174±0.006^{Ba}	0.225±0.006^{Ca}	0.151±0.006^{Aa}	0.169±0.006^{Ba}
	Vanillic acid	1.990±0.013^{Ea}	2.095±0.010^{Fa}	1.621±0.010^{Ca}	1.820±0.010^{Da}	1.420±0.008^{Aa}	1.582±0.010^{Ba}
	Syringic acid	0.230±0.006^{CDa}	0.233±0.001^{Da}	0.204±0.020^{BCDa}	0.202±0.010^{Ca}	0.162±0.008^{ABa}	0.185±0.012^{Ba}
	P-coumaric acid	0.500±0.008^{Da}	0.505±0.006^{Da}	0.472±0.008^{Ca}	0.506±0.010^{Da}	0.362±0.014^{Aa}	0.406±0.001^{Ba}
	Ferulic acid	**3.170±0.006^{Ea}**	**3.530±0.008^{Fa}**	**3.016±0.008^{Ca}**	**3.127±0.006^{Da}**	**2.620±0.004^{Aa}**	**2.820±0.011^{Aa}**
	Synapic acid	0.330±0.008^{Ba}	0.330±0.002^{Ba}	0.296±0.008^{Aa}	0.327±0.006^{Ba}	0.292±0.004^{Aa}	0.285±0.006^{Aa}
	total	6.500±0.020^{Ea}	7.040±0.020^{Fa}	5.820±0.030^{Ca}	6.270±0.010^{Da}	5.020±0.030^{Aa}	5.480±0.030^{Aa}
25°C	Galic acid	0.053±0.006^{a}	0.050±0.006^{a}	0.0500±0.004^{a}	0.052±0.004^{a}	0.043±0.006^{a}	0.049±0.006^{b}
	P- oh-benzoic acid	0.412±0.004^{Db}	0.389±0.008^{Cb}	0.355±0.006^{ABb}	0.536±0.007^{Eb}	0.295±0.008^{Ab}	0.340±0.010^{Bb}
	Vanillic acid	2.830±0.010^{Db}	2.63±0.0100^{Cb}	2.620±0.010^{Cb}	3.140±0.000^{Eb}	2.142±0.008^{Ab}	2.250±0.008^{Bb}
	Syringic acid	0.360±0.010^{Eb}	0.339±0.006^{Db}	0.308±0.008^{Cb}	0.480±0.000^{Fb}	0.227±0.006^{Ab}	0.252±0.008^{Bb}
	P-coumaric acid	0.638±0.000^{Db}	0.602±0.000^{Cb}	0.602±0.000^{Cb}	0.680±0.000^{Eb}	0.508±0.000^{Ab}	0.580±0.000^{Bb}

	Ferulic acid	**5.047±0.000^{Eb}**	**4.890±0.000^{Db}**	**4.820±0.010^{Cb}**	**5.420±0.010^{Fb}**	**4.490±0.004^{Ab}**	**4.620±0.014^{Bb}**
	Synapic acid	0.532±0.000^{Db}	0.455±0.010^{Cb}	0.458±0.006^{Cb}	0.720±0.010^{Eb}	0.332±0.008^{Ab}	0.372±0.008^{Bb}
	total	9.900±0.010^{Eb}	9.350±0.020^{Db}	9.190±0.030^{Cb}	10.99±0.040^{Fb}	8.040±0.020^{Ab}	8.450±0.030^{Bb}
30^{o}C	Galic acid	0.055±0.008^{a}	na	0.054±0.006^{a}	0.047±0.008^{a}	0.047±0.010^{a}	0.049±0.002^{b}
	P- oh-benzoic acid	0.726±0.012^{Bc}	na	0.730±0.008^{Bc}	0.730±0.016^{Bc}	0.612±0.004^{Ac}	0.730±0.012^{Bc}
	Vanillic acid	4.010±0.008^{Dc}	na	3.960±0.0140^{Cc}	4.850±0.012^{Ec}	3.420±0.010^{Ac}	3.498±0.008^{Bc}
	Syringic acid	0.618±0.001^{Cc}	na	0.628±0.001^{Dc}	0.636±0.001^{Ec}	0.550±0.000^{Ac}	0.601±0.001^{Bc}
	P-coumaric acid	0.840±0.001^{Dc}	na	0.768±0.012^{Cc}	1.070±0.006^{Ec}	0.698±0.010^{Ac}	0.719±0.001^{Bc}
	Ferulic acid	**7.420±0.010^{Dc}**	**na**	**6.820±0.006^{Cc}**	**8.370±0.010^{Ec}**	**6.250±0.008^{Ac}**	**6.370±0.004^{Bc}**
	Synapic acid	0.810±0.066^{Dc}	na	0.750±0.006^{Cc}	0.894±0.010^{Ec}	0.728±0.004^{Ac}	0.735±0.010^{Bc}
	total	14.48±0.040^{Dc}	na	13.70±0.040^{Cc}	16.60±0.030^{Ec}	12.31±0.010^{Ac}	12.69±0.010^{Bc}

^{a,b,c} Different small letters superscripts indicate significant differences in the same column in the same dependent variable ($P < 0.05$).

^{A,B,C,D,E,F} Different capital letters superscripts indicate significant differences in the same row ($P < 0.05$). na = data not available.

Bold numbers indicate the most abundant individual phenolic acid in free fraction of wheat genotypes grown at specified temperature.

Table 6.2. Individual bound phenolic acids (µg/g dry matter) extracted from six wheat varieties grown in controlled environments at 20°C, 25°C and 30°C.

Growth Temp	Bound phenolic acid	Wheat Genotypes					
		AC Crystal	AC Navigator	Carberry	Kennedy	Fango60	EGA Gregory
20 °C	P- oh-benzoic acid	12.74±0.300^{Ba}	17.34±1.670^{Ca}	11.78±1.040^{Ba}	14.07±1.130^{Ba}	7.740±0.500^{Aa}	9.540±0.990^{Aa}
	Vanillic acid	14.20±0.200^{Ca}	14.20±0.720^{Ca}	15.40±0.000^{Da}	15.68±0.110^{Da}	9.060±0.420^{Aa}	13.00±0.400^{Ba}
	Syringic acid	11.53±1.300^{CDa}	13.20±0.350^{Da}	9.070±0.810^{BCa}	10.94±0.580^{Ca}	6.020±0.350^{Aa}	8.140±0.240^{Ba}
	P-coumaric acid	24.74±0.300^{Ca}	29.74±0.940^{EDa}	27.80±1.210^{Da}	30.67±0.300^{Ea}	18.84±0.730^{Aa}	22.68±0.310^{Ba}
	Ferulic acid	**587.3±33.84^{Ca}**	**716.9±41.56^{Da}**	**436.1±11.20^{Ba}**	**548.9±51.39^{Ca}**	**312.9±29.60^{Aa}**	**400.4±40.00^{ABa}**
	Synapic acid	50.80±6.700^{BCa}	59.20±9.530^{Ca}	46.07±3.580^{BCa}	54.06±3.350^{BCa}	29.74±2.670^{Aa}	40.07±0.500^{ABa}
	total	701.3±36.60^{Ca}	850.6±50.90^{Da}	546.2±16.27^{Ba}	674.3±55.67^{Ca}	384.5±32.33^{Aa}	493.8±41.91^{ABa}
25 °C	P- oh-benzoic acid	14.14±1.610^{ABCa}	16.54±1.660^{BCa}	12.94±1.100^{ABab}	17.74±2.140^{Cab}	10.13±0.940^{Aab}	10.80±1.210^{Aab}
	Vanillic acid	14.54±0.410^{ABCa}	15.06±0.420^{ABCa}	16.67±0.230^{BCb}	17.27±2.200^{Ca}	12.60±0.720^{Ab}	14.80±0.800^{ABCb}
	Syringic acid	12.80±0.920^{BCa}	14.34±2.780^{Ca}	9.930±1.330^{ABa}	14.60±0.870^{Cb}	7.730±0.750^{Aa}	8.200±1.050^{Aa}
	P-coumaric acid	29.20±0.200^{Bb}	31.47±1.89^{Ba}	24.00±0.340^{Ab}	31.20±0.530^{Ba}	24.67±0.640^{Ab}	22.94±0.300^{Aa}
	Ferulic acid	**595.2±82.20^{BCa}**	**685.6±89.24^{Ca}**	**505.5±60.98^{ABab}**	**757.6±40.02^{Ca}**	**368.9±38.10^{Aab}**	**426.4±53.60^{ABa}**
	Synapic acid	57.74±0.120^{Ca}	57.07±0.500^{Ca}	45.07±0.800^{Ba}	57.20±1.250^{Ca}	41.34±0.500^{Ab}	43.27±1.970^{ABa}

	total	723.6±84.31^{Ca}	820.0±95.10^{CDa}	614.1±62.60^{BCab}	895.6±46.15^{CDb}	465.4±39.30^{ABab}	526.4±58.36^{ABa}
30 ^{o}C	P- oh-benzoic acid	15.67±1.400^{Ca}	na	14.80±0.530^{BCb}	20.00±1.500^{Db}	11.40±1.440^{Ab}	12.40±0.800^{ABb}
	Vanillic acid	18.14±1.700^{BCb}	na	20.33±0.230^{Cc}	20.80±0.200^{Cb}	16.07±1.33^{ABc}	15.60±0.400^{Ab}
	Syringic acid	12.27±0.640^{Ba}	na	10.87±0.610^{ABa}	15.94±1.130^{Cb}	9.46±0.83^{Ab}	10.07±1.480^{Aa}
	P-coumaric acid	32.46±1.810^{Bc}	na	32.67±0.500^{Bc}	39.73±0.410^{Cb}	26.74±1.92^{Ab}	24.34±0.230^{Ab}
	Ferulic acid	**604.2±39.50^{Ba}**	**na**	**565.0±31.40^{ABb}**	**813.8±84.40^{Cb}**	**444.87±30.64^{Ab}**	**487.6±50.10^{ABa}**
	Synapic acid	60.83±6.200^{Ba}	na	54.54±0.700^{ABb}	80.73±0.750^{Cb}	49.14±2.47^{Ac}	50.86±2.230^{Ab}
	total	743.6±47.30^{Ca}	na	698.2±31.97^{BCb}	991.0±87.30^{Db}	557.66±37.9^{Ab}	600.8±55.02^{ABa}

^{a,b,c} Different small letters superscripts indicate significant differences in the same column in the same dependent variable ($P < 0.05$).

$^{A,B,C,\ D}$ Different capital letters superscripts indicate significant differences in the same row ($P < 0.05$). na = data not available.

Bold numbers indicate the most abundant individual phenolic acid in free fraction of wheat genotypes grown at specified temperature.

Bold numbers indicate the most abundant individual phenolic acids in bound fraction of wheat genotypes grown at specified temperature.

6.4.2 FREE AND BOUND PHENOLIC ACIDS INHIBIT GLUCOSE UPTAKE INTO CACO-2 CELLS CONCENTRATION DEPENDENT

Extracts of the free phenolic acids from the whole grains of all wheat genotypes inhibited glucose uptake into CaCo-2 cell monolayers to varying degrees. Notably, this effect positively correlated with the growth temperature (**Figure 6.1**), which also elevated phenolics levels (Tables 6.1 and 6.2). For example, extracts from Kennedy grown at 30^{o}C showed the highest inhibition of 49.5 % (**Figure 6.1 A**), followed by AC Crystal with 42.7 % (**Figure 6.1 B**). These effects were observed for all wheat genotypes (**Figures 6.1A-F**).

Extracts of the bound phenolic acid from all wheat genotypes also inhibited glucose uptake, and overall this effect positively correlates with the growth temperatures (**Figure 6.2**). However, the effect of the temperature was not seen in extracts of bound phenolic acid from AC Navigator, which may to be related to the fact that the total amount of phenolic acids was lower for AC Navigator grown at 25°C compared to 20°C.

The degree of inhibition of glucose uptake positively correlated with the total free and bound phenolic acids concentrations in the extracts, as depicted in **Figure 6.3** (R^2=0.9769 and R^2=0.917 for free and bound extracts, respectively; $P < 0.05$).

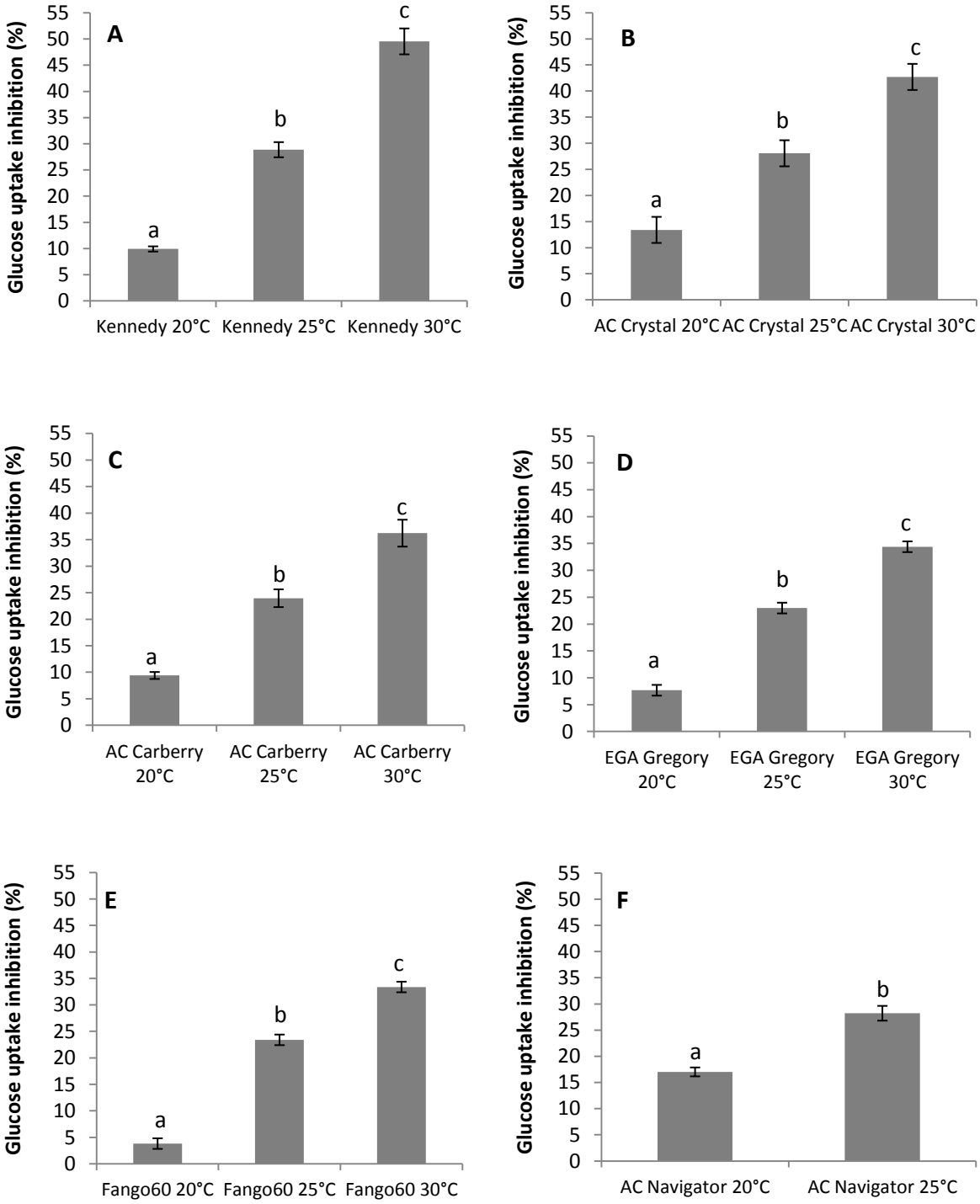


Figure 6.1. Relative inhibition of the uptake of [3H] 2-Deoxyglucose into CaCo-2 monolayers caused by extracts of free phenolic acids obtained from six wheat genotypes (panels A-F) grown in controlled environments at 20ºC, 25ºC and 30ºC. ^{a,b,c} Different small letter superscripts indicate significant differences ($P < 0.05$).

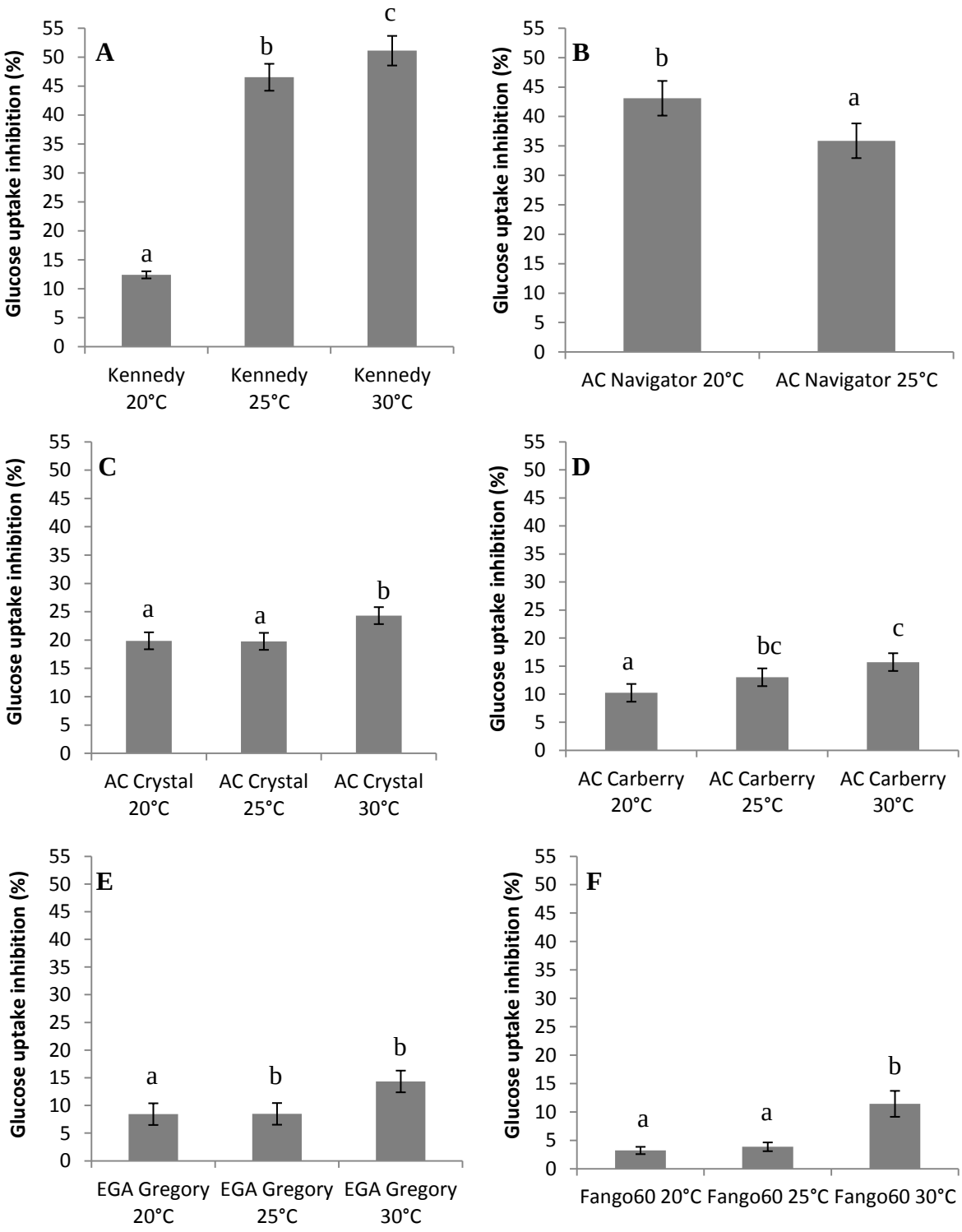


Figure 6.2. Relative inhibition of the uptake of [3H] 2-Deoxyglucose into CaCo-2 monolayers caused by extracts of bound phenolic acids obtained from six wheat genotypes (panels A-F) grown in controlled environments at 20ºC, 25ºC and 30ºC. [a,b,c] Different small letter superscripts indicate significant differences ($P < 0.05$).

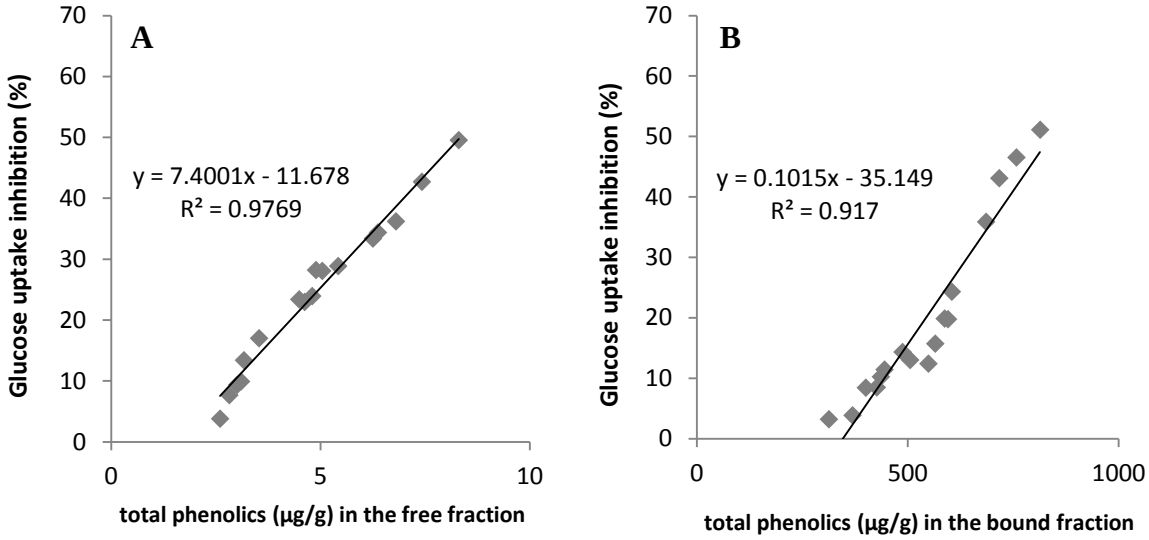


Figure 6.3. Correlation between the relative inhibition of glucose uptake into CaCo-2 monolayers and free ferulic acid (A), and bound ferulic acid (B) contents in extracts of six wheat genotypes grown in controlled environments at 20ºC, 25ºC and 30ºC.

6.5 DISCUSSION

Phenolic compounds are not classical nutrients, such as vitamins and minerals, but evidence increasingly indicates that humans benefit from consuming these secondary plant metabolites through mechanisms yet to be determined [4-8]. This study shows that phenolics from wheat inhibit glucose transport into the absorbing intestinal epithelial cell, providing basic experimental evidence that the glycemic response can be improved through the addition of whole wheat products in the regular diet. We propose that this mechanism can explain the preventative effects of whole wheat consumption on type 2 diabetes, offering an alternative to the antioxidant hypothesis. The antioxidant activity of polyphenolic compounds had been repeatedly demonstrated *in vitro*, but if they retain those features *in vivo* is uncertain, due to their low bioavailability [9]. In contrast, to inhibit luminal facing intestinal glucose transporters, the bioactives do not need to be absorbed. The presented data support the notion that non-absorbable

phenolics inhibit intestinal glucose transporters since this phenomenon was observed for extracts of the free and bound fractions, showing that even compounds bound to dietary fibers could interact with membrane transporter proteins. The abundance of phenolic compounds in wheat ensure efficacious concentrations in the intestinal tract, which can be as low as 50 micromole/liter for specific molecules [10-12]. For example, it has been suggested that daily dietary uptake of 77 mg of the main phenolic compound in wheat, ferulic acid, may effectively suppress hyperglycemia [10]. Wheat bran as one of the main food sources of ferulic acid contains 5mg/g [26], while whole wheat bread contains 330.1 μg/g [27], making it feasible that beneficial luminal concentrations are reached.

The contents of phenolic compounds in wheat positively correlated with the growth temperatures of 20°C, 25°C and 30°C in environmental chambers, and depended on the genotype. It has been established that the genotype influences the levels of polyphenols of wheat cultivars [28], but data on the impact of growth temperatures were lacking. In line with our result, the accumulation of phenolic compounds under heat stress has been reported for other plants [18,19,29]. It is suggested that rising temperatures, as an abiotic stress, cause the plant to produce extra polyphenolic compounds as a defense strategy [18]. Our data suggest that polyphenols levels in whole wheat, as in other plants, increase with rising air temperatures.

Considering that we currently experience globally rising temperatures, it can be speculated that phenolic compounds in wheat will increase in coming years. Provided that whole wheat products, as recommended by all major scientific bodies [30,31], will be consumed, increased health

benefits through the reduction of incidences of type 2 diabetes and other non-communicable diseases might be expected.

6.6 CONCLUSIONS

With globally rising temperatures the associated increases in secondary plant bioactives in wheat grains could translate into increased nutritional values and health benefits through an amelioration of postprandial hyperglycemia. This could be relevant to prevent type 2 diabetes or individuals risk for obesity.

6.7 ACKNOWLEDGEMENTS

Authors would like to thank Dr. Rale Gjuric, Dr. Debbie Jones, Jill Lockery for their assistance with growth and harvesting the wheat grains, Dr. Henry Robert, Dr. Agnelo Furtado, Dr. Parimalan Rangan for providing Australian wheat grains, and Dennis Labossiere for his technical assistance.

6.8 REFERENCES

1 Krishnamurthy, P. K., Lewis., K. & Choularton., R. J. Climate impacts on food security and nutrion: A review of existing knowledge. *United Nations World Food Programme*, 1-22 (2012).

2 Luber, G. *et al.* in *Climate Change Impacts in the United States: The Third National Climate Assessment, J. M. Melillo, Terese (T.C.) Richmond, and G.W. Yohe, Eds., U.S. Global Change Research Program, 220-256.* (2014).

3 Mpofu, A., Sapirstein, H. D. & Beta, T. Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. *J Agric Food Chem* **54**, 1265-1270, doi:10.1021/jf052683d (2006).

4 Jacobs, D. R., Meyer, K. A., Kushi, L. H. & Folsom, A. R. Is whole grain intake associated with reduced total and cause-specific death rates in older women? The Iowa Women's Health Study. *American Journal of Public Health* **89**, 322-329 (1999).

5 Jacobs, D. R., Jr., Meyer, H. E. & Solvoll, K. Reduced mortality among whole grain bread eaters in men and women in the Norwegian County Study. *Eur J Clin Nutr* **55**, 137-143 (2001).

6 Liu, S. *et al.* Whole-grain consumption and risk of coronary heart disease: results from the Nurses' Health Study. *Am J Clin Nutr* **70**, 412-419 (1999).

7 Liu, S. *et al.* A prospective study of whole-grain intake and risk of type 2 diabetes mellitus in US women. *Am J Public Health* **90**, 1409-1415 (2000).

8 Meyer, K. A. *et al.* Carbohydrates, dietary fiber, and incident type 2 diabetes in older women. *Am J Clin Nutr* **71**, 921-930 (2000).

9 Chiva-Blanch, G. & Visioli, F. Polyphenols and health: Moving beyond antioxidants. *Hournal of Berry Research* **2**, 63-71 (2012).

10 Malunga, L. N., Eck, P. & Beta, T. Inhibition of Intestinal alpha-Glucosidase and Glucose Absorption by Feruloylated Arabinoxylan Mono- and Oligosaccharides from Corn Bran and Wheat Aleurone. *Journal of Nutrition and Metabolism*, doi:10.1155/2016/1932532 (2016).

11 Kwon, O. *et al.* Inhibition of the intestinal glucose transporter GLUT2 by flavonoids. *Faseb Journal* **21**, 366-377, doi:10.1096/fj.06-6620com (2007).

12 Song, J. *et al.* Flavonoid inhibition of sodium-dependent vitamin C transporter 1 (SVCT1) and glucose transporter isoform 2 (GLUT2), intestinal transporters for vitamin C and Glucose. *The Journal of biological chemistry* **277**, 15252-15260, doi:10.1074/jbc.M110496200 (2002).

13 Ludwig, D. S. & Eckel, R. H. The glycemic index at 20 y? *American Journal of Clinical Nutrition* **76**, 264S-265S. (2002).

14 Scalbert, A., Morand, C., Manach, C. & Remesy, C. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed Pharmacother* **56** (2002).

15 Belobrajdic, D. P. & Bird, A. R. The potential role of phytochemicals in wholegrain cereals for the prevention of type-2 diabetes. *Nutrition Journal* **12**, 62, doi:10.1186/1475-2891-12-62 (2013).

16 Scalbert, A. & Williamson, G. Dietary intake and bioavailability of polyphenols. *The Journal of nutrition* **130**, 2073s-2085s (2000).

17 Ramakrishna, A. & Ravishankar, G. A. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior* **6**, 1720-1731, doi:10.4161/psb.6.11.17613 (2011).

18 Wahid, A. Physiological implications of metabolite biosynthesis for net assimilation and heat-stress tolerance of sugarcane (Saccharum officinarum) sprouts. *Journal of plant research* **120**, 219-228, doi:10.1007/s10265-006-0040-5 (2007).

19 Wang, S. Y. & Zheng, W. Effect of plant growth temperature on antioxidant capacity in strawberry. *Journal of Agricultural and Food Chemistry* **49**, 4977-4982, doi:10.1021/jf0106244 (2001).

20 Jeong, S. W. *et al.* The effects of different night-time temperatures and cultivation durations on the polyphenolic contents of lettuce: Application of principal component analysis. *Journal of Advanced Research* **6**, 493-499, doi:http://dx.doi.org/10.1016/j.jare.2015.01.004 (2015).

21 Wu, G. *et al.* Growth temperature and genotype both play important roles in sorghum grain phenolic composition. *Sci Rep* **6**, 21835, doi:10.1038/srep21835 (2016).

22 Högy, P. & Fangmeier, A. Effects of elevated atmospheric CO2 on grain quality of wheat. *Journal of Cereal Science* **48**, 580-591, doi:http://dx.doi.org/10.1016/j.jcs.2008.01.006 (2008).

23 Taub, D. R., Miller, B. & Allen, H. Effects of elevated CO2 on the protein concentration of food crops: a meta-analysis. *Global Change Biology* **14**, 565-575 (2008).

24 Wieser, H., Manderscheid, R., Erbs, M. & Weigel, H. J. Effects of elevated atmospheric CO2 concentrations on the quantitative protein composition of wheat grain. *J Agric Food Chem* **56**, 6531-6535, doi:10.1021/jf8008603 (2008).

25 Shamloo, M. *et al.* Effects of genotype and temperature on accumulation of plant secondary metabolites in Canadian and Australian wheat grown under controlled environments *Scientific Reports* **Accepted** X(2017).

26 Boz, H. Ferulic acid in cereals – a Review. *Czech Journal of Food Sciences* **33**, 1-7 (2015).

27 Yu, L., Nanguet, A.-L. & Beta, T. Comparison of antioxidant properties of refined and whole wheat flour and bread. *Antioxidants* **2**, 370-383, doi:10.3390/antiox2040370 (2013).

28 Hernandez, L., Afonso, D., Rodriguez, E. M. & Diaz, C. Phenolic compounds in wheat grain cultivars. *Plant Foods for Human Nutrition* **66**, 408-415, doi:10.1007/s11130-011-0261-1 (2011).

29 Wu, G. *et al.* Growth temperature and genotype both play important roles in sorghum grain phenolic composition. *Scientific Reports* **6**, doi:10.1038/srep21835 (2016).

30 U.S. Department of Health and Human Services and U.S. Department of Agriculture. 2015–2020 Dietary Guidelines for Americans. 8th Edition. December 2015. Available at http://health.gov/dietaryguidelines/2015/guidelines/.

31 Health Canada, Eating Well with Canada's Food Guide –A Resource for Educators and Communicators. 2011, Available at: http://www.healthcanada.gc.ca/foodguide.

CHAPTER 7

OVERALL CONCLUSION

7.1 SUMMARY AND IMPLICATIONS

In order to prove that concentrations of bioactives in wheat a determined by the genotype, but also by environmental factors, a two-phase project was designed under special considerations of increasing growing temperature due to global climate change.

In the first phase, the effects of genotype, location and crop year on secondary metabolites of Canadian wheat grains grown under field conditions were determined. Here, the profiles and concentrations of phenolic acids, flavonoids, fatty acids and plant sterols of Canadian wheat varieties were shown to be significantly influenced by genotype, crop year, and growth locations. Phenolic acids, flavonoids and fatty acids of wheat grains were most influenced by the effect of the environment, however, the wheat plant sterols were determined by the genotype. Increased growing temperature resulted in a significantly higher amount of total flavonoids contents. Plant sterols in red spring wheat varieties were significantly higher than white spring wheat varieties.

Phase one's results informed on the selection of the wheat genotypes containing higher amounts of secondary metabolites in order to conduct the second phase, where selected wheat varieties were grown under controlled environments where the only changing factor was temperature , in order to investigate the global climate change effect on wheat secondary metabolites.

In increased the growing temperatures, elevated total phenolic acids, total flavonoid contents, palmitic acid (C16:0) and oleic acid (C18:1n9) were observed. Moreover, reductions in the amounts of linoleic acid (C18:2n6), linolenic acid (C18:3n3) and campesterol content of wheat varieties were detected.

To translate the observed changes of secondary metabolites into potential into mechanisms of health benefits, the inhibition of cellular glucose uptake was assessed in the CaCo-2 model of intestinal glucose uptake. These inhibitions correlated positively with the phenolics contents of a variety of wheat extracts.

The inhibition of intestinal glucose uptake represents one of the latest concepts in diabetes and obesity research and offers the possibility to control postprandial blood glucose spikes via dietary components. This work demonstrates that phenolic acids in whole wheat extracts exert inhibitions and therefore are prime candidates to investigate these effects in more advanced animal models and human intervention trials.

Additionally, this study demonstrates elevated levels of potential beneficial bioactives when growing temperatures are elevated. It is predicted that global temperature increase by 1.8-4ºC by 2100 [4], and it is currently estimated that this will negatively impact food security and nutrient contents of crops. However, it should be noted that in the case of wheat elevated levels of bioactives might result from future growing conditions and this recognition might help the food industry to enhance beneficial dietary compounds.

Lastly, taken the results of two phases together, the present research provides useful information and/or implications for wheat breeders to select wheat varieties containing higher amounts of secondary bioactives.

7.2 LIMITATIONS

In the present research, the following areas would be considered as the main limitations:

1) The results of phase 1 of this study mainly represent the changes of secondary metabolites of Canadian wheat varieties grown in different locations in Canada, hence selecting larger sets of wheat varieties and growing them in the international agricultural centers could provide a more comprehensive data set.

2) In phase 2 of the present study, while growing Canadian and Australian wheats in the growth chambers, the only variable parameter was the temperature and it can be expected that other environmental factors such as water deprivation, elevated CO_2, and UV light can induce a different physiological response in wheat plants. Thus our present results are lacking information regarding the combined effect of these abiotic factors on wheat secondary metabolites.

7.3 FUTURE DIRECTIONS

Future studies should, therefore, focus on simultaneously testing the effects of multiple environmental factors to gain a more realistic perspective of how global climatic changes may impact the production of secondary bioactive metabolites of wheat grains and their related health

properties. Further research is needed to investigate the effects of wheat extracts on inhibition of glucose uptake through animal studies and human intervention trials.

7.4 FINAL REMARKS

Although the current research cannot help us to explain the exact underlying mechanism of how polyphenols inhibit the glucose uptake, it offers a novel hypothesis as an alternative to the antioxidant hypothesis on the mechanism of preventive effects of whole wheat consumption on type 2 diabetes. Based on these observations it can be speculated that rising global temperatures could increase the nutritional value of whole wheat, considering that elevated phenolics will lead to a better control of postprandial hyperglycemia. Since postprandial hyperglycemia is associated with obesity and type 2 diabetes, this effect might contribute to the mitigation of these major non-communicable diseases.

7.5 REFERENCES

1 Fernandez-Orozco, R., Li, L., Harflett, C., Shewry, P. R. & Ward, J. L. Effects of environment and genotype on phenolic acids in wheat in the HEALTHGRAIN diversity screen. *Journal of agricultural and food chemistry* **58**, 9341-9352, doi:10.1021/jf102017s (2010).

2 Mpofu, A., Sapirstein, H. D. & Beta, T. Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. *Journal of Agricultural and Food Chemistry* **54**, 1265-1270, doi:10.1021/jf052683d (2006).

3 Maki, K. C. & Phillips, A. K. Dietary substitutions for refined carbohydrate that show promise for reducing risk of type 2 diabetes in men and women. *The Journal of nutrition* **145**, 159s-163s, doi:10.3945/jn.114.195149 (2015).

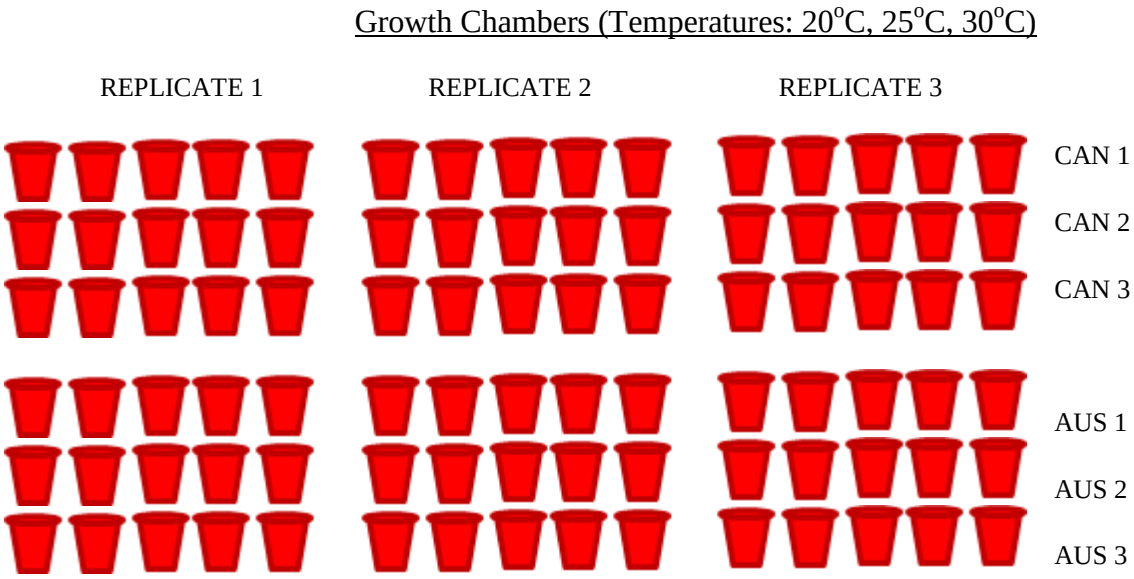
4 *IPCC. Contribution of Working Group I To The Fourth Assessment Report of The Intergovernmental Panel On Climate Change. Cambridge University Press, Cambridge, United Kingdom (2007).*

5 Vickers, C. E., Gershenzon, J., Lerdau, M. T. & Loreto, F. A unified mechanism of action for volatile isoprenoids in plant abiotic stress. *Nat Chem Biol* **5**, 283-291 (2009).

6 Ramakrishna, A. & Ravishankar, G. A. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior* **6**, 1720-1731, doi:10.4161/psb.6.11.17613 (2011).

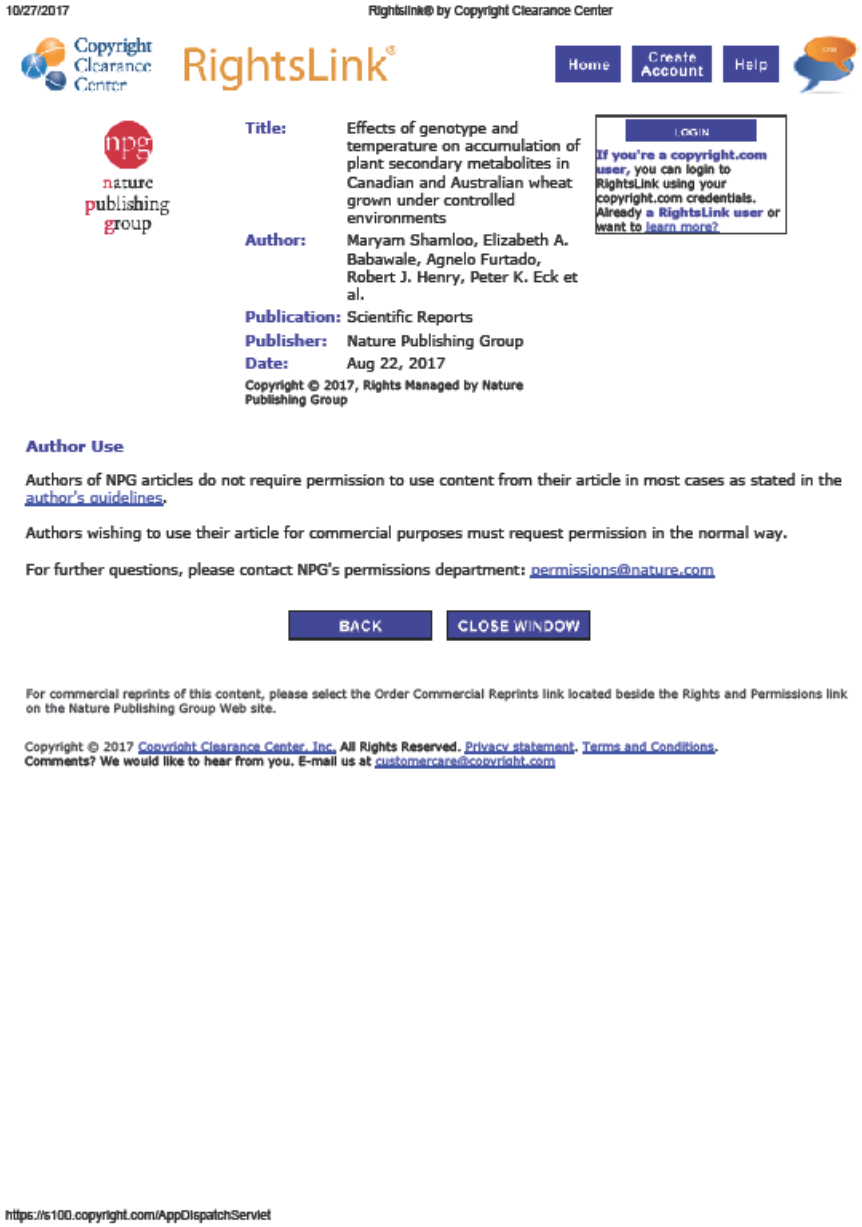
APENDICES

APPENDIX 1: PICTORIAL REPRESENTATION FOR THE LAYOUT OF THE TEMPERATURE REGIMES EXPERIMENT IN GROWTH CHAMBERS



NOTE: Six varieties of 3 Australian (AUS) and 3 Canadian (CAN) within each replicate were arranged in a random fashion in accordance with the statistical procedures for the design of experiment as per Randomized Block Design (RBD).

APPENDIX 2: COPYRIGHT LICENSES FOR PREVIOUSLY PUBLISHED MATERIALS



10/27/2017 RightsLink® by Copyright Clearance Center

Copyright Clearance Center RightsLink®

Home | Create Account | Help

nature publishing group

Title: Effects of genotype and temperature on accumulation of plant secondary metabolites in Canadian and Australian wheat grown under controlled environments

Author: Maryam Shamloo, Elizabeth A. Babawale, Agnelo Furtado, Robert J. Henry, Peter K. Eck et al.

Publication: Scientific Reports

Publisher: Nature Publishing Group

Date: Aug 22, 2017

Copyright © 2017, Rights Managed by Nature Publishing Group

LOGIN

If you're a copyright.com user, you can login to RightsLink using your copyright.com credentials. Already a RightsLink user or want to learn more?

Author Use

Authors of NPG articles do not require permission to use content from their article in most cases as stated in the author's guidelines.

Authors wishing to use their article for commercial purposes must request permission in the normal way.

For further questions, please contact NPG's permissions department: permissions@nature.com

BACK | CLOSE WINDOW

For commercial reprints of this content, please select the Order Commercial Reprints link located beside the Rights and Permissions link on the Nature Publishing Group Web site.

Copyright © 2017 Copyright Clearance Center, Inc. All Rights Reserved. Privacy statement. Terms and Conditions.
Comments? We would like to hear from you. E-mail us at customercare@copyright.com

https://s100.copyright.com/AppDispatchServlet 1/1