THE EFFECTS OF CRANBERRY SEED OIL, FLAXSEED MEAL, AND SOYBEAN OIL ON PLASMA EICOSAPENTAENOIC ACID, DOCOSAHEXAENOIC ACID, AND ALPHA-LINOLENIC ACID CONCENTRATIONS IN HORSES

By

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ABSTRACT

A completely randomized design experiment was conducted to examine the effects of cranberry seed oil, flaxseed meal, and soybean oil on plasma eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), alpha-linolenic acid (ALA), and sum fatty acid concentrations in horses. Nine geldings and three mares (13 to 28-yr-old) were equally divided into three groups of four and randomly assigned to one of three treatments: cranberry seed oil (CO), flaxseed meal (FM), and a control diet containing soybean oil (SO).

The 35-d trial consisted of a 7d diet adjustment period, followed by a 28-d feeding period. Beginning on d -7 and every 7d thereafter, venous blood was collected to determine plasma EPA, DHA, ALA, and sum fatty acid concentrations.

There was no main effect of treatment (P = 0.175), time (P = 0.465), or effect of the interaction of time x treatment (P = 0.191) observed on adjusted mean plasma EPA concentrations. There was a main effect of time (P = 0.005) on adjusted overall mean plasma DHA concentrations. Adjusted overall mean plasma DHA concentrations were higher at d 7 (P = 0.008), 14 (P = 0.001), and 21 (P = 0.005) as compared to d -7. Adjusted overall mean plasma DHA concentrations were lower at d 28 (P = 0.017) as compared to d 14. There was no main effect of treatment (P = 0.557), or effect of the interaction of time x treatment (P = 0.304) observed on adjusted mean plasma DHA concentrations.
concentrations. There was no main effect of treatment ($P = 0.390$), time ($P = 0.443$), or effect of the interaction of time x treatment ($P = 0.143$) observed on adjusted mean plasma ALA concentrations. There was a main effect of time ($P = 0.021$) observed on adjusted overall mean plasma sum fatty acid concentrations. There was a significant increase in overall adjusted mean plasma sum fatty acid concentrations at d 14 and 21 as compared to d -7, and a significant decrease at d 28 as compared to d 14. There was no main effect of treatment ($P = 0.314$), or effect of the interaction of time x treatment ($P = 0.100$) observed on adjusted mean sum fatty acid concentrations.

Results from this experiment indicate that aged horses fed plant-based n-3 fatty acid supplementation may have increased plasma n-3 fatty acid concentrations over time. Transient increases in n-3 fatty acid concentrations observed in this trial suggest the importance of proper handling and storage of fatty acid supplements to maintain n-3 fatty acid integrity.

Key words: n-3 fatty acid, cranberry seed oil, flaxseed meal, soybean oil, plasma, horse
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>Chapter</td>
<td></td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td>Fatty Acid Structure</td>
<td>3</td>
</tr>
<tr>
<td>Fatty Acid Function</td>
<td>4</td>
</tr>
<tr>
<td>Omega-3 and Omega-6 Polyunsaturated Fatty Acids</td>
<td>4</td>
</tr>
<tr>
<td>Fatty Acids in Forage</td>
<td>6</td>
</tr>
<tr>
<td>Fatty Acids in Cranberry and Benefits</td>
<td>6</td>
</tr>
<tr>
<td>Fatty Acid Oxidation</td>
<td>7</td>
</tr>
<tr>
<td>Fatty Acid Storage and Temperature</td>
<td>8</td>
</tr>
<tr>
<td>Omega-3 Fatty Acids in Humans</td>
<td>9</td>
</tr>
<tr>
<td>Omega-3 Fatty Acids in Dogs</td>
<td>10</td>
</tr>
<tr>
<td>Omega-3 Fatty Acids in Poultry</td>
<td>10</td>
</tr>
</tbody>
</table>
Omega-3 Fatty Acids in Swine 11
Omega-3 Fatty Acids in Cattle 11
Omega-3 Fatty Acids in Horses 12
Osteoarthritis in Horses 19
Omega-3 Fatty Acids in Arthritic Horses 20
Statement of the Problem 21

III. MATERIALS AND METHODS 23
Horses 23
Diets 24
Sample Collections 25
Laboratory Analysis 26
Gas Chromatograph 26
Plasma 27
Treatments and Hay 27
Quality Assurance of Chromatographs 28
Statistical Analysis 29

IV. RESULTS AND DISCUSSION 30
Adjusted mean plasma EPA concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil 30
Adjusted mean plasma DHA concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil 33
Adjusted mean plasma ALA concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil

Adjusted mean sum fatty acid concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil

Possible explanation for differences between studies

V. CONCLUSIONS AND IMPLICATIONS

LITERATURE CITED

APPENDIX FIGURES A, OVERALL ADJUSTED PLASMA FATTY ACID CONCENTRATIONS

APPENDIX FIGURES B, STANDARD CURVES

APPENDIX TABLES A, OVERALL SUMS BY TIME
LIST OF TABLES

1. Feed n-3 fatty acid concentrations 25

A-1 Least squares means (ng/µL) of plasma fatty acid concentrations in horses consuming all treatments (cranberry seed oil, flaxseed meal, and soybean oil) at d -7, 7, 14, 21, and 28 59

A-2 Adjusted mean plasma fatty acid concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil at d -7, 7, 14, 21, and 28 60
LIST OF FIGURES

1. Gas chromatography analysis of fatty acids in Standard 28

2. The interaction of time and treatment on adjusted mean plasma EPA concentrations 31

3. The effect of time on overall adjusted mean plasma EPA concentrations in horses 31

4. The interaction of time and treatment on adjusted mean plasma DHA concentrations 34

5. The effect of time on overall adjusted mean plasma DHA concentrations in horses 34

6. The interaction of time and treatment on adjusted mean plasma ALA concentrations 36

7. The effect of time on overall adjusted mean plasma ALA concentrations in horses 36

8. The interaction of time and treatment on adjusted mean plasma sum fatty concentrations 38
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.</td>
<td>The effect of time on overall adjusted mean plasma sum fatty acid concentrations in horses</td>
</tr>
<tr>
<td>A-1</td>
<td>Overall EPA concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil at d -7, 7, 14, 21, and 28</td>
</tr>
<tr>
<td>A-2</td>
<td>Overall DHA concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil at d -7, 7, 14, 21, and 28</td>
</tr>
<tr>
<td>A-3</td>
<td>Overall ALA concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil at d -7, 7, 14, 21, and 28</td>
</tr>
<tr>
<td>A-4</td>
<td>Overall sum fatty acid concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil at d -7, 7, 14, 21, and 28</td>
</tr>
<tr>
<td>B-1</td>
<td>Standard GC curve for EPA</td>
</tr>
<tr>
<td>B-2</td>
<td>Standard GC curve for DHA</td>
</tr>
<tr>
<td>B-3</td>
<td>Standard GC curve for ALA</td>
</tr>
</tbody>
</table>
Chapter 1

INTRODUCTION

Omega-3 series fatty acids have general anti-inflammatory, anti-thrombotic, anti-hypertensive, and anti-arrhythmic effects in the body (Simopoulos, 1999). Omega-3 supplementation may be beneficial to arthritic horses (Manhart et al., 2009). Studies have also shown marine-based n-3 supplementation to be beneficial in reducing inflammation and aiding in arthritic treatment. Marine-based n-3 supplements contain primarily n-3 fatty acids in the form of EPA and DHA. King et al. (2008) and Hess et al. (2012) reported an increase in plasma EPA and DHA concentrations when horses were fed fish oil. O’Connor et al. (2007) observed an increase in serum EPA, DHA, and total n-3 FA concentrations in horses fed fish oil. Vineyard et al. (2007) reported increased RBC and plasma concentrations of EPA and DHA in horses fed fish oil. Vineyard et al. (2010) observed fish oil supplementation increased RBC and plasma EPA, DHA, and total n-3 FA concentrations. Hall et al. (2004a) reported plasma concentrations of EPA and DHA increased in horses fed fish oil. When feeding stabilized EPA and DHA to horses, Woodward et al. (2005) observed an increase in DHA and total n-3 FA concentrations, and EPA concentrations tended to increase. Manhart et al. (2009) reported arthritic horses fed EPA and DHA supplementation had increased plasma DHA concentrations.
Previous studies with plant-based n-3 FA supplementation (e.g., flaxseed), which consisted primarily of n-3 FA source of ALA, have reported conflicting results. Hansen et al. (2002) reported an increase in plasma EPA and total n-3 FA concentrations in horses fed flaxseed. Vineyard et al. (2010) and Hess et al. (2012) reported no increase in plasma EPA, DHA, or total n-3 FA concentrations in horses fed flaxseed. Data from previous studies indicated important variability in availability and efficacy of FA supplements due to source. Also, studies feeding cranberry seed oil as a n-3 FA source have only been reported in rats and humans. The objective of the current study was to compare the effects of three different plant-based FA supplements on plasma EPA, DHA, ALA, and total n-3 FA concentrations in aged horses.
Chapter II
LITERATURE REVIEW

_Fatty Acid Structure_

Fatty acids consist of carbon, hydrogen, and oxygen molecules. Fatty acids can range from 2 to 24 carbons in length. A methyl group (-CH₃) is at one end of the chain, and at the other end is a carboxyl group (-COOH). Fatty acids are classified by the number of carbons and double bonds between carbons. Saturated fatty acids have no double bonds between carbons. A fatty acid with a chain with one double bond is classified as a MUFA. Polyunsaturated fatty acids contain two or more double bonds (Spallholz et al., 1999).

Numbering of fatty acids begins with the number of carbons followed by a colon and the number of double bonds. In determining the location of the first double bond, the number of carbons is counted away from the methyl group to the first carbon with a double bond. For example, alpha linolenic acid (ALA) is identified as 18:3 (n-3), with 18 as the number of carbons, 3 as the number of double bonds, and n-3 represents that ALA has the first double bond on the omega-3 carbon (Spallholz et al., 1999).
**Fatty Acid Function**

Fatty acids are critical nutritional components in the diet. There are essential and nonessential fatty acids. Essential fatty acids are not synthesized in the body, because of a lack of enzymes to produce double bonds before the ninth carbon from the methyl group (Hall et al., 2004a). Therefore, adequate amounts of essential fatty acids must be ingested for the body to function properly.

Fatty acids are the basic building blocks for fat in the body, and are required for brain development and maintenance of proper brain function (Lawrence, 2010). Fatty acids are precursors and antagonists of eicosanoid biosynthesis (Schoene, 1991). Eicosanoids function as chemical messengers that carry information of cell activation from one cell to another (Curtis-Prior, 2004). The four families of eicosanoids are; prostaglandins, leukotrienes, thromboxanes, and prostacyclins, which all have specific physiological and pathophysiological roles, and coordinate events between cells so that proper tissue function occurs (Curtis-Prior, 2004). Different eicosanoids are produced from various fatty acids.

**Omega-3 and Omega-6 Polyunsaturated Fatty Acids**

Omega-3 fatty acids, by definition, have a double bond on the third carbon from the methyl group. The essential fatty acid for the omega-3 family is alpha-linolenic acid (ALA), which is a precursor for the omega-3 series of fatty acids (Hall et al., 2004a). Alpha-linolenic acid goes through a series of desaturation and elongation processes to produce long-chain derivatives (Dubois et al., 2007). The most important derivatives produced are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Alpha-
linolenic acid is desaturated and elongated into EPA (20:5), and can be further elongated into DHA (22:6). Eicosapentaenoic acid and DHA are then incorporated into cell membrane phospholipids, and are stored until a chemical or physical insult causes the activation of these fatty acids to mobilize from the cell membrane phospholipids, and metabolize into eicosanoids (Hall et al., 2004a). Alpha-linolenic acid is primarily found in flaxseed, cranberry seed, and walnuts (Dubois et al., 2007). A common source of EPA and DHA is fish oil (Ackman et al., 1989). Omega-3 series fatty acids have general anti-inflammatory, anti-thrombotic, anti-hypertensive, and anti-arrhythmic effects in the body (Simopoulos, 1999).

Omega-6 fatty acids have the first double bond located on the sixth carbon from the methyl group. Vertebrates have a dietary requirement for linoleic acid in the omega-6 series. Linoleic acid is the precursor that is converted into arachidonic acid (20:4) through processes of desaturation and elongation (Dubois et al., 2007). Arachidonic acid is also stored in cell membrane phospholipids, until it is metabolized into eicosanoids (Hall et al., 2004a). Primary sources of linoleic acid are vegetable oils, such as corn, sunflower, and soybean (Carlier et al., 1991). Omega-6 fatty acids have pro-inflammatory, pro-thrombotic, and pro-aggregatory effects on the body (Simopoulos, 1999).

The omega-3 and omega-6 derivates compete with one another at the cyclooxygenase and lipoxygenase cellular metabolic pathways for prostaglandin and leukotriene synthesis (Simopoulos, 2002). Arachidonic acid metabolizes cyclooxygenase to produce 2-series eicosanoids (e.g., PGE$_2$) and lipoxygenase yields 4-series eicosanoids when further metabolized from arachidonic acid (Hall et al., 2004a). Series 2 and 4
Eicosanoids have pro-inflammatory effects. Eicosapentaenoic acid and DHA produce 3 and 5-series eicosanoids, which are less inflammatory (Hall et al., 2004a). While both are important in the body’s reaction to chemical or physical insult, the correct balance of fatty acids to be maintained in the body is important. Although an ideal dietary fatty acid ratio for horses has not been determined, in humans the ideal dietary ratio for omega-6 to omega-3 fatty acids is 1:1, though typical western human diets have a ratio of 15:1 (Simopoulos and Cleland, 2003). Cattle fed a typical concentrate in feed yards have a dietary omega-6 to omega-3 ratio close to 20:1 (Sretenovic et al., 2009). Excess of one of the series fatty acids causes a significant decrease in the conversion yield of the other (Brody, 1999).

**Fatty Acids in Forage**

Dewhurst et al. (2001) and Clapham et al. (2005) reported that ALA was the dominant fatty acid in all species of fresh forages, averaging 62% of the total fatty acids. Additionally, Dewhurst and King (1998) stated that green plants were the primary source of beneficial n-3 fatty acids, and forage processing decreases overall fatty acid content. After extended periods of forage wilting, the concentrations of polyunsaturated fatty acids were significantly lower than in corresponding fresh forage. However, after processing, the percentage ALA in silage was still higher than linoleic acid (Dewhurst and King, 1998).

**Fatty Acids in Cranberry and Benefits**

Berry seed oils have a favorable n-6: n-3 fatty acid compared to vegetable oils (Van Hoed et al., 2009). Van Hoed et al. (2009) reported that cranberry seed oil had a n-
6: n-3 fatty acid of 1.25. The cranberry seed oil was 37.68% linoleic acid and 30.09% ALA. Parry et al. (2006) reported that cranberry seed flour contained 30.9 g of ALA per 100 g fat, and the n-6: n-3 was 1.2:1. Yang et al. (2011) reported that cranberry seed oil consisted of 70% ALA and LA, with an approximate n-6: n-3 of 1:1. Helbig et al. (2008) reported the lowest n-6: n-3 of 0.72:1 in cranberry seed oil. Cranberry seed oil contained 31% linoleic acid and 43% ALA (Helbig et al., 2008).

Health benefits have been reported from feeding cranberry seed products. Parry et al. (2006) reported that cranberry seed flour significantly inhibited HT-29 colon cancer cell proliferation in humans. Nayak et al. (2011) observed that rats fed cranberry seed flour had increased rate of wound healing. Animals treated with cranberry oil exhibited an 88.1% reduction in the wound area compared to control of 78.4% reduction on d 13. Hydroxyproline content of the granulation tissue was significantly higher in animals treated with cranberry as well (Nayak et al., 2011).

Fatty Acid Oxidation

Fatty acids undergo enzymatic lipid oxidation, in which enzymes catalyze and promote oxygenation. Hydroperoxides are produced that can be degraded further by enzymes. The resulting products are often aroma-active aldehydes, for example, (2E, 6Z) - nonadienal is a degradation product of ALA. Lipid auto-oxidation also occurs when fatty acids react with oxygen. Lipid auto-oxidation can occur at room temperature, but is accelerated at higher temperature. The aldehydes produced depend on the precursor fatty acid. Polyunsaturated fatty acids are the most susceptible to fat oxidation, because the double bonds make the fatty acids less stable. Alpha-linolenic acid is the most susceptible
to oxidation because the carbon chain consists of three double bonds. Omega-6 fatty acids, with two double bonds, the second most susceptible to oxidation, followed by oleic acid, a MUFA, and stearic acid, a SFA (Cerny, 2010).

**Fatty Acid Storage and Temperature**

Fatty acids in oils are very volatile due to their higher level of unsaturation. Rufian-Henares et al. (2005) conducted a study to assess lipid oxidation by measuring the progression of FA profiles. Polymeric liquid enteral formulas, which consisted of all proteins, dextrinomaltose, vegetable oils, vitamins, and minerals were stored for short periods at high temperatures (1 to 4 wk at 32 to 55°C), or for long periods at low temperatures (12 to 36 wk at 4°C, 20°C, or 30°C). Unsaturated fatty acids decreased significantly in all long period treatments when stored at 4°C. As time and temperature increased, loss of essential fatty acids increased. The n-6: n-3 was increased by storage throughout all temperatures. The authors stated that enteral formula fat was oxidized during storage, leading to a loss of nutritional value, and should be stored under refrigeration to minimize losses. (Rufian-Henares et al., 2005)

Verardo et al. (2009) reported that oxidation of n-3 PUFA increased in fortified spaghetti under light exposure over 12 mo period, and as well from temperature of 55°C in 27 d. Encapsulation of flaxseed was reported to have an increased efficiency of 84% of a protective effect against the production of primary and secondary oxidative products versus non-encapsulated oil during 25 d of room temperature storage (Liu et al., 2010).
**Omega-3 Fatty Acids in Humans**

Extensive research has been performed on the benefits of n-3 fatty acids in humans. Studies have reported that populations eating more fish, which have a higher concentration of n-3 fatty acids, have lower incidence of cardiovascular diseases. Commonly, western civilization diets consist of n-6: n-3 ratios of 15: 1 (Stretenovic et al., 2009). Imbalance of n-6 to n-3 fatty acids leads to an overproduction of the pro-inflammatory prostaglandins and cytokines. Experimental studies have provided evidence that incorporation of omega-3 fatty acids into the diet modifies inflammatory and immune reactions (Simopoulos, 2002). Omega-6 to omega-3 ratios of 2:1 to 4:1 were related to reduced mortality caused by cardiovascular diseases, reduced inflammatory process in rheumatoid arthritis, and reduced risk of breast cancer (Stretenovic et al., 2009).

Simopoulos (2002) reported that ALA, EPA, and DHA are involved in immune function. Fifteen g/d of supplemental ALA will suppress human IL-1 and tumor necrosis factor-alpha, which are cytokines that cause pro-inflammatory effects and apoptosis. Supplemental n-3 fatty acids also helped to suppress production of Cyclooxygenase-2, which is over expressed in colon cancer cells (Simopoulos, 2002).

Ren and Chung (2007) observed that ALA had a strong inhibitory effect on the production of nitric oxide, Cyclooxygenase-2, and tumor necrosis factor-alpha, by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells and mitogen-activated protein kinase activations in lipopolysaccharide-stimulated cells. The complex nuclear factor kappa-light-chain-enhancer of activated B cells has a role in regulating the
immune response to infection, and incorrect function has been linked to cancer, inflammatory, autoimmune, and immune diseases. Mitogen-activated protein kinases have a pro-inflammatory effect. There is a possibility that this is the basis for the anti-inflammatory effect of ALA (Ren and Chung, 2007).

**Omega-3 Fatty Acids in Dogs**

Fritsch et al. (2010) observed that dogs with osteoarthritis benefited from n-3 supplementation. In this study, 109 dogs with radiographically-confirmed osteoarthritis of the hip or stifle were supplemented n-3 fatty acids for 12 wk. During the study, dogs received Caprofen™, a non-steroidal anti-inflammatory drug, to control pain level. Dogs receiving n-3 supplementation required significantly smaller dosages of Caprofen™ and dosages were reduced more quickly as compared to control for pain management (Fritsch et al., 2010).

**Omega-3 Fatty Acids in Poultry**

Baird et al. (2008) reported that laying hens fed flax oil had thicker cortical bones as compared to non-supplemented hens. Hens fed flax oil also had an increase in bone mineral content and ash weight (Baird et al., 2008).

Jia et al. (2008) observed that hens fed flaxseed meal produced eggs that had increased concentrations of n-3 fatty acids. When compared with control hens fed canola seed, hens fed flaxseed had increased content of total n-3 fatty acids in the eggs. The omega-6 to omega-3 fatty acid ratio also was significantly lower in eggs produced by hens fed flaxseed (Jia et al., 2008).
Omega-3 Fatty Acids in Swine

The effects of omega-3 fatty acids on fatty acid concentrations in meat have been studied in swine. Musella et al. (2009) reported that finishing pigs fed linseed, which is high in n-3 fatty acids, had increased total n-3 fatty acids and decreased total n-6 fatty acids in the ham. Supplemental linseed reduced the n-6: n-3 in the trimmed fat of both green and dry-cured ham. The authors reported an overall increase in ALA, EPA, and DHA in the trimmed fat, semimembranosus muscle, subcutaneous adipose tissue, and the whole slice of ham (Musella et al., 2009). Further, Romans et al. (1995) observed an increase in ALA, arachidonic acid, EPA, and DHA in hams from finishing pigs fed supplemental flaxseed. In addition, flaxseed-fed pigs had increased ALA and EPA, and decreased arachidonic acid in the longissimus thoracis and liver, while DHA increased in the longissimus thoracis (Romans et al., 1995).

Smits et al. (2011) studied the effects of n-3 supplementation on reproductive parameters in sows. The authors reported sows fed 3 g of supplemental fish oil had significantly greater litter size (one piglet per litter) as compared to non-supplemented sows (Smits et al., 2011).

Omega-3 Fatty Acids in Cattle

Farran et al. (2008) observed an increase in plasma total n-3 fatty acids when steers were fed a basal diet of ground flaxseed. Control cattle, fed a basal diet of rolled soybeans, had increased total plasma n-6 concentrations as compared to flaxseed-fed cattle. The authors also reported that flaxseed-fed steers injected with Escherichia coli
O55:B5 had lower rectal temperatures after lipopolysaccharide-challenge as compared to steers fed tallow with a 7.7:1 ratio of n-6: n-3 fatty acids (Farran et al., 2008).

Wistuba et al. (2006) reported that cattle fed supplemental fishmeal had decreased feed intake, but similar ADG as compared to non-supplemented cattle. However, when carcasses were evaluated, fishmeal-supplemented cattle had a lower HCW as compared to control cattle. Additionally, cattle fed supplemental fish oil had significantly increased plasma n-3 FA concentrations as compared to control (Wistuba et al., 2006).

White et al. (2012) observed that non-lactating mature Angus cows fed supplemental fishmeal had significantly higher plasma EPA and DHA concentrations as compared to cows fed corn-gluten meal. Fishmeal supplemented cows had significantly increased n-3 fatty acid concentration and decreased arachidonic acid concentration in the corpus luteum. The authors stated that fertility in cattle may increase due to reduction of PGF$_2$-$\alpha$ intra-luteal synthesis after breeding (White et al., 2012). Likewise, Wamsley et al. (2005) reported that heifers with low luteal-phase progesterone fed supplemental fishmeal appeared to have decreased uterine PGF$_2$-$\alpha$ synthesis as compared to heifers fed supplemental corn-gluten meal. The authors reported no difference in uterine PGE$_2$-$\alpha$ synthesis between control and fishmeal fed heifers with normal concentrations of luteal-phase progesterone (Wamsley et al., 2005).

**Omega-3 Fatty Acids in Horses**

In a study conducted by Hess et al. (2012), 21 maiden mares were used to determine the effects of different sources of supplemental dietary n-3 fatty acids on plasma, red blood cell, and skeletal muscle fatty acid profiles. Mares were assigned to
one of three treatments, control, fish oil, or flaxseed meal for 90 d. Mares fed fish oil received a combination of 38 g/d of ALA, EPA, and DHA. The flaxseed meal treatment contained 38 g/d of ALA. Blood and muscle samples were taken on d 0, 30, 60, and 90. Plasma linoleic acid and ALA concentrations were significantly lower in mares fed fish oil as compared to mares fed flaxseed meal or control. Eicosapentaenoic acid and DHA were only detected in the plasma and red blood cells of mares fed fish oil, and EPA and DHA significantly increased in mares fed fish oil at d 90 as compared to d 30. Plasma ALA was significantly greater in the flaxseed meal group as compared to control at d 30, and also at d 90. The authors reported greater EPA and DHA in skeletal muscle of mares fed fish oil as compared to those fed flaxseed meal and control. The authors stated that n-3 fatty acid concentrations in the blood and muscle are directly correlated with dietary supplementation (Hess et al., 2012).

O’Connor et al. (2007) stated that serum n-3 and n-6 fatty acids were affected by marine-derived n-3 supplementation. Thirteen horses of Thoroughbred or Standardbred breeding were placed on one of two treatments, fish oil or corn oil. Each horse received 324 mg/kg BW of fish oil or corn oil for 63d. Horses fed fish oil had increased serum concentrations of EPA and DHA as compared to those fed corn oil. Horses fed fish oil had increased serum total n-3 fatty acids and decreased n-6 fatty acids as compared to those consuming corn oil, resulting in a lower serum n-6: n-3. Horses consuming corn oil had increased serum cholesterol and triglycerides concentrations as compared to those consuming fish oil (O’Connor et al., 2007).
In a study conducted by King et al. (2008), 16 mares were used to determine circulating fatty acid profiles, and acquisition and washout of fatty acids in response to marine-derived n-3 supplementation. Mares were fed one of four treatments; 0, 10, 20, or 40 g/d of n-3 supplementation for 28 d. Plasma was collected at -11, 3, 7, 10, 16, 23, 30, 37, 44, 70, and 87 d. Plasma EPA and DHA increased in a dose-responsive manner by d 3 of feeding and reached peak concentrations by d 7. Mares consuming 40 g/d of n-3 supplement had a 13-fold increase in plasma EPA and 10-fold increase in plasma DHA as compared to those coming 0 g/d. The authors reported that plasma EPA and DHA concentrations declined significantly after cessation of supplementation and were near pre-supplementation values by 42 d after the end of the trial. The authors stated that plasma n-3 concentrations may be influenced through targeted supplementation (King et al., 2008).

Vineyard et al. (2007) used 18 Thoroughbred yearlings to determine the effects of fish oil supplementation on innate and acquired immunity. Yearlings were fed one of two treatments, encapsulated fish oil, or no n-3 supplementation for 56 d. The fish oil treatment contained 4 g EPA and 4 g DHA/100 kg BW/d. Blood samples were obtained at 0, 28, and 56 d. Plasma and red blood cell fatty acid compositions, and neutrophil function and specific antibody production were analyzed. Yearlings fed fish oil had greater plasma and red blood cell EPA, DHA, and arachidonic acid concentrations as compared to non-supplemented yearlings. Yearlings consuming fish oil also had lower red blood cell linoleic acid, and tended to have lower plasma linoleic acid. The authors reported that fish oil supplementation had no effect on phagocytosis-induced oxidative
burst or antibody production. The authors concluded that fish oil neither enhanced nor impaired overall immune function (Vineyard et al., 2007).

Hansen et al. (2002) investigated the effects of n-3 fatty acid supplementation on plasma fatty acid profiles and platelet aggregation in healthy horses. Twelve horses were evenly divided and randomly assigned to either a diet with 10% flaxseed oil added or a control diet for 18 wk. Fasting venous blood samples were collected before 6am at week 0, 4, 8, 12, and 16. Horses consuming flaxseed oil had greater plasma n-6 and n-3 concentrations. Total n-3 concentrations increased more than n-6 concentrations in supplemented horses. Eicosapentaenoic acid and malondialdehyde concentrations significantly increased in horses fed flaxseed oil. There were no differences reported in DHA and arachidonic acid concentrations between treatment groups, or platelet aggregation (Hansen et al., 2002).

Vineyard et al. (2010) studied the effects of various dietary n-3 fatty acid supplements on plasma and red blood cell fatty acid profiles, and immune function. Eighteen Quarter Horse yearlings were assigned to one of three treatments; encapsulated fish oil, milled flaxseed, or control. The fish oil contained 15 g of EPA and 12.5 g DHA. The flaxseed meal contained 61 g of ALA/100g of fatty acids. Fish oil and flaxseed meal were mixed into the concentrate in amounts to provide 6 g of total n-3/100 kg BW. Treatments were fed for 70 d. Venous blood was drawn at d 0, 35, and 70. Peripheral blood mononuclear cells were isolated from blood samples to determine lymphocyte proliferation and PGE2 production. Yearlings fed fish oil had significantly higher plasma and red blood cell concentrations of EPA, DHA, arachidonic acid, and total n-3 as
compared to those consuming flaxseed meal and control. Plasma ALA and linoleic acid were lower in those consuming fish oil as compared to horses consuming flaxseed meal and control. Yearlings fed fish oil and flaxseed meal had a greater increase in skin thickness than control horses 4-h post-injection of intradermal phytohemagglutinin. The authors stated that of the two treatments, fish oil had the greatest impact on plasma and red blood cell concentrations of n-3 fatty acids, but both flaxseed and fish oil supplementation resulted in a more pronounced early inflammatory response compared to control horses (Vineyard et al., 2010).

Hall et al. (2004a) completed a 14-wk study on 10 mature, non-pregnant, light-breed mares. Mares were fed one of two treatments, fish oil or corn oil. Venous blood was collected at 0, 6, 8, and 12 wk. Mares fed fish oil had a decline in n-6: n-3 fatty acid from 0 to 6 wk; however, at wk 12, the fatty acid ratio was not different from baseline ratio. Plasma concentrations of arachidonic acid, EPA, and DHA increased in those mares consuming fish oil as compared to those consuming corn oil. Neutrophils from horses fed fish oil produced significantly more leukotrienes B_5 and B_4 than horses fed CO, and the ratio of leukotriene B_5: leukotriene B_4 concentrations were higher in the fish oil treatment group. The authors concluded that polyunsaturated fatty acids have an effect on the leukotriene inflammatory response in horses (Hall et al., 2004a).

In a companion study, Hall et al. (2004b) compared the effects of PUFA supplementation on selected immune responses. Horses fed corn oil had higher production of PGE_2 from lipopolysaccharide-stimulated bronchoalveolar lavage fluid cells as compared to horses consuming fish oil at 6 and 12 wk. Horses in both treatment
groups had higher tumor necrosis factor-α production from lipopolysaccharide-stimulated cells and phagocytic activity of bronchoalveolar lavage fluid cells at 8 and 12 wk as compared to pretrial values. No differences were observed in skin tests and antibodies throughout the study. The authors concluded that fatty acid supplementation increased the production of tumor necrosis factor-α, whereas only horses consuming corn oil had increased production of PGE₂. The authors theorized that fish oil, because it did not increase production of PGE₂, could have value in the treatment of equine inflammatory diseases (Hall et al., 2004b).

In a study using 10 Thoroughbred and Standardbred horses, O’Connor et al. (2004) studied the effects of fish oil supplementation on the metabolic response to a high-intensity exercise test. Horses were assigned to one of two treatments; fish oil or control. The fish oil contained 10.6% EPA and 8% DHA, and was top-dressed daily at 324 mg/kg BW. Horses received their respective diet for 63 d, and were exercised 5 d/wk. After 63 d, all horses performed a standardized exercise test. Horses receiving fish oil had a lower heart rate during exercise, and tended to have lower packed cell volume. During recovery, horses consuming fish oil had lower plasma glucose concentrations. There was also a tendency for serum insulin to be lower and glucose: insulin ratios to be higher in the fish oil-treated horses. Serum glycerol and cholesterol concentrations were lower in horses receiving fish oil as compared to those consuming control. The authors concluded that fish oil supplementation altered glucose metabolism in conditioned horses (O’Connor et al., 2004).
A study was conducted by Portier et al. (2006) to determine the effects of an oral antioxidant supplement enriched with n-3 fatty acids on erythrocyte membrane fluidity and fatty acid composition in Eventing horses. Twelve conditioned horses were evenly divided and randomly assigned to one of two treatments, an oral antioxidant cocktail enriched with EPA and DHA, or a placebo. The trial lasted 4 wk. At the end of the 4-wk trial, horses performed a standardized exercise test. Venous blood was drawn at d 0, immediately before the exercise test, and 15 min and 24 h post-exercise test. Horses consuming antioxidant n-3 supplement had significantly greater n-3 fatty acids, and n-3:n-6. Control horses had significantly decreased erythrocyte membrane fluidity post-exercise as compared to n-3 enriched antioxidant supplemented horses. During recovery time, erythrocyte membrane fluidity decreased significantly in n-3 supplemented horses as compared to placebo horses. The authors concluded that n-3 FA supplementation in an oral antioxidant supplement may be beneficial in inducing changes in red blood cell membrane composition (Portier et al., 2006).

Woodward et al. (2005) conducted a study to determine if long chain PUFA supplementation would increase n-3 fatty acid concentrations in the plasma and reduce joint pain, as measured by improved stride length. Twelve mature and six 2-yr-old horses were fed either a stabilized long chain PUFA supplement, containing 15 g/d of EPA and DHA, or a control diet containing corn oil, for 75 d. Horses were exercised 5 d/wk. Venous blood was drawn on d 0, 25, 50, and 75. Stride length at the walk and trot was measured on d 0 and 75. Total n-3 fatty acid and DHA concentrations were higher in the treatment group as compared to control. Plasma EPA concentrations tended to increase in
horses consuming long chain PUFA. Treatment horses had a lower plasma n-6: n-3 than control group. There was also a trend for longer trot-stride length in the treatment group. The authors concluded that n-3 concentrations increased in the plasma and tended to ease joint pain (Woodward et al., 2005).

Munsterman et al. (2005) conducted a study to determine the effects of ALA supplementation on equine synovial explants challenged with lipopolysaccharide. Eight mature horses were assigned to receive one of seven concentrations of ALA, ranging from 0 to 300 µg/ml. Cell inflammatory response was evaluated by measuring PGE_2 production. Challenge with lipopolysaccharide significantly increased production of PGE_2 and decreased production of hyaluronic acid in all horses receiving ALA. Horses receiving the highest dosage of ALA (300 µg/ml) had inhibited PGE_2 production. Treatment with ALA increased the percentage of ALA in the explanted cell membranes. The authors suggested that research is warranted for ALA as an anti-inflammatory medication for equine synovitis (Munsterman et al., 2005).

*Osteoarthritis in Horses*

Osteoarthritis is the most common type of arthritis, characterized by gradual wear and loss of cartilage in the joints resulting in friction between the bones, which leads to swelling and pain. Due to disruption of the cartilage collagen matrix, water increases in the cartilage. Together with the progressive loss of proteoglycans, the elasticity of the cartilage diminishes. Cartilage is lost and osteophytes and calcium deposits form. Osteophytes further limit flexibility of the joint. Osteoarthritis progression is associated
with synovial inflammation, joint swelling, stiffness and pain, leading to progressive functional impairment (Jerosch, 2011).

The incidence of osteoarthritis increases with the amount of physical activity exerted on the horse (Wallin et al., 2000; Brommer et al., 2003). The most common cause of lameness in horses is joint disease (Todhunter, 1992). Rossdale et al. (1985) reported that lameness is the most common reason for retirement in the equine industry. Leblond et al. (2000) looked at the causes of death of 448 horses in France and determined that older horses were more likely to have non-traumatic causes, such as degenerative joints, for fatal locomotor disease. The study observed that draft-type horses that pulled carts had increased non-traumatic lesions, which led to osteoarthritis. The authors also reported that locomotor disease was a significant cause of death in racehorses (Leblond et al., 2000).

Wallin et al. (2000) studied the longevity of Warmbloods and reasons for their culling or death. The authors reported that musculoskeletal diseases were the dominant cause of death. With regard to Warmbloods, 55% of deaths resulted from musculoskeletal disease. Joint diseases were associated with 45% of all deaths in this group, of which osteoarthritis was most frequent (Wallin et al., 2000).

**Omega-3 Fatty Acids in Arthritic Horses**

The effect of n-3 supplementation on inflammation markers in arthritic horses was evaluated by Manhart et al. (2009). Sixteen mature horses with arthritis in the knee, fetlock, hock, or stifle joints were blocked by severity of arthritis. The horses were randomly assigned to one of two treatments. Treatment horses received the same mixed
diet as control in addition to 300 g/d of two pelleted n-3 supplements to supply an additional 15 g EPA and 19.8g DHA. Horses received treatments for 90 d. Synovial fluid was collected from at least one affected joint of each horse on d 0, 30, 60, and 90. Blood samples were collected every 15 d beginning on d 0 through d 90. Horses receiving n-3 supplementation had increased DHA plasma concentration and decreased plasma linoleic acid. Treatment horses exhibited a greater decrease in synovial fluid white blood cell concentration, plasma PGE$_2$, and plasma fibrinogen concentrations as compared to control horses. Force-plate analysis revealed no significant difference due to treatment for horses to bear more weight on their arthritis-affected limb. The authors concluded that n-3 supplementation with EPA and DHA may be beneficial to arthritic horses (Manhart et al., 2009)

*Statement of the Problem*

Previous studies reported that marine based n-3 supplementation could increase n-3 fatty acids concentrations in the blood. Research has also shown marine based n-3 supplementation to be beneficial in reducing inflammation and aiding in arthritic treatment. However, there are palatability issues with feeding marine based n-3 supplementation, due to smell and taste. Studies with plant-based n-3 fatty acid supplementation (e.g., flaxseed), which consists primarily of n-3 fatty acid source of ALA, have reported conflicting results. When feeding flaxseed, Hansen et al. (2002) reported that plasma total n-3 fatty acid concentrations significantly increased. Hansen et al. (2002) also reported an increase in EPA plasma concentrations. Vineyard et al. (2010)
and Hess et al. (2012) did not report an increase in EPA or DHA in plasma from horses
fed flaxseed, and flaxseed had no effect on total n-3 fatty acid plasma concentrations.

Marine-based n-3 supplements contain primarily n-3 fatty acids in the form of
EPA and DHA. King et al. (2008) and Hess et al. (2012) reported an increase in plasma
EPA and DHA concentrations when horses were fed fish oil. O’Connor et al. (2007)
observed an increase in serum EPA, DHA, and total n-3 fatty acid concentrations in
horses fed fish oil. Vineyard et al. (2007) reported increased concentrations of red blood
cell and plasma EPA and DHA in horses fed fish oil. Vineyard et al. (2010) observed fish
oil supplementation increased red blood cell and plasma EPA, DHA, arachidonic acid,
and total n-3 fatty acid concentrations. Hall et al. (2004a) reported plasma concentrations
of arachidonic acid, EPA, and DHA increased in horses fed fish oil. When feeding
stabilized EPA and DHA, Woodward et al. (2005) observed an increase in DHA and total
n-3 FA concentrations, and EPA concentrations tended to increase. Manhart et al. (2009)
reported arthritic horses fed EPA and DHA supplementation had increased plasma DHA
concentrations.

Data from previous studies indicate variability in availability and efficacy of fatty
acid due to feed source. To date, research on the effect of feeding flaxseed has been
reported with healthy horses. Research with cranberry seed oil has only been reported in
rats and humans. The objective of the current study was to compare the effects of three
different plant-based fatty acid supplements on plasma EPA, DHA, ALA, and sum of n-3
fatty acid concentrations in horses.
Chapter III
MATERIALS AND METHODS

Horses

Twelve aged horses, three mares and nine geldings, of stock-horse and Thoroughbred type were used to determine the effects of three different plant-based n-3 fatty acid supplements on plasma EPA, DHA, ALA, and sum of n-3 fatty acid concentrations. Horses were stratified by weight and evenly assigned to one of three groups. Horses’ ages ranged from 13 to 28 yr of age with a mean age of 19 yr.

Horses were divided into four groups of three and housed in 6 x 20 m pens at the West Texas A&M University Horse Center. Throughout the trial, horses were exercised lightly. Horses were fed individually in 2 x 5 m stalls twice daily at 0600 and 1700, and were allowed 2 h to consume rations before being turned out in 6 x 20 m pens with two other horses. Routine farrier work, vaccinations and deworming were consistent with farm protocols. Body weight was measured in 7-d intervals on the same day jugular venous blood was drawn. Body condition score remained between 4 and 6.5 throughout the trial. Trial protocol was approved by WTAMU Institutional Animal Care and Use Committee.
**Diets**

All horses consumed a basal diet of alfalfa hay at 1.25 to 1.50% BW/d. Treatments consisted of three types of plant-based fatty acid supplements top-dressed or mixed into the basal hay diet. Horses were assigned to control diet, soy oil (SO; n = 4); flaxseed meal (FM; n = 4) or cranberry seed oil (CO; n = 4). At the beginning of the trial (d -7), venous blood was drawn and horses were weighed and weights recorded. During d -7 to 0, horses were introduced to their respective treatment diet. After the 7-d adjustment period, horses were fed their respective treatment diets for 28 d. Diets were fed in amounts to maintain BCS.

Treatment oils and flaxseed meal were weighed out prior to each feeding, and oils were poured over hay and allowed to absorb into the hay for about 1 hr. Intakes and orts were weighed at time of horses being turned out after meal time. Flaxseed meal, oils, and hay were sampled throughout the trial and stored for later analysis of n-3 fatty acid concentrations. Soybean and cranberry seed oils were delivered in 5-gal containers. Samples were obtained upon opening of each container (fresh), and at about 14 d after opening (exposed) to measure any differences in n-3 FA concentrations due to oxidation of the oils.
Table 1. Feed n-3 Fatty Acid Concentrations

<table>
<thead>
<tr>
<th>Feedstuff</th>
<th>ALA</th>
<th>EPA</th>
<th>DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hay</td>
<td>28.34</td>
<td>32.79</td>
<td>0.00</td>
</tr>
<tr>
<td>Flaxseed meal</td>
<td>9.38</td>
<td>1285.08</td>
<td>0.00</td>
</tr>
<tr>
<td>Soybean oil (fresh)</td>
<td>0.00</td>
<td>0.00</td>
<td>64.08</td>
</tr>
<tr>
<td>Soybean oil (exposed)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Cranberry seed oil (fresh)</td>
<td>120.59</td>
<td>23.11</td>
<td>46.49</td>
</tr>
<tr>
<td>Cranberry seed oil (exposed)</td>
<td>27.81</td>
<td>75.05</td>
<td>27.39</td>
</tr>
</tbody>
</table>

Beginning on d 0, horses consuming C and CO received 0.908 kg of soy oil and cranberry seed oil respectively, and alfalfa hay at 1.5% of BW as-fed to d 7. Due to a feeding miscalculation from d 0 to 7, C and CO horses received 0.454 kg oil/d, and alfalfa hay at 1.25% as-fed of BW/d from d 7 to 28. Horses consuming FLAX received 1.816 kg of flaxseed meal and alfalfa hay at 1.5% of BW as-fed from d 0 to 7. From d 7 to 28, hay was fed at 1.25% as-fed of BW/d.

**Sample Collections**

Venous blood samples were collected 6 h after morning feeding on d -7, 0, 7, 14, 21, and 28 via jugular veni-puncture into 10 mL green-top Vacutainer™ tubes containing Na-heparin. Sample tubes were slowly inverted eight times, placed on ice, and transported to the WTAMU CORE laboratory. All samples were cooled on ice and whole blood was transferred to 1.5 mL micro centrifuge tubes. The 1.5 mL samples were centrifuged at 1061.67 x g for 20 min. After separation, plasma was pipetted into labeled
1.5 mL micro-centrifuge tubes, where they were closed and stored upright at -80°C until analysis.

**Laboratory Analysis**

*Gas Chromatograph (GC).* A 6890 GC (Agilent Technologies, Santa Clara, CA) was used to determine concentrations of plasma and feed fatty acid samples. The SGE BP20 capillary column of the GC was used for fatty acid analysis. The temperature of the GC inlet column at time of injection was +50°C and maximum GC temperature was 300°C. Column flow rate was 1.0 mL/min at a pressure of 10 psi. Total run time per sample was about 35.0 min.

The Supelco 37 FAME mix (Sigma-Aldrich®, St. Louis, MO) was used as a standard. To develop a standard curve for each fatty acid of interest, various known concentrations of the standard were injected in the GC. The concentrations were 0.1 µL standard and 0.9 µL methanol, 0.5 µL standard and 0.5 µL methanol, and 1.0 µL standard. After the standard was analyzed, a line graph was produced with a linear trend line and R² value added to obtain an equation to calculate each fatty acid concentration. Alpha-linolenic acid linear equation was calculated as:

\[
C_{18:3n3} = 5E^{-9}x - 0.009, \text{ with } R^2 = 0.7536.
\]

The linear equation for DHA was calculated as:

\[
C_{22.6} = 2E^{-8}x - 0.0485, \text{ with } R^2 = 0.9586.
\]
Eicosapentaenoic acid concentration equation was calculated as:

$$C_{20.5} = 1 \times 10^{-8}x + 0.0415, \text{ with } R^2 = 0.8883.$$

The linear equation was then utilized to calculate the concentrations (ng/µL) of the fatty acids in the plasma and feed samples.

**Plasma.** Plasma samples were analyzed for EPA, DHA, and ALA fatty acid concentrations using a gas chromatograph (GC; WTAMU CORE Laboratory, Canyon, TX). In preparation for analysis, 0.065 mL H$_2$SO$_4$ and 0.585 mL methanol (CH$_3$OH) were pipetted into 2 ml screw-top micro-centrifuge tubes. Plasma samples were added to the 10% H$_2$SO$_4$ mixture at a level of 0.1 mL. Prepared samples were then heated for 1 h in an 80°C water bath. After heating, tubes were allowed to cool to room temperature and 0.25 ml of hexanes and .975ml 0.9% NaCl was added. Tubes were then centrifuged at 16002 x g for 5 min. Of the upper organic solvent layer, 0.2 mL was transferred to a GC vial. Prepared GC vials were stored at -20°C until analysis. Samples were injected in 1 µL amounts into the GC when prompted.

**Treatments and Hay.** Treatment and hay samples were analyzed for EPA, DHA, and ALA concentrations using a gas chromatograph (WTAMU CORE Laboratory, Canyon, TX). In preparation for analysis, 0.065 mL H$_2$SO$_4$ and 0.585 ml CH$_3$OH were pipetted into 2 mL screw-top micro-centrifuge tubes. Cranberry seed and soy oil samples were added to the 10% H$_2$SO$_4$ mixture at amounts of 0.1mL. Flaxseed and forage
samples were added at amounts of 30 mg. Prepared samples were then heated for 1 h in an 80˚C water bath. After heating, tubes were cooled to room temperature and 0.25 mL of hexanes and .975ml 0.9% NaCl was added. Tubes were then centrifuged at 16002.2 x g for 5 min. Of the upper organic solvent layer, 0.2 mL was transferred to a GC vial. Prepared GC vials were stored at -20˚C until analysis. Samples were injected in 1.0 µL amounts into the GC when prompted.

*Figure 1. Gas chromatograph analysis of fatty acids in standard*

*Quality Assurance of Chromatographs.* Once samples were analyzed, chromatographs were quantified for each fatty acid. Each fatty acid had a particular retention time (min), which was established by the standard. When plasma was quantified by the GC however, certain fatty acids co-eluted. To ensure co-eluted peaks didn’t create a false-positive, each peak in question was evaluated, and corrected (Figure 1).
Statistical Analysis

Data for mean fatty acid concentrations were reported as adjusted means. These means were adjusted by subtracting d -7 values from all other days for each horse to determine changes in plasma fatty acid concentrations from d 0. In the current study, sum fatty acid concentrations refers to the sum of EPA, DHA, and ALA measured. Data for EPA, DHA, ALA, and sum fatty acid concentrations was analyzed using the PROC MIXED procedure of SAS (2011), with period and treatment as main effects. Significant differences between treatment means and time were declared at P ≤ 0.05. Trends for differences between treatment means and time were declared at P ≤ 0.15.
Adjusted means for plasma DHA, EPA, ALA, and sum fatty acid concentrations in horses consuming cranberry seed oil (CO), soybean oil (SO), or flaxseed meal (FM) at d -7, 7, 14, 21, and 28 d are shown in Appendix Figures A-1, A-2, A-3, and A-4; and Appendix Tables A-1 and A-2.

Adjusted mean plasma EPA concentrations of horses consuming cranberry seed oil, flaxseed meal, and soybean oil

Overall plasma EPA concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil is represented in appendix Figure A-1. There was no effect of the interaction (P = 0.191) of time x treatment observed on adjusted mean plasma EPA concentrations (Figure 2).

There was no main effect of treatment (P = 0.175) observed on adjusted mean plasma EPA concentrations (Figure 2). Although non-significant, plasma EPA concentrations appeared to be higher in horses consuming CO and FM as compared to horses consuming SO at d 7, 14, 21, and 28.
There was no main effect of time ($P = 0.465$) observed on adjusted overall mean plasma EPA concentrations (Figure 3 and Appendix Table A-1). Although non-significant, overall mean plasma EPA concentrations appeared to increase at d 7 and 14, but decrease below baseline concentrations at d 21 and 28.
There are no studies reporting the effects of cranberry seed oil on fatty acid concentrations in horses. In the current study, EPA concentrations in horses consuming CO and FM appeared to be increased at d 7, 14, 21, and 28 as compared to the baseline draw at d -7. In contrast, EPA concentrations in horses consuming SO appeared to be decreased at d 14, 21, and 28 as compared to d -7. Data from this study partially agree with that of Hansen et al. (2002), who reported detectable concentrations of EPA, and a significant difference in plasma EPA at wk 4, 8, 12, and 16 in horses fed flaxseed oil. These data, however, disagree with Vineyard et al. (2010) and Hess et al. (2012), who reported non-detectable concentrations of plasma EPA in horses fed flaxseed meal. In the current study, plasma EPA concentrations were at detectable concentrations at d 7 and remained in detectable concentrations throughout the study.

The transient increase in plasma EPA in horses consuming CO could have beneficial effects regarding osteoarthritis in horses (Manhart et al., 2009). However, the decrease in EPA concentrations by d 21 and 28 could be due to several factors. One factor could be that the cranberry seed oil used in this study had an expiration date approximately 24 mo prior to this trial starting. Further, the cranberry seed oil used was in 5-gal containers. After opening these containers, the level or extent of oxidation that occurred is unknown, thereby causing destruction of the unsaturated fatty acids, primarily the n-3 fatty acids contained in this oil. Rufian-Henares et al. (2005) observed unsaturated fatty acids exposed to high temperatures for short periods (1 to 4 wk at 32 to 55°C) or low temperatures for long periods (12 to 36 wk at 4°C, 20°C, or 30°C) decreased significantly. Lipid auto-oxidation can occur at room temperature, but is accelerated at
higher temperature. Polyunsaturated fatty acids are the most susceptible to fat oxidation, because the double bonds make the fatty acid less stable. Omega-3 fatty acids are the most susceptible to oxidation because it consists of three double bonds. (Cerny, 2010).

No studies were found reporting the effects of soybean oil on fatty acid concentrations in horses. In the current study, horses consuming SO appeared to have greatest increase of EPA concentrations at d -7, and appeared to decrease until d 28. Wistuba et al. (2006) reported non-detectable plasma EPA concentrations in steers a diet containing soybean meal. Likewise, Farran et al. (2008) reported no significant increase in plasma EPA concentrations in heifers fed rolled soybeans.

*Adjusted mean plasma DHA concentrations of horses consuming cranberry seed oil, flaxseed meal, and soybean oil*

Overall plasma DHA concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil is represented in Appendix Figure A-2. There was no effect of the interaction (P = 0.304) of time x treatment observed on adjusted mean plasma DHA concentrations (Figure 4).

There was no main effect of treatment (P = 0.557) observed on adjusted mean plasma DHA concentrations (Figure 4). Although non-significant, plasma DHA concentrations appeared to be higher in horses consuming CO and FM as compared to SO at d 7, 14, and 28.
There was a main effect of time (P = 0.005) on adjusted overall mean plasma DHA concentrations (Figure 5 and Appendix Table A-1). Overall mean plasma DHA concentrations were higher at d 7 (P = 0.008), 14 (P = 0.001), and 21 (P = 0.005) as compared to d -7. Overall mean plasma DHA concentrations were lower at d 28 (P = 0.017) as compared to d 14. Further, overall mean plasma DHA concentrations tended to be lower at d 28 as compared to d 7 (P = 0.078) and 21 (P = 0.055).
In the current study, plasma DHA concentrations were detectable throughout the trial. Data for plasma DHA from the current study partially agree with that of Hansen et al. (2002), who reported detectable concentrations of DHA, and no significant differences between horses fed flaxseed oil. These data, however, disagree with Hess et al. (2012), who reported non-detectable concentrations of plasma DHA in horses fed flaxseed meal. These results disagreed with Wistuba et al. (2006) and Farran et al. (2008) who reported no increase in plasma DHA concentration in cattle fed soybean products. A possible explanation for the different results from that of Vineyard et al. (2010) and Hess et al. (2012) is varying preparation methods and analysis procedures using gas chromatograph analysis.

*Adjusted mean plasma ALA concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil*

Overall adjusted mean plasma ALA concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil is represented in Appendix Figure A-3. There was no effect of the interaction (P = 0.143) of time x treatment observed on adjusted mean plasma ALA concentrations (Figure 6).

There was no main effect of treatment (P = 0.390) observed on adjusted mean plasma ALA concentrations (Figure 6). Although non-significant, adjusted mean plasma ALA concentrations appeared to be higher in horses consuming FM as compared to those consuming SO at d 7, 14, and 28; and appeared to be lower in horses consuming CO as compared to SO and FM at d 14, 21, and 28.
There was no main effect of time (P = 0.443) observed on adjusted overall mean plasma ALA concentrations (Figure 7; and Appendix Table A-1). Although non-significant, adjusted overall mean plasma ALA concentrations appeared to be higher at d 7, 14, 21, and 28 as compared to d -7.

Results from this study agreed with Hess et al. (2012) who observed that plasma ALA varied with time in horses fed flaxseed meal. Results from this study partially agree with Vineyard et al. (2010) who reported that horses fed milled flaxseed had increased
plasma ALA concentrations from d 0 to d 35; and Hansen et al. (2002) who reported that plasma concentrations of ALA were markedly increased in horses supplemented with flaxseed oil throughout a 16 wk study.

*Adjusted mean plasma sum fatty acid concentrations in horses consuming cranberry seed oil, flaxseed meal, and soybean oil*

Overall plasma sum fatty acid concentrations in horses consuming CO, FM, and SO is represented in Appendix Figure A-4. There was no effect of the interaction (P = 0.100) of time x treatment observed on adjusted mean plasma sum fatty acid concentrations (Figure 8).

There was no main effect of treatment (P = 0.314) observed on adjusted mean sum fatty acid concentrations. The effect of treatment on adjusted mean sum fatty acid concentrations is represented in Figure 8. Although non-significant, plasma sum fatty acid concentrations appeared to be higher in horses consuming CO and FM as compared to SO at d 7, 14, and 28.
There was a main effect of time (P = 0.021) observed on adjusted overall mean plasma sum fatty acid concentrations (Figure 9; and Appendix Table A-1). There was a significant increase in overall adjusted mean plasma sum fatty acid concentrations at d 14 (P = 0.004) and 21 (P = 0.019) as compared to d -7; and a significant decrease at d 28 as compared to d 14 (P = 0.019). There tended to be an increase in adjusted overall plasma sum n-3 fatty acid concentrations at d 7 as compared to d -7 (P = 0.062); and a decrease at d 28 (P = 0.082) as compared to d 21.

Figure 8. The interaction of time (d -7, 7, 14, 21, and 28) and treatment (CO, SO, and FM) on adjusted mean plasma sum fatty acid concentrations (P = 0.100)
Results from this study partially agree with Vineyard et al. (2010) who reported that horses fed milled flaxseed had increased plasma total n-3 fatty acid concentrations from d 0 to d 35.

Possible explanation for differences between studies

Possible explanations for the different results observed in the current study and that of previous studies are the source, storage temperature, and storage time of the flaxseed used in the current study. Hansen et al. (2010) fed flaxseed oil, whereas Vineyard et al. (2010) and Hess et al. (2012) fed flaxseed meal. The flaxseed meal used in this study was stored for 6 mo prior to the trial beginning, with temperatures that exceeded 50°C. Although encapsulated fatty acids in flaxseed meal are more stable than oils, encapsulation of flaxseed was reported to have an increased efficiency of 84% of a protective effect against the production of primary and secondary oxidative products versus non-encapsulated oil during 25 days of room temperature storage (Liu et al. 2010).
However, this does not take into account the possibility of oxidization of n-3 fatty acids during 6 mo of storage. Therefore, it is possible that much of the unsaturated fatty acids had oxidized during the long storage time along with high temperatures.

Possible oxidation of the fatty acids in the oils used in this study is another factor to be considered. Unsaturated fatty acids are very volatile. Rufian-Henares et al. (2005) conducted a study to assess lipid oxidation and reported that oxidation significantly decreased unsaturated fatty acid concentrations in as little as 1 wk at 32 to 55°C, and 12 wk when stored at 4°C. As storage time and temperature increased, loss of EFA increased. Although encapsulation of fatty acids provides protection, oxidation still may occur.
Chapter V

CONCLUSIONS AND IMPLICATIONS

Results from this experiment indicate that horses fed plant-based n-3 fatty acid supplementation may have increased plasma n-3 fatty acid concentrations over time. Transient increases in n-3 fatty acid concentrations observed in this trial suggest the importance of proper handling and storage of fatty supplements to maintain n-3 fatty acid integrity. Plasma fatty acid data from this trial indicate that feeding plant-based n-3 fatty acid supplementation may increase circulating EPA, DHA, ALA, and sum fatty acid concentrations in aged horses.

There was no main effect of treatment (P = 0.175), time (P = 0.465), or effect of the interaction of time x treatment (P = 0.191) observed on adjusted mean plasma EPA concentrations. There was a main effect of time (P = 0.005) on adjusted overall mean plasma DHA concentrations. Overall mean plasma DHA concentrations were higher at d 7 (P = 0.008), 14 (P = 0.001), and 21 (P = 0.005) as compared to d -7. Overall mean plasma DHA concentrations were lower at d 28 (P = 0.017) as compared to d 14. There was no main effect of treatment (P = 0.557), or effect of the interaction of time x treatment (P = 0.304) observed on adjusted mean plasma DHA concentrations. There was no main effect of treatment (P = 0.390), time (P = 0.443), or effect of the interaction of time x treatment (P = 0.143) observed on adjusted mean plasma ALA concentrations.
There was a main effect of time (P = 0.021) observed on adjusted overall mean plasma sum fatty acid concentrations. There was a significant increase in overall adjusted mean plasma sum fatty acid concentrations at d 14 and 21 as compared to d-7; and a significant decrease at d 28 as compared to d 14. There was no main effect of treatment (P = 0.314), or effect of the interaction of time x treatment (P = 0.100) observed on adjusted mean sum fatty acid concentrations.

Data reported from previous studies and the current study on cranberry seed oil and flaxseed meal is conflicted. However, further research is merited using fresh sources of these products to determine their effects on aged or arthritic horses.

Data from this trial suggests that proper handling and storage of fatty acid supplements and source is critical to maintaining the integrity and efficacy of polyunsaturated fatty acids, especially the n-3 series, due to their inherent instability. Oxidation of polyunsaturated fatty acids prior to feeding will result in destruction of the fatty acids and will remove any added benefits that n-3 fatty acids may have had in the horse.
LITERATURE CITED


APPENDIX FIGURES A

OVERALL ADJUSTED PLASMA FATTY ACID CONCENTRATIONS GRAPHS
APPENDIX FIGURES B

STANDARD CURVES
APPENDIX TABLES A

OVERALL MEANS BY TIME
Table A-1. Least squares means (ng/µL) of plasma fatty acid concentrations in horses consuming all treatments (cranberry seed oil, flaxseed meal, and soybean oil) at d -7, 7, 14, 21, and 28

<table>
<thead>
<tr>
<th></th>
<th>Draw Day</th>
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<tbody>
<tr>
<td></td>
<td>-7</td>
</tr>
<tr>
<td>Overall DHA</td>
<td>0.00(^a)</td>
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<tr>
<td>Overall EPA</td>
<td>0.00</td>
</tr>
<tr>
<td>Overall ALA</td>
<td>0.00</td>
</tr>
<tr>
<td>Overall sum fatty acid</td>
<td>0.00(^{a,e})</td>
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</tbody>
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\(^{a, b, c, d}\) Means within rows with differing superscripts differ at P < 0.05

\(^{e, f, g, h}\) Means within rows with differing superscripts differ at P < 0.15
Table A-2. Adjusted mean plasma fatty acid concentration in horses consuming cranberry seed oil, flaxseed meal, and soybean oil at d -7, 7, 14, 21, and 28

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<thead>
<tr>
<th></th>
<th>CO</th>
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<th>SO</th>
<th></th>
<th>CO</th>
<th>FM</th>
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<tr>
<td>d -7</td>
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<td>0.00</td>
<td>0.00</td>
<td>d 7</td>
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<td>30.35</td>
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