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Increased Growing Temperature Reduces Content of Polyunsaturated Fatty Acids in Four Oilseed Crops

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List of Abbreviations: Oleic acid = C18:1; Linoleic acid = C18:2; Linolenic acid = C18:3; Gas Chromatography or Gas Chromatograph = GC; Flame Ionization Detector = FID

Abstract

Environmental temperature directly influences the lipid profile produced by oilseeds. If growing temperatures increase, as is predicted by current models, the precise profile of lipids produced are likely to change. This paper develops models to predict lipid profiles as a function of growing temperature. Data relating to lipid profiles of soybean (*Glycine max*), spring canola (*Brassica napus*), spring camelina (*Camelina sativa*), and sunflower (*Helianthus annuus*) were gathered from the literature and evaluated to examine the influence of temperature on relative production of oleic, linoleic, and linolenic acid. For each crop, a set of linear regressions was used to correlate temperature during the grain fill, defined as 30 days before harvest, with the molar percentages of oleic, linoleic, and linolenic acid present. An increase in temperature from 10 to 40°C resulted in an increase in the production of oleic acid and a decrease in the production of linoleic and linolenic acid in soybeans, canola, and sunflowers. Over the range of data available, the lipid profile of camelina was temperature insensitive. To test the validity of the correlations, the four crops were grown in a field study in Manhattan, Kansas simultaneously, in the same environment, in 2011. The correlations accurately predicted the field data for soybean, canola, and camelina but not for sunflower. The correlation for sunflower under-predicted the molar amount of oleic acid and over-predicted the molar amount of linoleic acid. This study indicates increasing growing temperatures from 10 to 40°C will result in more monounsaturated oils and less polyunsaturated oils in soybean, canola, and sunflower.

Keywords: Fatty acid profile; unsaturated oils; *Glycine max*; *Brassica napus*; *Camelina sativa*; *Helianthus annuus*

1. Introduction

Plant lipids are important because of their use as food, fuel, and chemicals. Lipids also have uses as starting materials for surfactants, lubricants, epoxides, coatings, inks, polymers, and other products in the chemical industry (Metzger and Bornscheuer, 2006). The lipid profile of a seed can affect its end use. In oils for human consumption, linoleic acid is valued for its health benefits but linolenic acid results in oil having a poor oxidative stability and shortened shelf life (Singh et al., 2010). For biodiesel production, it is desirable to have a lipid profile that is highly saturated to minimize oxidation of double bonds because oxidized methyl esters can form polymers that plug fuel filters and damage engine performance (Monyem and Gerpen, 2001). Specific lipid profiles also influences reactivity. Multiply unsaturated lipids have been shown to have a higher reactivity than monounsaturated species (Singh et al., 2009, 2011).

Fatty acid profiles are influenced by plant type, genotype, temperature, environmental conditions, and agricultural practices (Harris et al., 1978). Several studies have examined the effect of temperature on fatty acid composition of the grain (Canvin, 1965; Aksouh et al., 2001; Ren et al., 2009). Many studies examining how temperature influenced the resultant seed lipid profile were performed in greenhouses but greenhouses can only approximate growing conditions in the field and cannot give a complete picture of how crops will respond to different temperatures (Canvin, 1965; Aksouh et al., 2001; Ren et al., 2009). Conversely, data from field studies only encompass a relatively narrow temperature range (Nagao and Yamazaki, 1983; Putnam et al., 1991; Gugel and Falk, 2006; Gao et al., 2009). Most studies found that as temperatures rise, the percentage of polyunsaturated lipids, linoleic (C18:2) and linolenic (C18:3) in particular, decreases

while the percentage of oleic acid (C18:1) increases. Yet each of the prior studies is limited in scope, typically including only one crop grown in a handful of locations, resulting in growing conditions across a limited temperature range. The general consensus from these studies is that growing temperature and genotype are the main factors contributing to the large variation within a crop's lipid profile (Lajara et al., 1990).

In the current work, the literature was reviewed to determine if temperature is the single dominant factor influencing lipid composition. In this paper, 25 studies of oil profiles for crops grown in fields and greenhouses were compiled with temperatures ranging between 10 and 40°C to provide a more complete understanding of how lipid profiles are affected by temperature. Temperature during the grain fill, defined as 30 days before harvest, was correlated with the percentage of major lipids contained in soybean, canola, camelina and sunflower. Then the oilseed crops were grown and the lipid profile of their seeds was determined and compared to the literature to demonstrate the validity of the determined correlations.

Oilseed crops were chosen for their ability to grow in the Midwest and their potential for use as a feedstock for production of biodiesel or other biochemicals. Soybean (*Glycine max*) is the most valuable oilseed crop in the United States in terms of production and economic value as it accounts for over 90 percent of U.S. production of biodiesel and is a dominant food product (Gao et al., 2009). Over 50 million metric tons of canola (*Brassica napus*) is produced annually, making it the world's third most important oilseed crop behind palm and soybean (Downey, 1990). Camelina (*Camelina sativa*) is a relatively new oilseed crop that because of its low agricultural inputs and

ability to grow on marginal lands, could play an important role in food and fuel production in the future (Budin et al., 1995). Sunflower (*Helianthus annuus*) is one of the five largest oilseed crops in the world with over 1.5 million acres of sunflower planted in the US in 2011 ("Economic Research Service, USDA. 'Table 20: Sunflowerseed: Acreage planted, harvested, yield, production and value, U.S., 1980-2011'," n.d.). Compared to previous multi-crop studies on seed oil compositions, the current study is distinct in that it includes camelina with the traditional crops (Werteker et al., 2010).

Our objectives were to gather literature data on lipid profiles over a large range of growing temperatures and correlate the temperature during the grain fill to the molar amount of lipid contained in the seeds. The correlations were compared to field studies to demonstrate their validity.

2. Materials and methods

2.1. Collecting lipid profiles from literature

For field studies, literature was included in this review if the study included the location where the crops were grown and harvest date (or sufficient data with which to make a reasonable estimate of the date of harvest). If only the planting date was given, the harvesting date was assumed to be the average of the recommended days to allow the plant to grow in the field. For soybean the assumed harvest date was 100 days after planting while for the short season crops, canola, camelina, and sunflower, the assumed harvest date was 92 days after planting. The mean monthly maximum temperature for each location was found in the National Oceanic and Atmospheric Administration's National Climatic Data Center. If the grain filling days spanned two months, the average mean maximum temperature of that period was calculated, accounting for the days of

grain filling in each month. For greenhouse studies, literature was included in this review if temperature data was given. A list of the literature included in this review can be found in Table 1.

Genotype has been documented to have an effect on the oilseed profile, so an attempt was made to control for genotype in the collected literature. Only literature studies with genotypes that matched the oilseed crops grown as validation studies were used. Since few studies specifically articulated the genotype of the seeds, categorization strategies were employed. Soybean cultivars have been considerably modified due to genetic engineering and can have a wide variety of lipid profiles. Studies with more oleic acid than linoleic acid were neglected because soybean in the field studies had twice as much linoleic acid than oleic. The commercial canola evaluated is significantly different from the wild *Brassica napus* varieties. By definition, canola is a *Brassica napus* hybrid or variety with less than 5% erucic acid, therefore *Brassica napus* oils with more than 5% erucic acid were neglected. Because it has not yet reached use maturity, camelina has experienced limited genetic modifications, thus all data from the literature was included. Commercially available sunflower hybrids have a wide variety of lipid profiles and are classified by percentage of oleic acid. The sunflower hybrid used in this study was classified as a mid-oleic line with oleic acid percentages between 55-75% and linoleic acid percentages between 20-42% (Grompone, 2005). Therefore, only literature studies with these properties were included.

A linear regression was used to correlate the molar amount of each fatty acid as a function of the mean maximum temperature during the grain fill. SAS software ("SAS Version 8. SAS Institute Inc.," 2006) was used to determine the parameter estimates for

137 the linear regression. Residuals for each regression were plotted to determine that errors
138 were normally distributed and that the mean of the errors was zero.

139 **2.2. Agricultural practices**

140 A randomized complete block with four replications was used to plant maturity
141 4.7 ('KS 4702' Kansas St. Univ. Manhattan, KS) soybean, canola '1651h Clearfield'
142 (Cropland Genetics St. Paul, MN), camelina (Cheyenne, Blue Sun Biodiesel Golden,
143 CO), and sunflowers '559 CL, DMR, NS' (Cropland Genetics St. Paul, MN), in
144 Manhattan, KS (98.3°W, 39.14°N) in 2011. The soybean line was chosen because its
145 maturity matched the growing season. Cropland Genetics' canola and sunflower lines
146 were chosen for their herbicide resistance. The sunflower line is resistant to drought,
147 making it suitable for planting in central and western Kansas. The camelina line was
148 chosen because it is one of the few that is tailored for dryland farming and commercially
149 available. These crops were grown simultaneously in the same location to minimize
150 variations in weather patterns and soil types. Canola and camelina were planted 11 Mar.
151 2011 and harvested 9 July 2011 and 29 June 2011 respectively. The soybean and a 95-
152 day relative maturity sunflower were planted as full season crops on 17 May 2011. The
153 sunflower crop was harvested at the end of August while the soybean crop was harvested
154 at the end of September. The non-legume crops received 112 kg*ha^{-1} of (N) as urea
155 ($(NH_2)_2CO$) 15 - 20 days after planting. The brassica crops received 22.4 kg * ha^{-1} of
156 sulfur (S) from gypsum ($CaSO_4(H_2O)_2$) simultaneously with the N broadcast application.
157 Following harvest, all crops were dried to 3% moisture prior to oil extraction.

158 **2.3. Extraction of lipids**

Each extraction started with 100 mg of grain. An overview of the procedure can be found in Figure 1. The extraction and fatty acid synthesis procedure is a modification from previous work by the same lab (Kim et al., 2013). The seeds were heated at 75°C for 15 min in 0.01 wt% BHT in isopropanol to inactivate lipolytic enzymes. The mixture was transferred to a homogenizer to crush the seeds. To separate the triglycerides from the protein solids, 1.0 mL chloroform, 1.0 mL methanol and 0.8 mL of water were added. The mixture was shaken for 30 seconds and centrifuged for 10 minutes at 10,000 rpm to facilitate phase separation. The chloroform layer, containing the triglycerides, was transferred to a separate vial and saved. The extraction was repeated three times, each time adding more chloroform to the aqueous phase, with the triglyceride fraction collected in a common vial. To remove any water that might have been carried over from the extraction, 0.5 mL of 1 M KCl was added as a desiccant to the triglyceride solution and the mixture was shaken and centrifuged. The upper aqueous layer was removed and discarded. To remove any remaining proteins, 1.0 mL of water was added and the mixture was shaken and centrifuged. The aqueous layer was discarded and the triglyceride solution was then dried under nitrogen and redissolved in 1000 µL of chloroform.

For the synthesis of methyl esters, 25 µL of the triglycerides solution and 50 µL of internal standard, pentadecanoic acid in chloroform, were mixed in a screw-cap tube. The chloroform was evaporated and 1 mL of 3 M methanolic hydrochloric acid was added to each tube. The mixture was bubbled with nitrogen to remove oxygen. The tubes were heated at 78°C for 30 minutes to synthesize the methyl esters. To isolate the methyl esters from water soluble compounds, 2 mL of water and 2 mL of hexane:chloroform

(4:1, v/v) were added to the tubes and then shaken for 30 seconds and centrifuged for 2 minutes. The upper layer, containing methyl esters in hexane:chloroform, was pipetted to a separate vial. This separation was repeated three times, each time adding more hexane:chloroform to the remaining aqueous layer, with the methyl ester fraction collected in a common vial. The organic layer was dried under nitrogen. The sample was then dissolved in 100 µL of hexane and transferred to gas chromatograph (GC) vials.

2.4. Analysis of lipids

The GC-FID (Flame Ionization Detector) analysis was performed at the Kansas Lipidomics Research Center with a 6890N GC (Agilent Technologies, Santa Clara, CA) coupled to an FID. The GC was fitted with a HP-88 capillary column with a bis (cyanopropyl) polysiloxane stationary phase (column length: 100 m, internal diameter: 250 µm, film thickness: 0.25 µm). Helium was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. The injector port was maintained at 275°C. An Agilent 7683 autosampler was used to inject 1 µL of the sample in the split mode with a split ratio of 10:1. The GC temperature ramp was operated as follows, initial temperature of 70 °C, ramp 1 at 15 °C min^{-1} to 175°C, ramp 2 at 1 °C min^{-1} to a final temperature of 235°C. The FID was operated at 260°C. The hydrogen flow to the detector was 30 mL min^{-1} and air flow was 400 mL min^{-1}. The sampling rate of the FID was 20 Hz. The data were processed using Chemstation software.

3. Results and discussion

3.1. Results from literature review

The molar percentage of oleic, linoleic and linolenic acids were plotted versus the temperature during the grain fill for each of the four crops are presented in Figures 2, 3,

4, and 5, respectively. The dotted line represents the best linear fit (minimized residuals), while the solid lines represent the 95% confidence intervals based on the estimation of the standard deviation. The slope and y-intercepts for each of the linear regressions and their respective standard deviations were determined using SAS. SAS was also used to confirm that the residuals were approximately normal and the use of a linear regression was appropriate for the literature values collected.

In soybean, canola, and sunflower, as the temperature increased, the percentage of oleic acid increases while the percentage of linoleic and linolenic acids decreased. These results agree with other studies that were conducted in greenhouses over broad temperature ranges with canola and sunflower (12 to 27 °C) (Tremolieres et al., 1982) and with studies performed in fields with soybean, canola, and sunflower over smaller temperature ranges (12 to 17 °C) (Werteker et al., 2010). Camelina was unique in that its lipid profile was nearly independent of growing temperature over the range investigated. Other authors have found that the effect of temperature on fatty acid composition was small for nine varieties of camelina, although they noted that during a particularly warm year the different varieties produced 2% less linolenic acid than the same varieties during a normal year (Crowley and Frohlich, 1998). Soybean and sunflower exhibited the strongest trends towards more monounsaturated and less polyunsaturated fatty acids with increasing temperature while canola and camelina changed minimally with increasing temperature. Camelina has not been extensively studied and had fewer data points over a smaller temperature range than the other crops. The data collected had temperatures during the grain fill between 19°C and 28°C. This is a relatively small range compared with the other three crops studied which had data from approximately 10°C to 40°C.

More work needs to be completed, with camelina grown in both cooler and warmer temperatures to gain a more complete understanding of the effect of temperature during the grain fill on the molar amounts of lipids present.

3.2. Field studies

For oilseeds grown near Manhattan, KS, the oil profiles varied considerably between crops (Table 2). The oil profiles varied considerably between crops. Oleic acid was the primary fatty acid in canola and sunflower seeds. The soybean varieties had far more linoleic acid than oleic or linolenic acid. Camelina was highly unsaturated, having the most linolenic acid of any of the oil seeds grown. Camelina was the only crop with significant amounts of fatty acids with 20 carbons.

To compare percentage of fatty acid predicted by the regression to the experimentally determined percentage of fatty acid contained in the seed, the temperature during the grain fill must be known. The temperature during the grain fill for the Manhattan, KS crops were calculated based on the growing season temperatures (Table 3). The experimentally determined molar percentages of the crops were compared to the predicted value of the molar percentage of lipids from the regressions (Figure 6). Points closest to the diagonal line represent an agreement between the lipid profile determined from the plants grown in Manhattan, KS and the value predicted by the correlation from the literature values. For soybean, canola and camelina, the developed correlations accurately predicted the molar percentage of lipids within the confidence intervals. The sunflower regression under predicted the amount of oleic acid and over predicted the amount of linoleic acid. Sunflower was the only plant that did not contain linolenic acid. The planted sunflower hybrid was classified as mid-oleic or having between 55 and 75%

oleic acid, but the grain from this trial, grown under high temperatures, had 77% oleic acid. This was perhaps due to high temperatures increasing the oleic seed content above typical levels. The discrepancy between the experimentally determined amount of lipids and the values predicted by the regressions might have resulted because the literature review purposefully excluded studies with oleic acid outside the mid-oleic range in an attempt to control for genetic differences. Differences could also be explained by other factors which are known to affect the fatty acid profile such as precipitation or genotype (Rao et al., 1998; Gao et al., 2009). Some literature suggests that agricultural practices can also affect oil profiles (Vera et al., 2007).

Enzymes that promote the formation of lipids are similar in all higher plants but temperature affects lipid profiles to different degrees. Previous research documents that the lipid profiles of all four studied crops are affected by temperature and the amount of oleic acid increases while the amount of linoleic and linolenic acids decrease with increasing temperature (Tremolieres et al., 1982; Wolf et al., 1982; Lajara et al., 1990; Zubr and Mattha, 2002). There are two accepted explanations for how temperature causes changes in the lipid profile. The earliest literature suggests that oilseeds produce more linoleic acid at lower temperatures because oxygen is a necessary reactant for desaturase enzyme activity and oxygen is more soluble in water at lower temperatures (Harris and James, 1969). Later literature confirmed that the activity of oleoyl-phosphatidylcholine desaturase, an important enzyme in the desaturation of oleic acid into linoleic acid, is highly dependent on the amount of available oxygen in sunflowers (Rolletschek et al., 2007). It has also been suggested that higher temperatures directly affect the lipid profile by destabilizing the enzyme (Martinez-Rivas et al., 2003).

4. Conclusions

The lipid profile of a crop determines its ability to be used in industrial and nutritional applications. The linear regressions from previously published results suggest that the molar percentage of oleic, linoleic and linolenic acids contained in soybean, canola, and sunflower depend on the temperature during grain fill. The molar amounts of oleic, linoleic and linolenic acids in the soybean, canola, and camelina crops grown in Manhattan, KS were within the 95% confidence interval of each of their respective regressions. Higher temperatures will result in lower amounts of polyunsaturated lipids and higher amounts of monounsaturated lipids in soybean, canola, and sunflower. As average temperatures across the planet rise, oilseed crops are going to produce more monounsaturated fats and less polyunsaturated fats.

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Figure 1: The procedure for extraction and separation of triglycerides from seed proteins, synthesis of methyl esters, and subsequent separation from water-soluble organics from oilseed crops grown for analysis by GC.

Figure 2: Linear regressions of the molar amount of the three most common lipids in soybean gathered the literature plotted versus the mean high temperature during the grain fill. See Table 1 for references. a) oleic acid b) linoleic acid c) linolenic acid

Figure 3: Linear regressions of the molar amount of the three most common lipids in canola gathered the literature plotted versus the mean high temperature during the grain fill. See Table 1 for references. a) oleic acid b) linoleic acid c) linolenic acid

Figure 4: Linear regressions of the molar amount of the three most common lipids in camelina gathered the literature plotted versus the mean high temperature during the grain fill. See Table 1 for references. a) oleic acid b) linoleic acid c) linolenic acid

Figure 5: Linear regressions of the molar amount of the two most common lipids in sunflower gathered the literature plotted versus the mean high temperature during the grain fill. See Table 1 for references. a) oleic acid b) linoleic acid

Figure 6: Comparison of predicted values from linear regression of literature values versus data from crops grown in Manhattan, KS. The error bars in the x and y-direction are the 95% confidence intervals for the 20 samples collected for each lipid for each crop

435 grown in Manhattan, KS and for the values collected from the literature, respectively. a)

436 oleic acid b) linoleic acid c) linolenic acid.

437

438 Table 1: Published data included in this study.

Citation (Author, Year)	Location	Plants Grown
(Aksouh et al., 2001)	Greenhouse	Canola
(Aksouh-Harradj et al., 2006)	Greenhouse	Canola
(Angelini et al., 1997)	Central Italy	Camelina
(Bhardwaj and Hamama, 2008)	Virginia	Canola
(Budin et al., 1995)	Minnesota	Camelina
(Canvin, 1965)	Greenhouse	Sunflower
(Gao et al., 2009)	Michigan	Soybean
(Gugel and Falk, 2006)	Saskatoon and Scott, Saskatchewan and Beaverlodge, Alberta	Camelina
(Iqbal et al., 2011)	Greenhouse	Canola
(Larson et al., 2002)	Gnhouse	Canola
(Lu and Kang, 2008)	Greenhouse	Camelina
(Maestri et al., 1998)	Cordoba, Argentina	Soybean
(Martınez-Force et al., 1998)	Greenhouse	Sunflower
(Nagao and Yamazaki, 1983)	Okayama, Japan	Sunflower
(Putnam et al., 1991)	Rosemount, MN	Soybean, Canola, Camelina
(Rao et al., 1998)	Fort Valley, GA	Soybean
(Ren et al., 2009)	Greenhouse	Soybean
(Rennie and Tanner, 1989)	Greenhouse	Soybean
(Robertson et al., 1971)	Tifton, GA, Baton Rouge, LA, College Station, TX	Sunflower
(Shafiullah et al., 1994)	Islamabad, Pakistan	Sunflower
(Tremolieres et al., 1982)	Greenhouse	Canola and Sunflower
(Unger and Thompson, 1982)	Bushland, TX	Sunflower
(Vantoai et al., 2012)	Columbia and Portageville, MO	Soybean
(Wolf et al., 1982)	Greenhouse	Soybean
(Zubr and Mattha, 2002)	Mullhein, Paderborn, Carlow, Germany and Uppsala, Sweden	Camelina

439 Table 2: Lipids contained in four oilseed crops grown in Manhattan, KS in 2011 listed as average of twenty samples.

Crop	Lipid (mol of lipid/total mol of identified lipids)								Sum	Harvest Date
	C16:0 Palmitic	C18:0 Stearic	C18:1 Oleic	C18:2 Linoleic	C18:3 Linolenic	C20:1 Eicosenoic	C20:2 Eicosadienoic	C20:3 Eicosatrienoic		
Soybean	11 ± 0.5	4 ± 0.5	22 ± 1.1	55 ± 1.8	8 ± 0.5	<1	ND	ND	100	Sept 20
Canola	6 ± 0.7	2 ± 0.2	62 ± 1.0	22 ± 0.5	6 ± 0.2	1 ± 0.1	<1	ND	100	July 9
Camelina	7 ± 0.9	2 ± 0.4	17 ± 1.9	21 ± 2.4	31 ± 3.4	13 ± 0.6	2 ± 0.7	1 ± 0.1	94†	June 29
Sunflower	6 ± 0.5	3 ± 0.3	77 ± 4.8	14 ± 4.8	ND	<1	ND	ND	100	Aug 25

440 ND: Not detected

441 † The molar percentages for camelina do not sum to 100 because camelina also produced C18:3n6 and C20:0 fatty acids (~3%) and

442 detectable amounts of C22:0, C20:3n6, C22:2, C24:0 and C24:1 fatty acids.

443

444 Table 3: Monthly temperature for Manhattan, Kansas

	Monthly average high temperature (°C)	
Month	2011	30 year average
March	12.3	14.5
April	19.6	20.3
May	23.6	25.1
June	31.2	30.6
July	36.7	33.3
August	33.6	31.6
September	26.8	26.4

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