Peace Country Initiative

Evaluation of Fatty Acid Composition and Lignan Content of Peace Country Flax Seed

Jian Zhang, Alberta Research Council

Rong Cao, Guelph Food Research Centre
Agriculture and Agri-Food Canada

March, 2009

ABSTRACT

This project is a collaboration among the Branding Peace Country Canada, the Alberta Research Council and the Guelph Food Research Center of Agriculture and Agri-Food Canada. The main objective of the project was to document scientifically the quality characteristics of Canada's Peace Country commodity flax. Mature flax seeds were collected from the Peace Country and central Alberta locations. The fatty acid composition and lignan (SDG, secoisolariciresinol diglucoside) content of collected flax seeds were analysed at the Alberta Research Council and Guelph Food Research Center, Agriculture and Agri-Food Canada, respectively. The results showed higher levels of α-linolenic acid and lignan in flax seeds grown in the Peace Country than in those grown in central Alberta and elsewhere. However, the research team recognizes the limitations of sample size and short study period of this project and therefore recommends a multi-year, multi-location and multi-variety study to confirm the superior quality of Peace Country flax.

Jian Zhang, MSc. PhD
Research Scientist
Alberta Research Council

Rong Cao, PhD
Research Scientist
Guelph Food Research Centre
Agriculture Agri-Food Canada

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX

Abbreviations

ALA	**alpha-linolenic acid**
BPCA	**Branding the Peace Country Association**
DHA	**docosahexaenoic acid**
ED	**enterodiol**
EL	**enterolactone**
EPA	**eicosapentaenoic acid**
FAMEs	**fatty acid methyl esters**
GC	**gas chromatography**
HPLC	**high-performance liquid chromatography**
SDG	**secoisolariciresinol diglucoside**

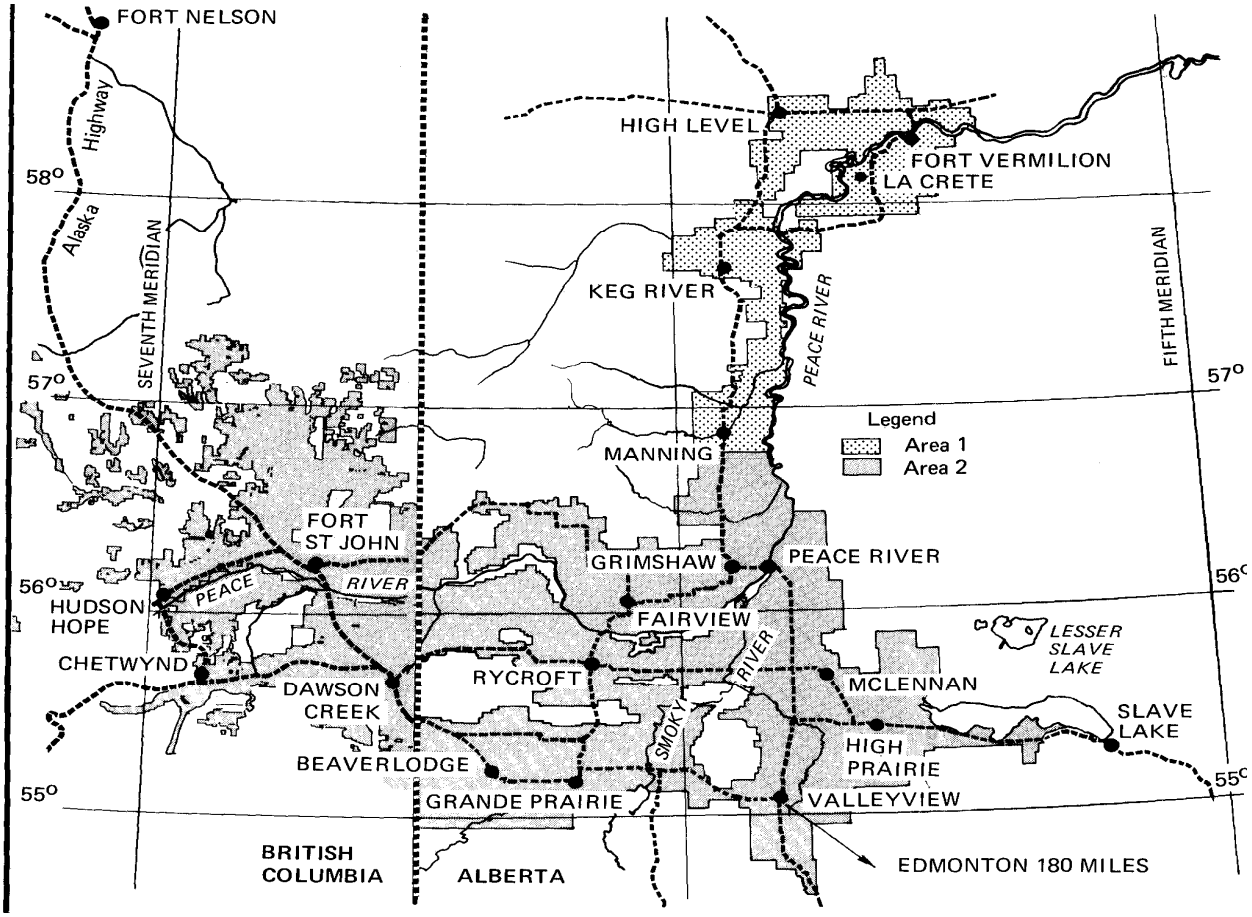
Chapter 1. Introduction and Background

1.1 Background of the project

Anecdotes from the farmers of Canada's Peace Country, the most northern agricultural region in Northern America, have long referred to "*northern vigour" as though it were a* unique phenomenon. Nestled in a valley along the foothills of the Rocky Mountains and fed with the pure waters of the Peace River watershed system, the geographic location of Canada's Peace Country has unique growing advantages (Fig.1.1). A combination of long freezing winters and short intense summers provides this region with favoured climatic conditions for growing high quality commodities. Branding the Peace Country Association (BPCA), a registered non-profit organisation launched in 2004, is a result of the vision of a group of commodity farmers in Canada's Peace Country who, in 1999, were determined to produce a collective "farmers' wisdom". Many farmers in the Peace Country viewed this wisdom - "that certain crops grown in the Peace Country are superior in quality than those of the international competitors" – with scepticism because of the lack of hard proof of the superiority of their crops.

Branding the Peace Country Association has developed a unified marketing strategy that captures these qualities of the Peace Country commodities. BPCA has identified that clarifying the definition of "high quality" is their top priority. To implement this top objective, the Association began a research project titled "*Documenting Peace Potency – Identifying the Nutritional, Chemical, and/or Growing Superiority of Agricultural Commodities Grown in Canada's Peace Country"* in 2008 in collaboration with Alberta Research Council and Agriculture & Agri-Food Canada. The Association is currently collecting agricultural production data for the Peace Country and national and international production data, and identifying the most "marketable" traits of the commodities grown in the Peace Country. BCPA has identified two questions that need to be answered for a commodity to be included in a regional marketing strategy. First: what is its unique quality? Second, who would be willing to pay for the premium of such high quality commodities? BCPA has determined that commodities to be studied in the research project will have one or more favourable quality characteristics such as nutritional, chemical, or growth superiority.. These characteristics are potentially of high values to retailers, consumers and international commodity buyers.

Preliminary research conducted by the BPCA highlights seven commodities that have potential for the Peace Country through a regional marketing strategy. These commodities are: flax, alfalfa, honey, timothy hay, and canola, seed potatoes and oat. BPCA's intent is to identify a minimum of one unique quality trait per commodity, relative to those from the international competitors, and then design a marketing strategy utilizing the findings of this special trait.



The agricultural area of the Peace River Region in Alberta and British Columbia. Area 1 extends from **10** miles south of Manning, Alberta, north to the High Level and Fort Vermilion area. Area 2 consists of the remaining parts of the Peace River Region. Adopted from McKenzie and Davidson, 1975,

Figure 1.1 Peace Country Agricultural Regions

Flax (*Linum usitatissimum* L) is a commercially important oilseed crop world wide. According to Flax Canada 2015 vision, the flax acreage will be approximately 7 millions acres by 2015. The flax industry contributed $300 million to the Canadian economy with a potential of $1.5 billion by 2015. Flax oil has both industrial and food applications, while flax meal is used as ruminant feed. Flax fibres are used for the manufacturing of high quality linen fabrics

and other industrial products. Flax seed has also been increasingly used as a nutraceutical in recent years, since it contains exceptionally high concentration of phytoestrogenic compounds known as lignans and dietary fibres in additions to its high content of ω-3-fatty acid. Traditional linseed oil contains high levels of α-linolenic acid (18:3, Fig 1.2) and is mainly used for industrial purposes, such as protective coatings (paints, varnishes and lacquers), linoleum, inks and ingredients of cosmetics. Newly-developed solin flax oil that contains a low level of α-linolenic acid and high level of linoleic acid has been mainly used for food applications such as margarine, salad dressing and other food products. Moreover, through genetic engineering, both linolenic acid and linoleic acid are ideal precursors for the production of very long chain polyunsaturated fatty acids with important health benefits and the production of industrial fatty acids of significant commercial applications. Furthermore, flax may be used as bio-vehicle for the expression of high value pharmaceutical proteins.

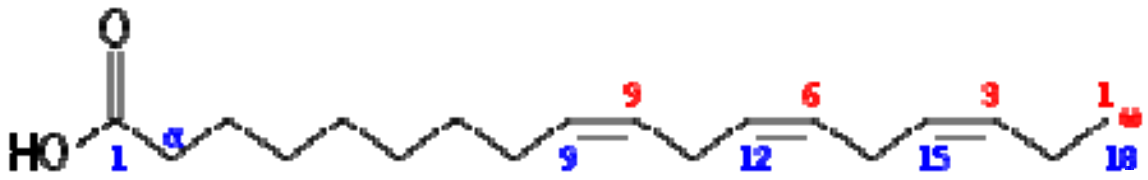


Figure 1.2. Chemical structure of alpha-linolenic acid (ALA), an essential −3 fatty acid, (18:3Δ9c,12c,15c, which means a chain of 18 carbons with 3 double bonds on carbons numbered 9, 12 and 15).

Shirouchi reported that Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) have cholesterol lipid-lowering effects in animal models and human studies (Shirouchi et al, 2007). Lands reported how *ω*-3 fatty acids can diminish competitively the intensity of *ω*-6 eicosanoid formation and the rate-limiting events in thrombosis (Lands, 1991). These competitive actions link the inadvertent dietary choices of *ω*-3 and *ω*-6 polyunsaturated fatty acids to the frequency of thrombotic deaths, and they illustrate the vital importance of the *ω*-3 and *ω*-6 fatty acids as precursors for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Fig 1.3). Lohner reported that increased dietary intake of *ω*-3 fatty acids may benefit persons with increased cardiovascular risk of obese subjects (Lohner et al, 2007).

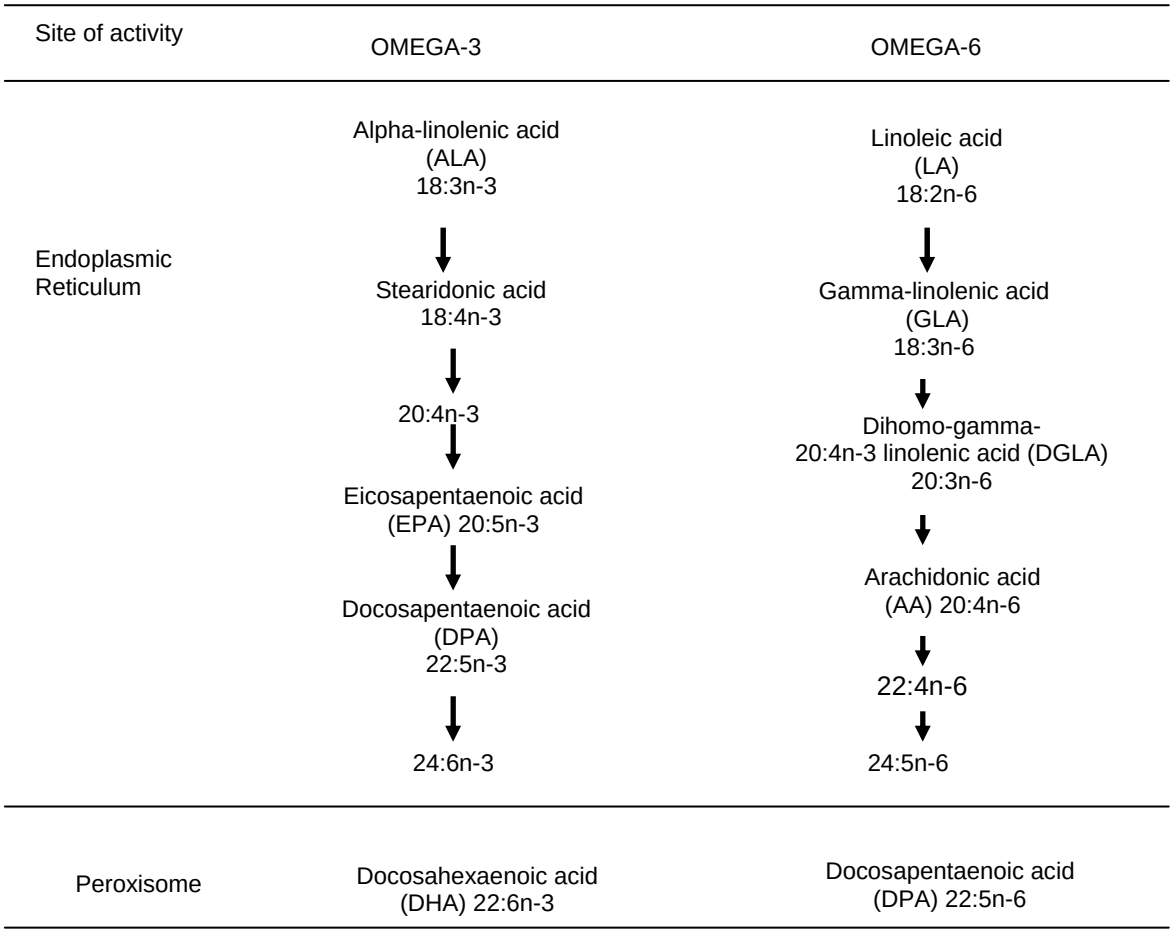


Figure 1.3 Metabolic pathways of the omega-3 and omega-6 fatty acids

Lignans are a group of phenolic compounds mainly found in plants, particularly in flax seed. The principal lignan found in flax seed is secoisolariciresinol diglucoside (SDG, Fig 1.4). Epidemiological and experimental studies strongly suggest that lignans have a potential role in the prevention of menopausal symptoms, hormone-dependent cancers (e.g. breast and prostate cancer), cardiovascular disease, and possibly osteoporosis. The beneficial effects of flax seed are mediated mainly by its mammalian lignan precursor SDG , which upon the action of colonic microflora is converted to mammalian lignans, enterolactone (EL) and enterodiol (ED), and subsequently absorbed and undergo enterohepatic circulation (Borrielo et al, 1985). Mammalian lignans (SDG metabolites) are positively linked to several bioactivities including antiestrogenic, anticarcinogenic, and antioxidant activities. Other research has shown that

antioxidant activities of SDG and its mammalian lignan metabolites can be stronger than vitamin E. Therefore lignan content (SDG concentration) can be one of the most important indicators of potential health benefit of flax in addition to ω-3 fatty acids. In other words, flax with high SDG content is potentially healthier.

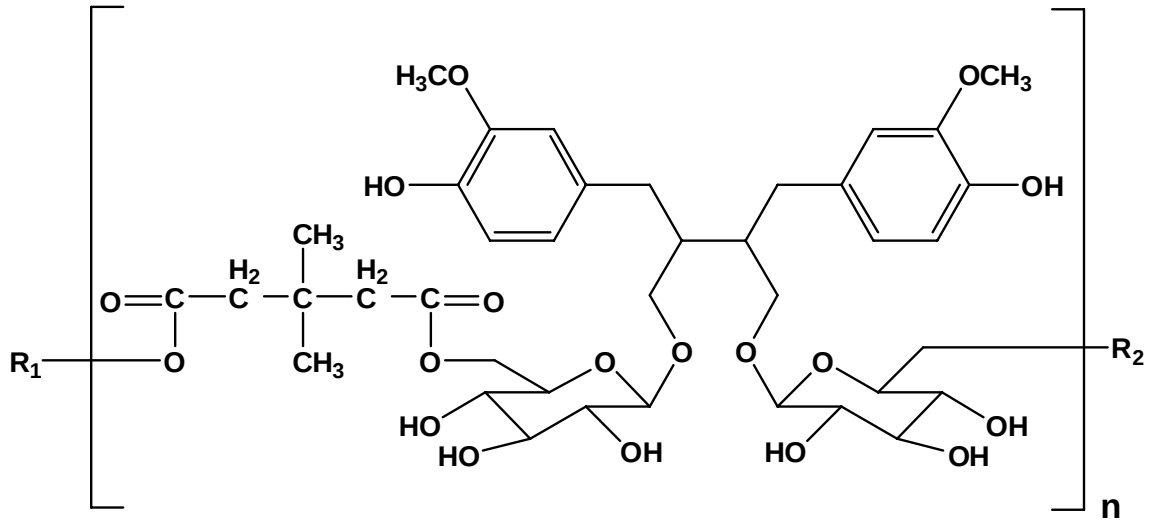


Figure 1.4 Structure of the lignan macromolecule from flaxseed: R1 = H or SDG, R2=OH

1.2 The objectives of this project are:

1. To analyse and evaluate the fatty acid composition of Peace Country flax seeds.
2. To analyse and evaluate the lignan content of Peace Country flax seeds.
3. To compare the ALA and lignan contents from the Peace Country flax with those from other flax-growing regions

Chapter 2. Methods and Materials

2.1 Fatty acid analysis

Fatty acid composition of total acyl lipid from mature seeds was determined following the International Organization for Standardization method reference number ISO 5508:1990 (E), "Animal and vegetable fats and oils—Analysis by gas chromatography of methyl esters of fatty acids". Between 50 and 100 mg of seeds were homogenized in 1 mL of petroleum ether in a 5 mL polypropylene vial using a steel rod. After allowing the meal to settle, 0.5 mL of the supernatant was transferred to a glass tube containing 1.2 mL of methylating solution (2% sodium methoxide in methanol). After thorough mixing, the solution was incubated at room temperature for 30 minutes. One mL of ddH_2O was added to the solution, mixed well and left for 10 minutes at room temperature for the phases to separate. After separation, 200 µL from the upper layer was diluted with 300 µL of petroleum ether in a GC autosampler vial and 2 µL was injected into a GC column.

Separation of fatty acid methyl esters (FAMEs) was performed on a flame ionization gas chromatograph (model 6890, Hewlett Packard, Mississauga, ON) fitted with a 30-m X 0.25 mm (i.d.) column (HP-INNOWAX, crosslinked polyethylene glycol) with helium as the carrier gas at a flow rate of 28.0 mL/minute. The oven temperature was from 180°C to 230°C at a rate of 5°C/minute and then held at 230°C for 13 minutes. Peaks were assigned by comparing retention time of those in the FAME standards (Nu-Chek Prep, Elysian MN, USA) and relative proportions of FAMEs were determined as percentages of summed peak areas.

2.2 Lignan analysis

An SDG working standard was accurately prepared at a concentration of 0.25mg/ml in methanol/water 40/60 (v/v). Ground flax seed samples (225 mg± 50 mg) were weighed and added to 4.0 ml isopropyl alcohol (IPA) in a clean dry 50 ml volumetric flask. The mixture was allowed to stand for 20 minutes and added 8.0 ml 2N NaOH. The mixture was then sonicated for 60 minutes in an ultrasonic bath at 60^0C. Once cooled to ambient temperature, the mixture was added 6.0 ml diluted phosephoric acid (1/5, v/v), and methanol/water 40/60 (v/v) to the volume (50 ml). The supernatant was filtered through a 0.45µm syringe filter prior to analysis. An Agilent Technology Series 1100 high-performance liquid chromatography (HPLC) system with an auto sampler and a diode array detector (monitored at 280 nm for quantification) was used for the analysis. Column: Phenomenex C18(2) column, 5µm, 250 x 4.6 mm; flow rate: 1.15 ml/min; mobile phase: A, 0.2% H_3PO_4 water; B: Acetonitrile. Gradient Program: 0-1 min, 90% A; 16 min,

70%A; 22 min, 10%A; 30 min, 0%A, 35 min, 90%A. The injection volumes were 10 µl. The retention time of SDG: 21.4 min.

2.3 Statistical analysis

SAS (SAS Institute Inc., 100 SAS Campus Drive, Cary, NC, 27513-2414, USA) software was used to perform the statistical analysis. The programs are shown below**:**

The SAS program

```
option formdlim='-';
data jian;
input loc$ sample content;
cards;
Veg 1       54.71
Veg 2       53.74
Veg 3       56.65
Veg 20      53.97
Veg 21      57.56
Veg 23      55.80
Veg 24      55.74
Veg 25      56.38
peace 1     61.72
peace 2     60.38
peace 3     61.53
peace 21    60.72
peace 22    60.94
peace 23    60.46
peace 24    61.67
peace 25    61.39
run;
proc univariate data=jian normal;
class loc;
var content;
run;
*they are normal so I can proceed with the t test;

ods rtf file='X:\Jian Zhang\SAS\results.rtf' STARTPAGE=NO;;
Proc ttest data=jian;
class loc;
var content;
run;
ods rtf close;

*the ANOVA;
proc glm data=jian;
class loc;
model content=loc;
lsmeans loc/pdiff stderr;
run;
quit;
```

Chapter 3 Results and Conclusions

3.1 ALA analysis on Peace Country Flax vs. Central Alberta flax

GC analyses were conducted with Peace Country (brown flax, PC Milling's Grains, Vimy, collected in 2008) and central Alberta samples (brown flax, Vegreville, CDC Bethune, collected in 2007). Eight replicate testing reactions were performed by using brown flax seed collected from two different locations. Peaks of different fatty acids were identified, and the proportions of alpha-linolenic acid (ALA) were calculated (Appendix 1). Percent ALA in total FA of the 8 replicates are shown in Fig 3.1.

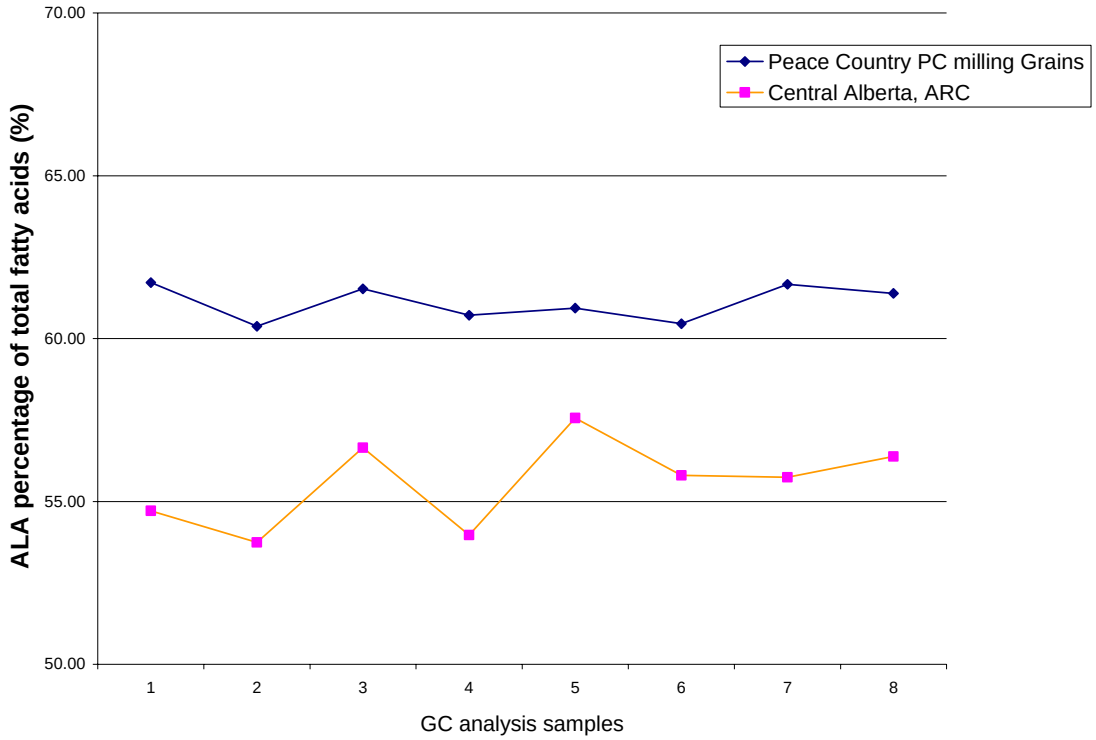


Figure 3.1 Alpha-linolenic acid (ALA) fatty acid amount comparisons between samples collected from Peace Country and Central Alberta

Statistical analysis was conducted using the SAS program. The results showed the ALA amounts are significantly higher in the brown flax (PC Milling's grains, Vimy) grown in Peace Country than that in Central Alberta (CDC Bethune) ($P<0.05$).

3.2 Lignan (SDG) analysis on Peace Country Flax vs. Central Alberta flax

Lignan analysis was conducted with samples collected from the Peace Country and central Alberta together with two samples from local Bulk Barn store in Guelph, Ontario. The results are summarized in Table 3.1. The SDG concentrations of flax grown in the Peace Country were 27-66% higher than those in central Alberta. Peace Country grown flax also showed 15-55% higher SDG content than flax samples purchased from the supermarket (Bulk Barn).

Table 3.1 SDG contents among flax samples

Flax collected different locations	SDG%
Peace Yellow , Yellow Gold (Peace Country)	**1.68% ± 0.02**
Linola1084 (Vegreville)	1.32% ± 0.04
Yellow Flaxseed (buy from Bulk Barn)	1.45% ± 0.04
Peace Brown, Vimy (Peace Country)	**1.82% ± 0.01**
CDC Bethune (Vegreville)	1.09% ± 0.03
Brown Flaxseed (buy from Bulk Barn)	1.19% ± 0.04

The brown flax (Vimy) grown in Peace Country contained 1.82% SDG as compared to 1.09% in the brown flax variety (Bethune) grown in central Alberta. For the yellow flax, the SDG concentrations were 1.68% and 1.32 %, respectively. The results suggest that Peace Country provides favourable growing environment for lignan production in flax (Fig. 3.2).

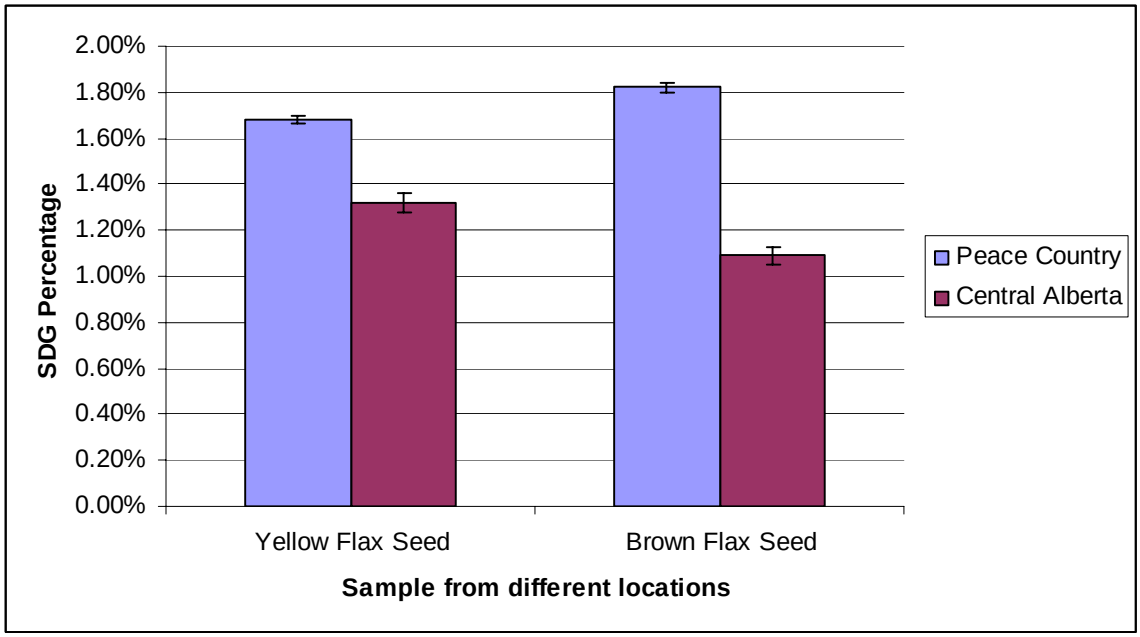


Figure 3.2 Comparison of lignan between Peace Country grown flax and central Alberta grown flax

3.3 Conclusions and Recommendations

Analysis of our preliminary data showed higher ALA and Lignan in Peace Country flax samples. Fatty acids analyses of the of brown flax, Vimy from PC Milling grains of Peace Country and the brown flax, CDC Bethune grown in central Alberta by gas chromatography showed an increase of 9.9% higher level of ALA in the former. The SDG content in flax grown in the Peace Country was 15-66% higher as compared to those in the flax grown in central Alberta.

We believe this to be a significant finding. The elevated concentrations of ALA and SDG in flax grown in the Peace Country indeed support the legend of the "*Northern Vigour*". However, cautions must be taken in the interpretation. First of all, albeit nutritional contents such as ALA and SDG are most important indicators of flax product quality, other components such as soluble dietary fibre and protein may also be useful quality traits; secondly, we have based the results we obtained in this study on limited varieties and number of samples. A solid conclusion can only be drawn when a systematically designed experiment that tests the effect of genetics (variety), geographic location and season is implemented. Nonetheless, our results have laid an initial foundation toward proving the superiority of Peace Country crops such as flax seed.

The research team for this project, including researchers from Agriculture and Agri-Food Canada, Alberta Research Council, and Branding Peace Country Association strongly believes that a multi-year, multi-location and multi-variety study is essential to prove our belief and the Peace Country claims. Analysis of the two most important nutritional markers, i.e. ALA and SDG from samples in Northern and central Alberta, will be central to such a study. Comparing data with those obtained elsewhere and by other researchers will also assist the team in making more scientifically sound conclusions.

References

Borrielo SP, Setchell KDR, Axelson M, Lawson AM. (1985) Production and metabolism of lignans by the human faecal flora. J Appl Bacteriol 58: 37-43.

Lands WEM. (1991) Biochemistry and physiology of n-3 fatty acids. *The FASEB Journal* 6:2530-2536

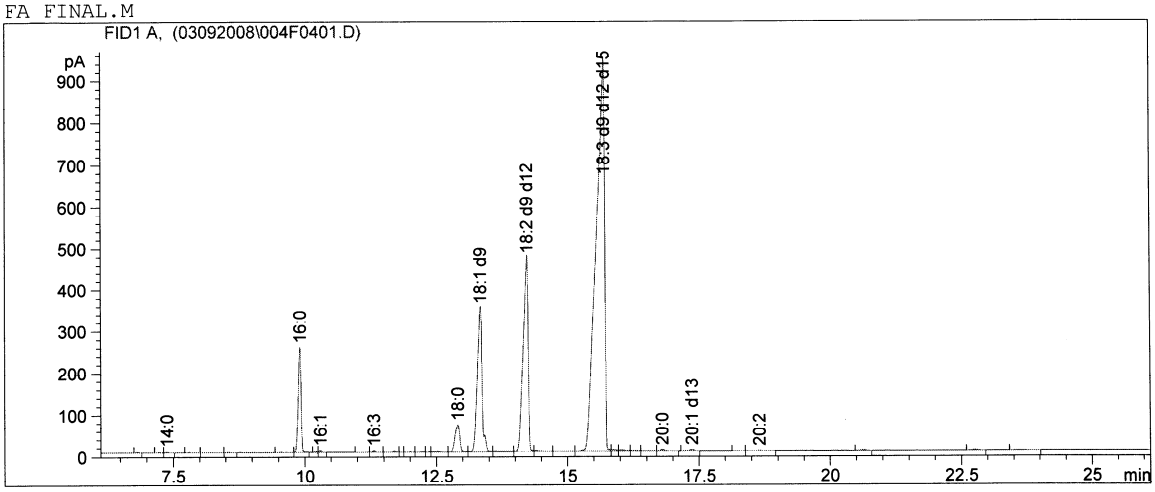
Lohner S, MarosvolgyiT, Burus I,Schmidt J, Molnar D, Decsi T. (2007) Dietary supplementation of obese children with 1000 mg alpha-linolenic acid per day: a placebo-controlled double blind study. *Orvosi Hetilap* 148:1499

Shirouchi B, Nagao K, Inoue N, Ohkubo T, Hibino H, Yanagita T. (2007) Effect of Dietary Omega 3 Phosphatidylcholine on Obesity-Related Disorders in Obese Otsuka Long-Evans Tokushima Fatty Rats. *Journal of Agricultural and Food Chemistry* 55:7170-7176

Appendix 1. Example of GC chromatographs (flax from Peace Country, central Alberta)

Data File C:\HPCHEM\1\DATA\03092008\004F0401.D Sample Name: Peace brown

```
======================================================================
Injection Date  : 9/3/08 8:01:28 PM              Seq. Line :   4
Sample Name     : Peace brown                        Vial :   4
Acq. Operator   : Jian                               Inj :   1
                                                 Inj Volume : 1 µl
Different Inj Volume from Sequence !     Actual Inj Volume : 2 µl
Acq. Method     : C:\HPCHEM\1\METHODS\FA_FINAL.M
Last changed    : 8/18/08 11:36:47 AM by Jian
Analysis Method : C:\HPCHEM\1\METHODS\FA_FINAL.M
Last changed    : 9/19/08 4:05:27 PM by Jian
                  (modified after loading)
FA_FINAL.M
```



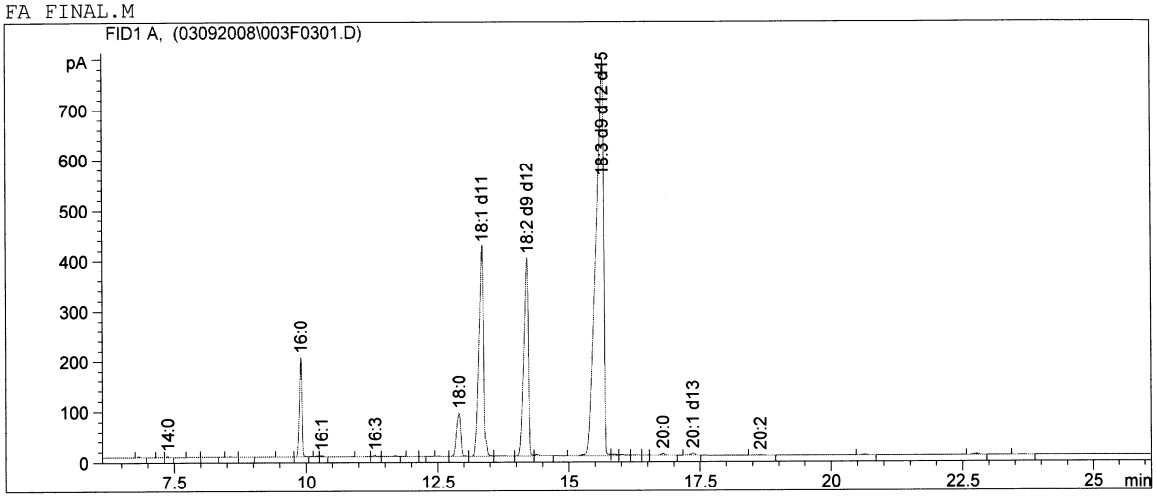

```
======================================================================
                      Normalized Percent Report
======================================================================

Sorted By             :      Signal
Calib. Data Modified  :      9/19/08 4:05:27 PM
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: FID1 A,
```

RetTime [min]	Type	Area [pA*s]	Amt/Area	Norm %	Grp	Name
7.381	VB	5.62782	1.00000	0.033100		14:0
9.912	VB	894.02954	1.00000	5.258198		16:0
10.295	VP	10.53933	1.00000	0.061987		16:1
11.309	VV	8.96637	1.00000	0.052735		16:3
12.909	VV	390.45126	1.00000	2.296423		18:0
13.342	VV	2136.00537	1.00000	12.562827		18:1 d9
13.350		-	-	-		18:1 d11
14.233	VV	3047.62012	1.00000	17.924452		18:2 d9 d12
14.400	VV	15.89179	1.01900	0.095243		
15.121		-	-	-		Peak ?
15.700	VV	1.04490e4	1.00000	61.455114		18:3 d9 d12 d15
16.800	VB	18.49230	1.00000	0.108762		20:0
17.318		-	-	-		20:1 d11
17.366	BP	18.19538	1.00000	0.107015		20:1 d13
18.649	VB	7.50584	1.00000	0.044145		20:2
19.770		-	-	-		21:0
22.362		-	-	-		Peak ?
22.938		-	-	-		22:0
23.878		-	-	-		22:1 d13

Instrument 1 9/19/08 4:05:27 PM Jian Page 1 of 2

Data File C:\HPCHEM\1\DATA\03092008\003F0301.D Sample Name: CDC brown

```
==========================================================================
Injection Date  : 9/3/08 7:21:34 PM              Seq. Line :   3
Sample Name     : CDC brown                         Vial :   3
Acq. Operator   : Jian                               Inj :   1
                                                 Inj Volume : 1 µl
Different Inj Volume from Sequence !     Actual Inj Volume : 2 µl
Acq. Method     : C:\HPCHEM\1\METHODS\FA_FINAL.M
Last changed    : 8/18/08 11:36:47 AM by Jian
Analysis Method : C:\HPCHEM\1\METHODS\FA_FINAL.M
Last changed    : 9/19/08 4:05:27 PM by Jian
                  (modified after loading)
FA_FINAL.M
```




```
==========================================================================
                      Normalized Percent Report
==========================================================================

Sorted By             :      Signal
Calib. Data Modified  :      9/19/08 4:05:27 PM
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: FID1 A,
```

RetTime [min]	Type	Area [pA*s]	Amt/Area	Norm %	Grp	Name
7.381	VP	5.03657	1.00000	0.035654		14:0
9.905	VB	654.53998	1.00000	4.633524		16:0
10.295	VP	6.81097	1.00000	0.048215		16:1
11.309	VV	7.89323	1.00000	0.055877		16:3
12.907	VV	484.17432	1.00000	3.427496		18:0
13.345		-	-	-		18:1 d9
13.355	VV	2536.98242	1.00000	17.959435		18:1 d11
14.217	VV	2359.88086	1.00000	16.705724		18:2 d9 d12
14.395	VB	14.09585	1.01900	0.101681		
15.121		-	-	-		Peak ?
15.655	VV	7998.55908	1.00000	56.622231		18:3 d9 d12 d15
16.796	VB	22.67666	1.00000	0.160529		20:0
17.318		-	-	-		20:1 d11
17.365	BV	26.52708	1.00000	0.187787		20:1 d13
18.645	BP	8.73653	1.00000	0.061846		20:2
19.770		-	-	-		21:0
22.362		-	-	-		Peak ?
22.938		-	-	-		22:0
23.878		-	-	-		22:1 d13

Appendix 2. Statistical analysis results

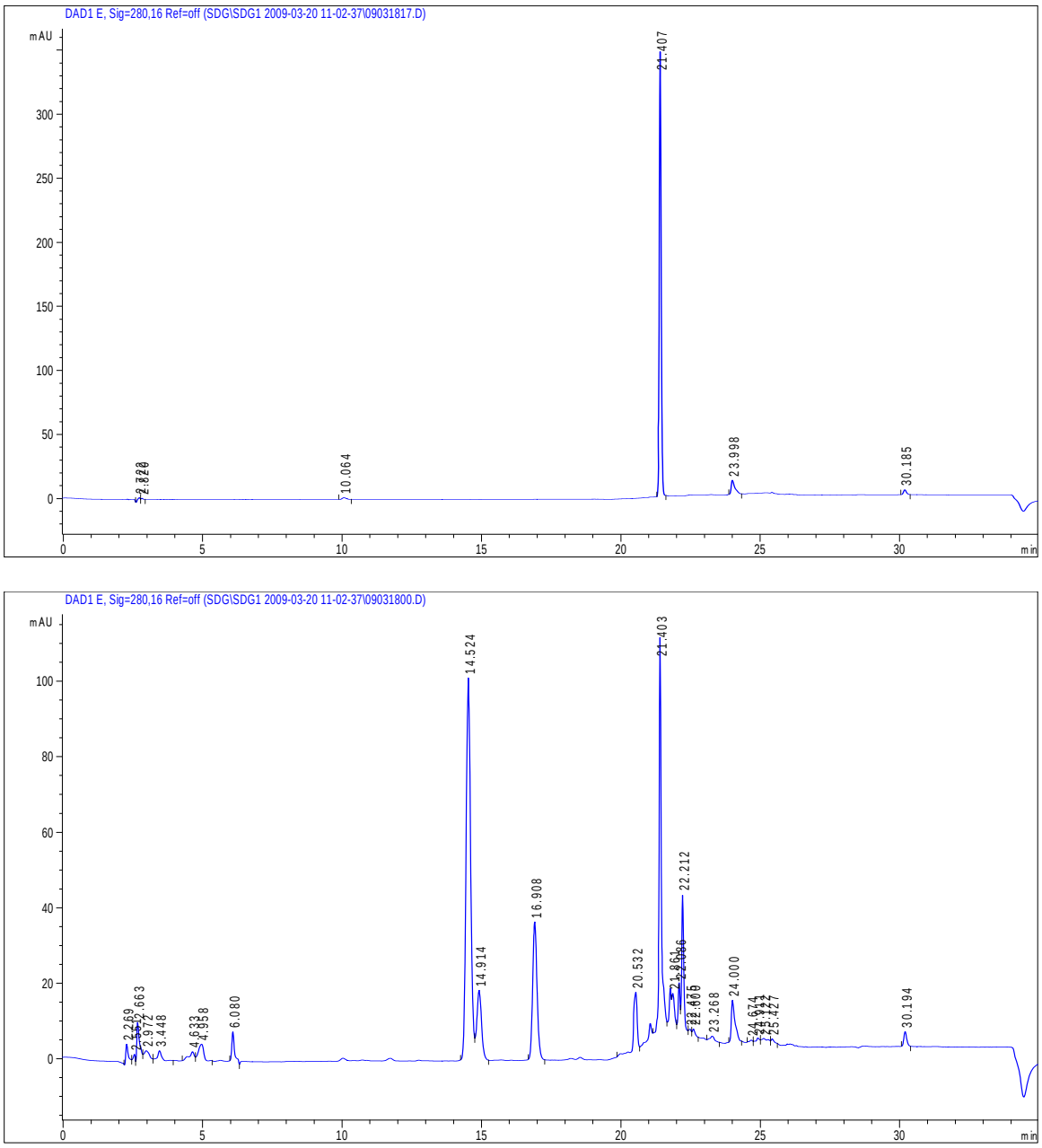
Statistics											
Variable	location	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev	Upper CL Std Dev	Std Err	Minimum	Maximum
content	veg	8	54.451	55.569	56.687	0.884	1.337	2.7211	0.4727	53.74	57.56
content	peace	8	60.646	61.101	61.557	0.3601	0.5446	1.1085	0.1926	60.38	61.72
content	Diff (1-2)		-6.627	-5.533	-4.438	0.7474	1.0208	1.6099	0.5104		

T-Tests					
Variable	Method	Variances	DF	t Value	Pr > |t|
content	Pooled	Equal	14	-10.84	<.0001
content	Satterthwaite	Unequal	9.26	-10.84	<.0001

Equality of Variances					
Variable	Method	Num DF	Den DF	F Value	Pr > F
content	Folded F	7	7	6.03	0.0303

The variances are unequal (p=0.0303 <0.05), therefore the t –test was done with unequal variances (Satterthwaite).

Appendix 3. HPLC analysis of SDG



HPLC Analysis of SDG. Top Panel: SDG standard (retention time: 21.0 min); Bottom Panel: hydrolysis mixture of a typical flax seed containing SDG (peak at 21.0 min).