Unraveling the Mystery of Peroxidized Lipids

A. R. Hanson[1], B. J. Kerr[2], and G. C. Shurson[1]

[1]Department of Animal Science, University of Minnesota, St. Paul, MN
[2]USDA-ARS, National Laboratory for Agriculture and Environment, Ames, IA

Take Home Message

Lipid peroxidation is a dynamic process which produces numerous compounds (Belitz et al., 2009) which have been shown to be associated with deleterious effects on animal health, metabolic oxidative status, and growth performance (Lykkesfeldt and Svendsen, 2007). Consequently, these effects can have significant negative impacts on costs associated with food animal production. However, accurate measurement of the extent of lipid peroxidation and its relationship to animal health and performance are major obstacles that must be overcome to optimize energy utilization efficiency in animal feeds. Currently, there are no universally accepted analytical standards for measuring lipid peroxidation, and various measures are used in different segments of the food, agriculture, and lipid industries. Animal nutritionists have historically assumed that peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) assays are reliable indicators of the extent of lipid peroxidation in feed fats and oils. However, a review of the scientific literature and recent studies indicate that the use of PV and TBARS does not accurately characterize the extent of lipid peroxidation as it relates to animal performance, and may often provide misleading results. Therefore, other analytical procedures or combinations of metrics must be identified and used to provide a more accurate assessment of peroxidation of lipids used in animal feeds, and determine threshold levels at which growth is impaired.

Background

The cost of dietary energy has increased dramatically in recent years, causing an increase in total feed costs for swine and poultry, and prompting nutritionists to continue to find ways to optimize efficiency of energy utilization. Lipids provide more energy per unit than carbohydrates, whereupon the efficiency of gain generally improves when lipids are added to swine and poultry diets (Engel et al., 2001). In addition, other commonly used feedstuffs such as dried distillers grains (DDGS) and animal protein by-products contain significant amounts of lipid. However, some lipid sources, as well as corn oil present in DDGS have relatively high levels of polyunsaturated fatty acids (PUFA) which are highly susceptible to peroxidation (Song and Shurson, 2013). For example, the polyunsaturated long chain fatty acids, linoleic and linolenic acid, are peroxidized at rates 12 and 25-times greater than oleic acid, a monounsaturated fatty acid (Belitz et al., 2009). Heat, light, moisture, oxygen, and the presence of transition metals increase the rate of lipid peroxidation, while antioxidants impede this process (Belitz et al., 2009). Feedstuffs and lipids are often exposed to pro-oxidation conditions during production, processing, and storage. Ultimately, lipid peroxidation degrades unsaturated fatty acids resulting in a reduction in energy value for non-ruminants (Wiseman, 1999).

Lipid peroxidation is a dynamic process, which simultaneously produces and degrades numerous compounds (Belitz et al., 2009) associated with deleterious effects on animal health,

metabolic oxidative status, and growth performance (Lykkesfeldt and Svendsen, 2007). These negative impacts can have significant economic effects on the cost of food animal production.

The Lipid Peroxidation Process

Lipid peroxidation is generally classified into three phases: initiation, propagation, and termination, with each step "consuming" and producing many compounds (Belitz et al., 2009; Figure 1). At least 19 volatile compounds are formed during peroxidation of linoleic acid, and these compounds may later be subsequently degraded (Belitz et al., 2009). Therefore, the complex and fluctuating nature of peroxidation, along with the numerous compounds produced during various stages, make it difficult to accurately quantify the extent of peroxidation of lipids in feed ingredients. For example, the unsaturated fatty acid content as measured by iodine value, generally declines (Johnson and Kummerow, 1957; Figure 2), and weight of a sample increases as oxygen is incorporated into fatty acids to yield lipid hydroperoxides (Shahidi and Wanasundara, 2008). This suggests that a change in sample weight or iodine value can indicate the extent of peroxidation. However, these methods require compositional knowledge of the original (fresh) lipid to ascertain the direction and magnitude of changes resulting from peroxidation. Therefore, such measures have limited practical utility for nutritionists. Furthermore, while iodine value may be a general indicator of peroxidative degradation, it is nonspecific. Therefore, additional assays must be utilized to identify specific products of peroxidation.

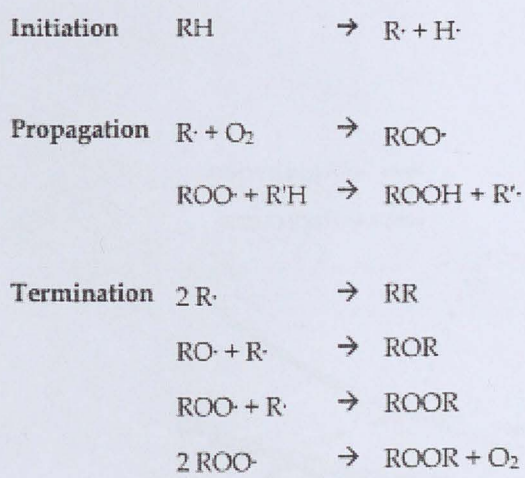

Figure 1. Free radical induced lipid peroxidation (Adapted from Seppanen, 2005).

A wide variety of analytical procedures exist to measure peroxidation products. The most commonly used measures of peroxidation used in the feed industry are peroxide value (PV), thiobarbituric acid reactive substances (TBARS), and anisidine value (AnV), but each analytical procedure only measures a fraction of the total types and amounts of peroxidation products. For example, the PV assay quantifies peroxides while TBARS and AnV estimate aldehydes. However, peroxides and aldehydes that are initially produced are ultimately degraded as peroxidation continues, resulting in underestimation of the extent of peroxidation in excessively peroxidized lipids (Fitch Haumann, 1993; Figure 3) .

Therefore, these commonly used analytical measures of the extent of lipid peroxidation may be misleading. To comprehensively describe the peroxidation status of a lipid, the NRC (2012) recommends the use of multiple measures. However, the ideal analytical procedures for

determining the maximum tolerable levels of peroxidized lipids in animal diets have not been established. Nutritionists need accurate, meaningful, and standardized analytical methods to quantify lipid peroxidation in feed ingredients before they will be able to effectively evaluate the impact of dietary lipid peroxidation on growth and metabolic oxidation status of animals.

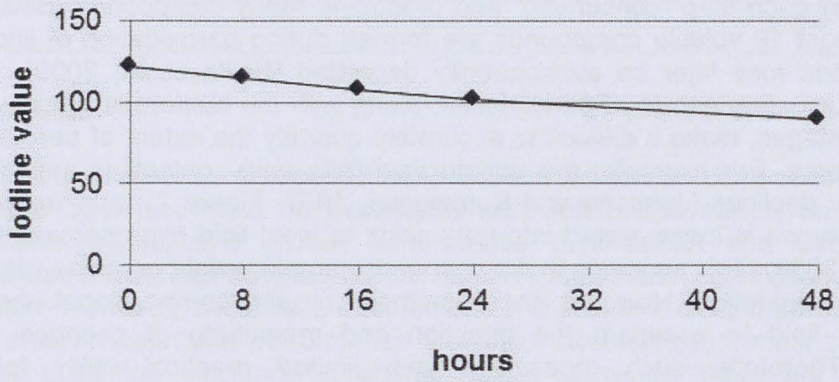

Figure 2. Iodine value declines over time in corn oil heated at 200° C resulting from degradation of unsaturated fatty acids and formation of peroxidation products (Adapted from Johnson and Kummerow, 1957).

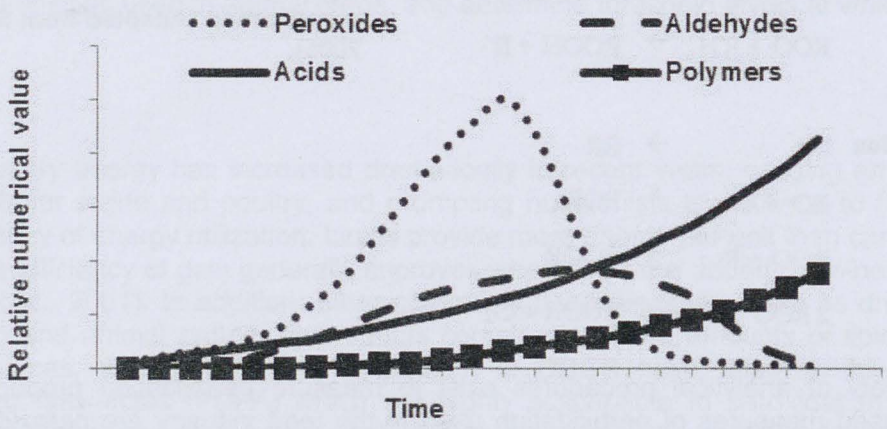

Figure 3. Various products are simultaneously produced and degraded throughout peroxidation. (Adapted from Fitch Haumann, 1993).

Dietary Peroxidized Lipids Impact Metabolic Oxidation Status and Health

Polyunsaturated fatty acids in cell membranes are susceptible to peroxidation, but these effects can be mitigated by adequate quantities of endogenous and dietary antioxidants. However, oxidative stress results from an imbalance between the extent of peroxidation and the capacity of antioxidants to overcome this peroxidation load. Metabolic oxidation status is often characterized by measuring TBARS and antioxidant concentrations in serum, liver, and other tissues. Researchers have consistently shown that consumption of peroxidized lipids reduce the antioxidant status of swine (Boler et al., 2012; Liu, 2012), broilers (Tavárez et al., 2011; Takahashi and Akiba, 1999), and rats (Liu and Huang, 1995) compared with animals fed diets containing unperoxidized lipids. High amounts of peroxidation products may overload the antioxidant defense system resulting in increased metabolic oxidative stress and reduced nutrient utilization efficiencies due to an animal's reduced capacity to cope with damage at the cellular and systemic levels (Lykkesfeldt and Svendsen, 2007; Figure 4). However, it is difficult to quantify the specific impacts of lipid peroxidation products and link them with the physiological changes in growth and health that occur. For example, increased liver size relative to body weight serves as a biological indicator of toxicity (Juberg et al., 2006). Research results have shown that feeding diets containing peroxidized lipids result in increased liver size (Liu, 2012; Eder, 1999; Huang et al., 1988), and this response may be a result of increased synthesis of microsomal enzymes to mitigate toxicity (Huang et al., 1988). However, the practical significance of such changes for nutrient metabolism, growth and health of animals is not clear.

Changes in gut barrier function have also been shown to have significant implications for animal health. The intestine acts as the first line of defense against a plethora of dietary components, toxins, bacteria, viruses and antigens (Turner, 2009; Walker and Sanderson, 1992). However, intestinal epithelial cell membranes are composed of PUFA, which are susceptible to peroxidation. Therefore, the function of the epithelial barrier may be compromised by peroxidative damage. Research results suggest that dietary peroxidized lipids induce metabolic oxidative stress in enterocytes, (Ringseis et al., 2007; Reddy and Tappel, 1974). Wang et al. (2000) suggested that dietary oxidants contribute to apoptosis of human intestinal epithelial cells *in vitro*, but little research has been conducted to demonstrate that a similar response occurs in animals. Dibner et al. (1996) showed histological evidence that the half-life of enterocytes was reduced in broilers fed diets containing peroxidized lipids, and suggested negative potential implications for the integrity of the gastrointestinal barrier and the immune system. However, results from our recent work showed that feeding diets containing 10% peroxidized corn oil, canola oil, beef tallow, and poultry fat had no effect on intestinal barrier function (Liu, 2012).

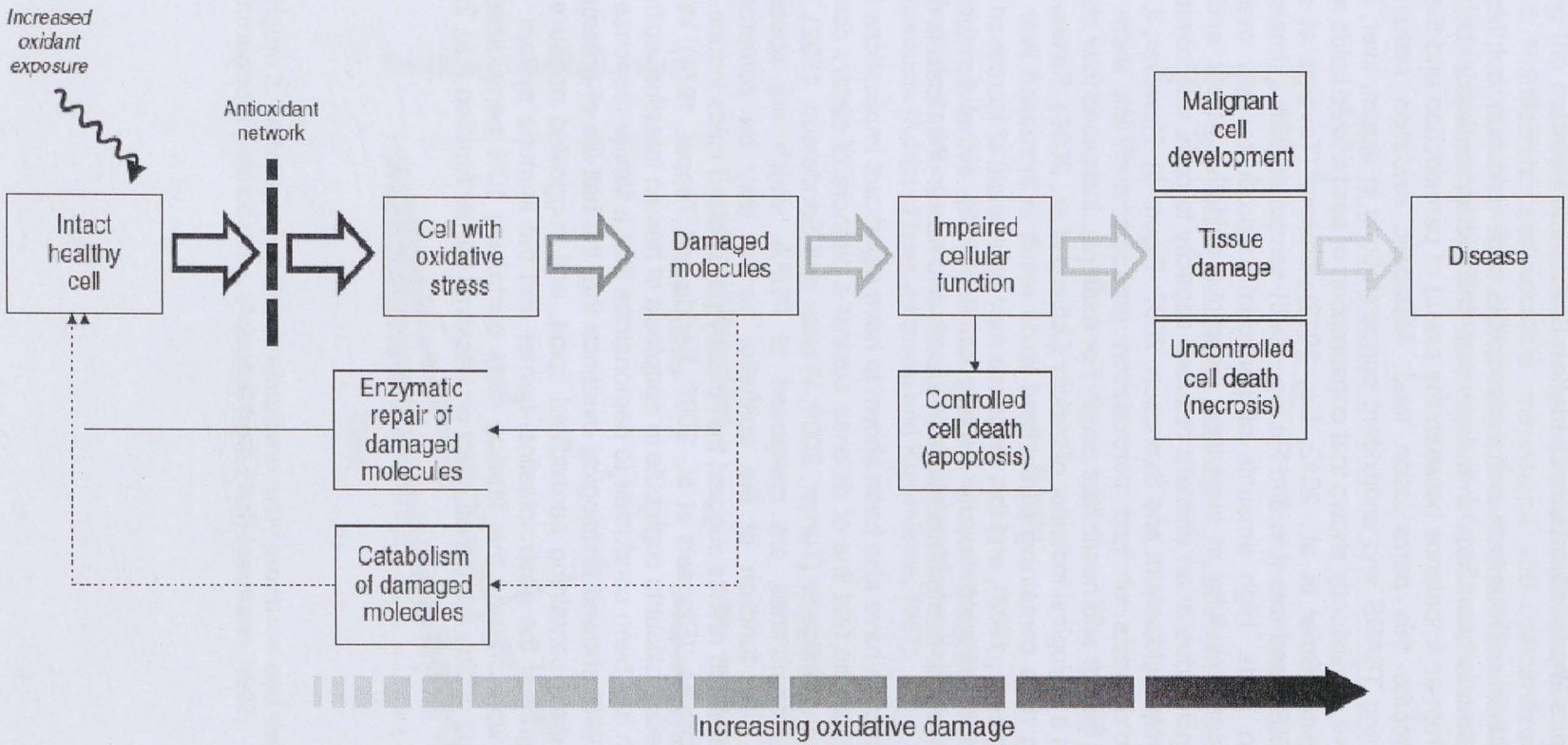

Figure 4. Oxidative stress contributes to metabolic damage and disease (Adapted from: Lykkesfeldt and Svendsen, 2007).

Dietary Peroxidized Lipids Reduce Growth Performance of Swine and Poultry

Researchers have reported reduced growth resulting from feeding peroxidized lipids to swine (Boler et al., 2012; Liu, 2012) and broilers (Tavárez et al., 2011; Takahashi and Akiba, 1999), which may be partially caused by a reduction in feed intake that often accompanies this response. However, metabolic oxidative stress and peroxidative damage may also further contribute to reduced growth, but the physiological mechanisms that cause this response have not been elucidated. Furthermore, nutritionists lack practical guidelines for maximum tolerable limits of adding peroxidized lipids to animal feeds because the level of dietary inclusion at which growth declines is not clear.

The establishment of maximal tolerable levels for peroxidized lipids is necessary to enable nutritionists to optimize the animal health and growth performance. DeRouchey et al. (2004) and Gray and Robinson (1941) suggested the maximum threshold level of PV is 2.4 mEq O_2/kg of diet and 20 mEq O_2/kg of lipid, respectively, but these proposed thresholds were not substantiated with experimental evidence. In one of our recent studies, we fed nursery pigs diets containing 6% peroxidized corn oil resulting in a diet PV of 8.09 mEq O_2/kg (134.9 mEq O_2/kg oil × 6% corn oil inclusion) and did not find any adverse effect on growth or survival of nursery pigs (Hanson et al., unpublished data). Yet, the total diet PV concentration used in this study far exceeded the maximal recommended levels suggested by DeRouchey et al. (2004) and Gray and Robinson (1941). These results confirm that using PV as a sole indicator of lipid or diet peroxidation may not be a meaningful way of characterizing the extent of lipid peroxidation of feeds and lipid sources.

Feed fats and oils vary in fatty acid composition, dietary inclusion levels, as well as the extent of heating and peroxidation. The current approaches being used to quantify lipid peroxidation among studies make it difficult to establish definitive, practical guidelines for maximum tolerable limits of dietary peroxidized lipids for animals.

Literature Summary of Growth Performance Responses from Feeding Peroxidized Lipids to Pigs and Broilers

To evaluate the impact of peroxidized lipids, and identify potential dietary and metabolic markers for reduced growth, we summarized data from 43 experiments involving the growth and metabolic oxidation status of pigs (n = 17) and broilers (n = 26) fed diets containing peroxidized lipids (Boler et al., 2012; Liu, 2012; McGill et al., 2011a,b; Tavárez et al., 2011; Harrell et al., 2010; Racanicci et al., 2008; Yuan et al., 2007; Anjum et al., 2004; DeRouchey et al., 2004; Anjum et al., 2002; Takahashi and Akiba, 1999; Engberg et al., 1996; Lin et al., 1989; Cabel et al., 1988; Inoue et al., 1984; Oldfield et al., 1963) . Only studies that compared supplemental lipid sources using diets that were formulated to be isocaloric across the two dietary treatments being compared were included in this summary. Dietary TBARS, PV, and AnV were obtained or calculated from the information provided in these published studies. Response parameters obtained or calculated from each study included: ADG, ADFI, G:F, and serum or plasma concentrations of vitamin E and TBARS. These data were analyzed using UNIVARIATE and CORR procedures of SAS (SAS Inst. Inc., Cary NC).

Across these studies, ADG declined 11.1%, ADFI declined by 7.4%, and G:F was reduced by 4.3% when substituting peroxidized lipid for fresh lipid in diets for swine and broilers (Table 1). Interestingly, the magnitude of change relative to fresh lipid control diets for ADG and ADFI were similar for swine and poultry, suggesting that the growth performance of both species is impacted to a similar extent when feeding peroxidized lipids (Table 1).

The difference in magnitude of change for ADG (12.2%) compared to ADFI (7.4%) suggests that factors in addition to feed or caloric intake responses contribute to reduced growth when feeding peroxidized lipids. We suspect that this additional reduction in growth is due to physiological and metabolic changes associated with feeding high amounts of lipid peroxidation products. To support this hypothesis, feeding diets containing peroxidized lipids resulted in a 46.3% decline in the serum content of vitamin E and 20.4% increase in TBARS relative to animals fed diets with fresh lipids, suggesting that peroxidized lipids induce metabolic oxidative stress. Furthermore, the serum concentrations of TBARS and vitamin E were moderately negatively correlated (r = -0.66, P = 0.02), and a similar relationship has been reported by others (Yanik et al., 1999; Liu and Huang, 1995; Hossein Sadrzadeh et al., 1994). In swine, the serum concentration of TBARS was positively associated with dietary TBARS (r = 0.64, P = 0.06), and PV (r = 0.78, P = 0.008), but this association was not observed for serum vitamin E content. Although these biomarkers may indicate that metabolic oxidative stress occurred, it is unclear if these biomarkers are associated with reduced growth performance. For example, serum content of vitamin E tended to be positively associated with ADG (r = 0.42, P = 0.07), and was positively associated with ADFI (r = 0.46, P = 0.05), but these impacts are a function of increased daily intake of vitamin E.

Dietary PV concentration was moderately negatively associated with ADG (r = -0.48, P = 0.002) among species, but this dietary measure seems to be a more useful potential indicator in broilers (r = -0.78, P < 0.001) because the relationship was not significant (r = 0.05, P = 0.86) in swine (Figure 5). These results are in agreement with results from previous research by our group where there was no correlation between ADG of nursery pigs and PV content of dietary lipid (Liu, 2012). This is not surprising because highly peroxidized lipids can have relatively low levels of peroxides, making PV an unreliable indicator of the extent of peroxidation.

The TBARS content of lipid sources was reported in only 11 of the 43 studies. These 11 evaluations were only with swine and, as a result, we were unable evaluate the value of dietary TBARS concentration for predicting reduced growth in broilers. Across these studies, there was no association (r = 0.01, P = 0.98) between dietary TBARS and ADG for swine. Results from previous work by our group showed a trend for a low negative correlation (r = -0.29, P = 0.09) between ADG and TBARS content of dietary lipid (Liu, 2012). Therefore, it appears that the use of TBARS as a measure of peroxidation of lipids in feed may not be a reliable indicator of changes in growth performance.

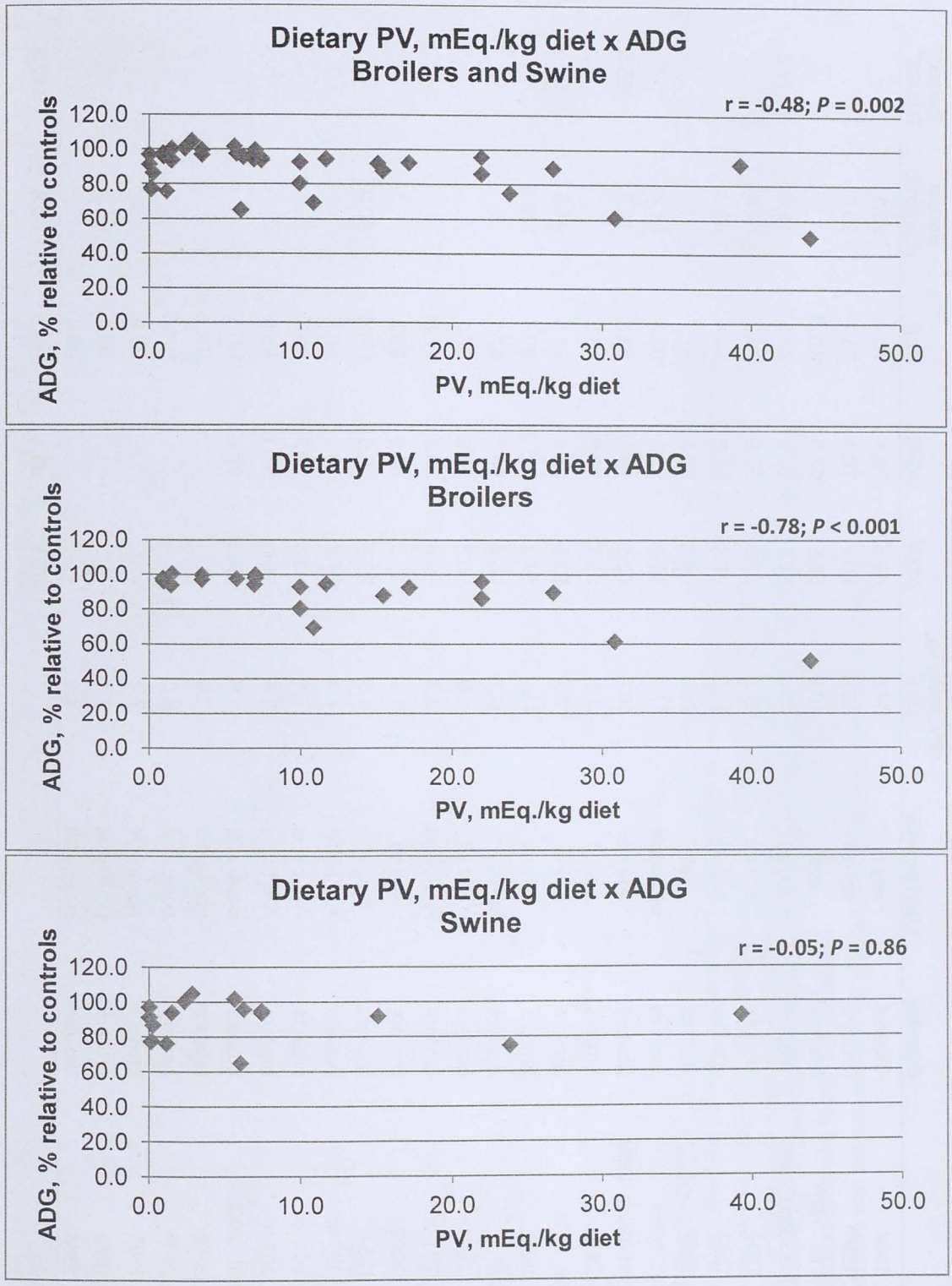

Figure 5. Dietary peroxide value (PV) is negatively correlated to the growth of broilers fed peroxidized lipids, but not for swine.

Table 1. Summary of 43 evaluations of dietary peroxidized lipids from broilers (n = 26) and swine (n = 16)

Citation	Species	Lipid source[1]	PV, mEq/kg diet[2]	ADG[3]	ADFI	G:F	Serum vitamin E	Serum TBARS[4]
Inoue et al., 1984	broilers	soy oil	44.0	49.8	70.1	70.4	23.1	.
Inoue et al., 1984	broilers	soy oil	30.9	60.7	76.4	78.7	50.0	.
L'Estrange et al., 1966	broilers	beef	10.9	68.9	67.8	101.2	.	.
Takahashi and Akiba, 1999	broilers	soy oil	10.0	80.2	90.6	88.5	19.6	174.8
Inoue et al., 1984	broilers	soy oil	22.0	85.8	92.8	92.0	47.8	.
Inoue et al., 1984	broilers	soy oil	15.5	87.4	92.8	94.4	62.3	.
Wang et al., 1997	broilers	fat	26.8	89.3	92.3	91.7	.	.
Engberg et al., 1996	broilers	vegetable	17.2	92.2	92.8	99.4	61.4	.
Takahashi and Akiba, 1999	broilers	soy oil	10.0	92.5	99.1	93.3	15.2	156.5
Anjum et al., 2002	broilers	soy oil	1.5	93.7	98.8	94.6	.	.
Tavárez et al., 2011	broilers	soy oil	7.0	93.9	98.4	95.5	41.5	97.0
Inoue et al., 1984	broilers	soy oil	11.7	94.2	103.2	90.4	105.8	.
Lin et al., 1989	broilers	sunflower	22.0	95.4	98.2	97.1	.	.
Anjum et al., 2004	broilers	soy oil	1.0	95.8	98.6	97.1	.	.
McGill et al., 2011a	broilers	A-V blend	3.5	96.8	99.3	97.8	.	.
Anjum et al., 2002	broilers	soy oil	1.0	97.3	98.9	98.2	.	.
Inoue et al., 1984	broilers	soy oil	5.9	97.5	96.0	101.9	60.9	.
McGill et al., 2011a	broilers	A-V blend	7.1	97.8	99.1	98.4	.	.
McGill et al., 2011b	broilers	A-V blend	7.1	99.3	99.3	98.4	.	.
McGill et al., 2011b	broilers	A-V blend	3.5	99.3	100.0	98.9	.	.
Racanicci et al., 2008	broilers	poultry fat	1.6	100.0	97.8	101.9	.	.
Upton et al., 2009	broilers	poultry fat	3.0	.[5]	.	101.5	.	.
Upton et al., 2009	broilers	poultry fat	3.0	.	.	104.6	.	.
Cabel et al., 1988	broilers	poultry fat	7.2	.	.	88.9	.	.
Cabel et al., 1988	broilers	poultry fat	2.1	.	.	99.4	.	.
Cabel et al., 1988	broilers	poultry fat	4.1	.	.	99.4	.	.
Mean, broilers				**88.9**	**93.4**	**95.1**	**48.8**	**142.8**

Table 1 (continued). *Summary of 43 evaluations of dietary peroxidized lipids from broilers (n = 26) and swine (n = 16)*

Oldfield et al., 1963	pigs	fish oil	6.1	64.6	77.1	83.5	.	.
Liu, 2012	pigs	canola oil	23.9	74.6	89.5	84.1	48.9	122.8
Liu, 2012	pigs	canola oil	1.2	75.7	78.8	96.8	60.0	120.4
Liu, 2012	pigs	corn oil	0.2	76.9	84.4	91.3	18.9	114.8
Liu, 2012	pigs	poultry fat	0.2	77.3	87.1	88.6	70.0	103.4
Liu, 2012	pigs	tallow	0.3	86.3	89.4	95.7	89.7	102.0
DeRouchey et al., 2004	pigs	ch. white grease	0.1	91.0	90.8	100.3	.	.
Liu, 2012	pigs	corn oil	15.1	91.6	88.8	102.9	29.0	121.0
Yuan et al., 2007	pigs	fish oil	39.3	91.6	96.6	94.8	.	129.4
Boler et al., 2012	pigs	corn oil	7.5	93.4	95.0	95.3	46.3	113.1
Oldfield et al., 1963	pigs	fish oil	1.6	93.7	90.0	104.0	.	.
Harrell et al., 2010	pigs	corn oil	7.5	94.7	94.8	99.8	.	.
DeRouchey et al., 2004	pigs	ch. white grease	6.3	95.4	92.1	103.6	.	.
DeRouchey et al., 2004	pigs	ch. white grease	0.1	96.8	91.1	106.3	.	.
DeRouchey et al., 2004	pigs	ch. white grease	2.4	100.2	98.1	102.1	.	.
Liu, 2012	pigs	poultry fat	5.7	101.4	102.0	97.1	73.6	107.0
Liu, 2012	pigs	tallow	2.9	104.6	109.8	94.3	96.6	103.1
Mean, swine				**88.8**	**91.5**	**96.5**	**59.2**	**113.7**
Mean, broilers and swine				**88.9**	**92.6**	**95.7**	**53.7**	**120.4**

[1]Fat = Wang et al.(1997) did not report the source of supplemental fat. A-V blend = blend of animal fat-vegetable oil.

[2]PV = peroxide value calculated as: [oil inclusion level, % x PV of fat source] or reported as stated by researchers in diets with peroxidized lipid.

[3]All response variables were calculated by dividing the mean of the animals fed diets with peroxidized lipids by those fed fresh lipids and multiplying by 100. Therefore, the values presented represent the change in value of the variable relative to controls.

[4]TBARS = thiobarbituric acid reactive substances.

[5]A period indicates that this variable was not reported or estimable from the research reports.

Indicators of Lipid Peroxidation

A wide variety of measures can be used to quantify lipid peroxidation products. Each assay has unique positive and negative aspects which must be considered prior to their use. Ultimately, it would be ideal to have one accurate, universally acceptable assay, but perhaps with the exception of measuring non-elutable material (as described later), none of the current peroxidation assays meet this goal.

Peroxide value

Peroxide value (PV) has been commonly used to evaluate lipids in animal feeds for many years. Peroxide value measures the concentration of primary lipid peroxidation products, including peroxides and hydroperoxides, formed during the initial phase of lipid peroxidation. Peroxide value may provide useful information for predicting animal growth performance when lipids of various degrees of peroxidation are fed because peroxides and hydroperoxides can be toxic (Kaneko et al., 1988). However, PV may also provide misleading results because peroxides are initially produced and later degraded throughout the peroxidation process (Fitch Haumann, 1993). Therefore, a highly peroxidized lipid may have deceptively low levels of peroxides. Other peroxidation measures such as thiobarbituric acid reactive substances (TBARS) and p-anisidine value (AnV) have been developed to quantify secondary peroxidation products, and when used in conjunction with PV, may provide more accurate measures of the degree of lipid peroxidation.

Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances analysis is commonly used to quantify malondialdehyde (MDA). The cytotoxic and mutagenic effects of MDA have been extensively studied. However, this assay is not specific for MDA because other carbonyl-containing secondary lipid peroxidation products may react under the conditions of the assay and inflate the TBARS value (Esterbauer et al., 1991). Regardless, the TBARS assay may provide useful information for evaluating lipid peroxidation because it provides a more accurate measurement of secondary peroxidation products produced during the propagation phase of peroxidation, which are not measured by PV. However, TBARS are also produced and degraded over time during heating, resulting in a bell shaped curve response, similar to that reported for PV (Liu, unpublished data; Figure 6).

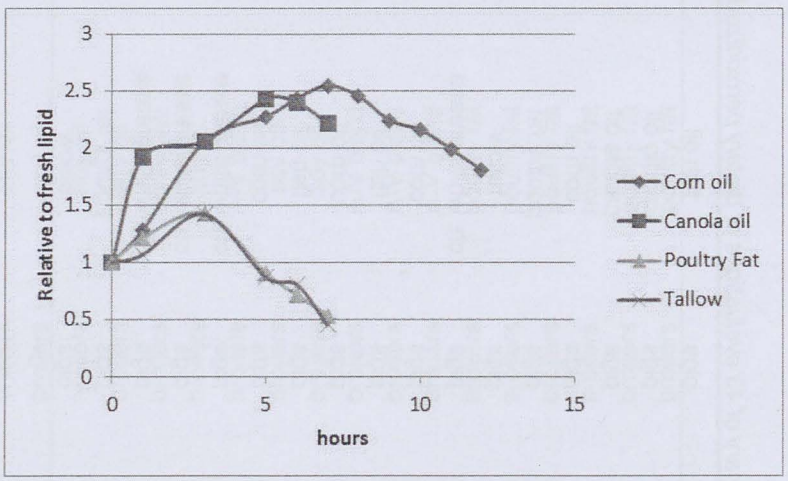

Figure 6. Thiobarbituric acid reactive substances are produced and degraded over time when heated at 185^{0}C (Liu, unpublished data).

Anisidine value

Anisidine value (AnV) is a measurement of the amount of high molecular weight saturated and unsaturated aldehydes in lipids. However, like the PV assay, a single measurement of AnV may not provide reliable information of the degree of peroxidation for lipids exposed to extreme peroxidation conditions because AnV measurements have been described to follow a bell shaped curve as a function of peroxidation time (DeRouchey et al., 2004).

Conjugated dienes

Conjugated dienes are produced during the formation of hydroperoxides from unsaturated fatty acids, and have been used to evaluate lipid peroxidation since the 1960's (Antolovich et al., 2002). This method is simple, fast, and only small amounts of sample are required. Good correlations between conjugated dienes and PV have been found (Wanasundara et al., 1995; Shahidi et al., 1994). However, this method can be less sensitive compared to direct PV analysis (Antolovich et al., 2002; Gordon, 2001). In fact, other compounds such as carotenoids may be detected, and thereby, lead to inaccurate results (Shahidi and Wanasundara, 2008). Therefore, caution must be taken when interpreting lipid peroxidation results using the conjugated dienes assay.

Hexanal value

Hexanal is one of the major secondary lipid peroxidation products generated during the termination phase of peroxidation of linoleic acid (C18:2 n-6) and other n-6 fatty acids. Hexanal content is directly proportional to off-flavors caused by lipid peroxidation (Kalua et al., 2007), and can be easily detected due to its low odor threshold (Van Ruth et al., 2000; Shahidi and Pegg, 1994). However, because hexanal is volatile at high temperatures, a low hexanal value does not necessarily indicate a low level of peroxidation in lipids because hexanal may degrade during peroxidation.

2,4-decadienal (DDE)

The compound 2,4-decadienal (DDE) is a by-product derived from linoleic acid during peroxidation. Compared to the PV, AnV, and TBARS analyses, the DDE assay is more specific, but it is also more complicated and expensive procedure requiring gas chromatography and mass spectrophotometry (Lasekan and Abbas, 2010; Selke and Frankel, 1987; Matthews et al., 1971). Low levels of DDE generate a deep-fried flavor, but excessive amounts of this specific dienaldehyde induce many undesirable effects, including peroxidative stress and proinflammatory reactions in human lung cells (Chang et al., 2005), cellular toxicity in liver and kidney tissues (Hageman et al., 1991), cellular proliferation in gastrointestinal epithelial cells, and carcinogenic effects in the gastrointestinal tract (Hageman et al., 1991). Thus, with respect to the considerable negative impact of DDE on animal health, quantification of DDE may provide valuable information regarding the quality of dietary lipids. However, to date, no research has been conducted to investigate the effect of DDE on growth performance and health status in swine or poultry.

4-Hydroxynonenal (HNE)

4-hydroxynonenal is one of the α, β-unsaturated lipophilic aldehydes formed during lipid peroxidation of n-6 polyunsaturated fatty acids, such as arachidonic and linoleic acid. This compound is well known for its cytotoxic and mutagenic effects (Esterbauer et al., 1991; Witz, 1989). Similar to the DDE assay, measurement of HNE is complicated and expensive (Zanardi et al., 2002). Previous studies demonstrated that the consumption of fat sources containing 4-HNE are likely to increase the oxidative load in the mammalian metabolic system because 4-HNE readily conjugates to glutathione, thus depleting this important antioxidant (Uchida, 2003; Seppanen and Csallany, 2002). Treating cells directly with 4-HNE increases the activation of stress pathways (Yun et al., 2009; Biasi et al., 2006) and alters immune signaling pathways. However, it is not clear if HNE affects growth performance and health status of animals.

Triacylglycerol dimers and polymers

The amount of dimers and polymers formed during lipid peroxidation increases as heating time is increased (Takeoka et al., 1997; Sánchez-Muniz et al., 1993). However, limited information is available regarding measurement and applicability of these compounds for determining the quality and feeding value of lipids used in animal feeds. Therefore, future research should focus on developing methods to quantify triacylglycerol dimers or triacylglycerol polymers and evaluate their relationships with negative impacts on animal health and growth.

Non-elutable material

As previously described, the PV, TBARS, and AnV assays can provide misleading results because highly peroxidized lipids can have relatively low detectable levels of peroxidation products making their individual usage unreliable to indicate the extent of peroxidation. In addition, free fatty acid content has been used to assess damage to lipids used in the human food industry, but this measure is not appropriate to use when evaluating animal feeds because lipids containing free fatty acids are acceptable to use in blends even though they have been shown to contain less energy than triglycerides (Wiseman, 2003). Therefore, additional analytical procedures must be considered to more accurately quantify the extent of peroxidation of dietary lipids. Wiseman (2003) suggested that estimating the total non-elutable material of a fat or oil through quantitative gas-liquid chromatography (Waltking et al., 1975; Edmunds, 1990) is a more meaningful measure of lipid damage. This procedure incorporates a correction for glycerol, which is present following hydrolysis and derivitization of fatty acids, so that it appears in the non-elutable material fraction and is not associated with damaged lipid products. As a result, this method collectively measures most degraded structures within a lipid and provides an indication if the lipid has been excessively heated.

Production of Peroxidation Compounds in Various Lipid Sources

To determine the impact of lipid composition and peroxidation conditions on the development of peroxidation products, corn oil, canola oil, poultry fat, or tallow were heated for 72 h at 95 ^{0}C (slow peroxidation; SO) and sampled every 8 h, or heated 12 h at 185 ^{0}C (rapid peroxidation; RO) and sampled every hour using constant compressed air flow at 12L/min (Liu, 2012). Samples were obtained after peroxidation and analyzed for PV, AnV, TBARS, hexanal, DDE, HNE, PUFA, and free fatty acids (Table 2).

Free fatty acids increased and PUFA content in all lipid sources decreased after heating. However the magnitude of change was different for each lipid source. For example, the PUFA content declined in both corn oil (9%) and tallow (35%) when exposed to RO conditions. The substantial difference in magnitude of change may be linked to initial PUFA content which is relatively greater in corn oil. Interestingly, PV substantially increased in lipids exposed to SO conditions, but levels increased to a lesser extent under RO conditions. This finding may indicate that high temperatures (i.e. 185^{0}C) expedite the catabolism of peroxides. Similar impacts of peroxidation conditions were evident for concentrations of TBARS, hexanal, and DDE. It is important to note, however, that the magnitude of differences elicited by RO and SO conditions varied with each lipid source. For example, the hexanal content of SO corn oil increased by 390-fold relative to fresh corn oil while tallow exposed to similar conditions increased by only 30-fold. Conversely, the magnitude of change relative to fresh lipids was greater for RO compared to SO for AnV and HNE, but only in the vegetable oils. In fact, the AnV and HNE content in lipids of animal origin was greater with SO relative to RO. This finding may indicate an interactive effect of lipid composition and peroxidation conditions on HNE and AnV. However, little research has investigated this connection.

Table 2. Lipid peroxidation measures in original lipids (OL) exposed to slow (SO) or rapid peroxidation (RO) conditions[1].

	Corn oil			Canola oil			Poultry fat			Tallow		
	OL	SO	RO	OL	SO	RO	OL	SO	RO	OL	SO	RO
PUFA	1.00	0.92	0.91	1.00	0.89	0.84	1.00	0.95	0.92	1.00	0.65	0.65
Free fatty acids	1.00	1.71	2.32	1.00	1.58	1.61	1.00	1.01	0.88	1.00	1.56	1.15
Peroxide value	1.00	151.00	2.00	1.00	239.00	12.00	1.00	57.00	2.00	1.00	29.00	3.00
Anisidine value	1.00	61.40	142.90	1.00	37.00	154.80	1.00	29.33	7.33	1.00	30.00	4.75
TBARS[2]	1.00	14.06	7.44	1.00	21.51	13.82	1.00	1.91	0.73	1.00	1.05	0.71
Hexanal	1.00	390.00	83.00	1.00	180.00	59.00	1.00	29.33	7.33	1.00	30.00	4.75
2,4-Decadienal	1.00	51.78	18.68	1.00	155.86	73.00	1.00	14.73	5.63	1.00	5.55	2.66
4-Hydroxynonenol	1.00	194.00	594.00	1.00	105.00	221.00	1.00	2.00	1.00	1.00	13.00	6.00

[1]OL = original fresh lipid; SO = heated at 95 ^{0}C for 72 h; RO = heated at 185 ^{0}C for 7 h. Both treatments maintained constant compressed air flow of 12L/min.

[2]TBARS = thiobarbituric acid reactive substances.

Production of Lipid Peroxidation Compounds Over Time

To evaluate the relative production and degradation of peroxidation compounds over time, corn oil was subjected to RO or SO conditions and sampled hourly or every 8 h, respectively. Free fatty acids, PV, AnV, TBARS, hexanal, and fatty acid profiles were compared at each time point (Figure 7).

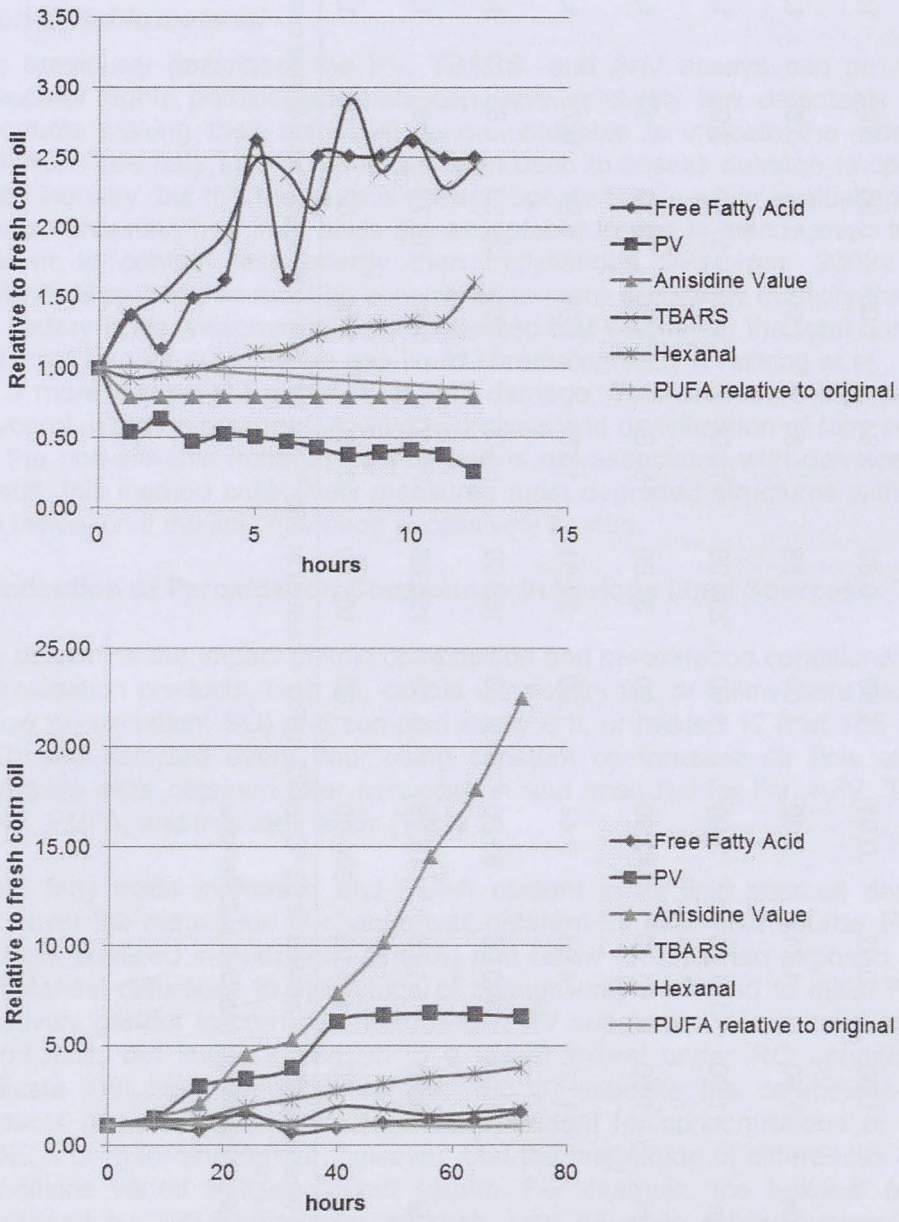

Figure 7. Relative concentrations of measures of lipid peroxidation over time in corn oil heated at 95 °C (top) or 185 °C (bottom) with constant compressed air flow of 12L/min.

Peroxide value of SO corn oil increased by 6.2-fold compared to the level found in fresh corn oil, and plateaued after 40h. However, the PV of RO corn oil declined by 74% by the end of the 12 h heating period. The difference in magnitude and direction of change indicates that PV may only serve as a good indicator of peroxidation for lipids heated at lower temperatures (i.e. 95 ^{o} C), and in the initial stages of peroxidation. The PUFA content in corn oil declined for both RO (16%) and SO (6%) relative to fresh oil PUFA concentrations. The difference in magnitude may suggest that the rate of peroxidation is impacted more by temperature than by time because the SO was heated for a 72 h period, which was 6 times longer than that used for producing RO. Free fatty acid content increased in SO, and RO. However, the magnitude of change after heat treatment was 63% for SO and 250% for RO indicating that temperature is a primary contributing factor to the rate of peroxidation. Similarly, the TBARS content increased for both SO (80%) and RO (244%). Interestingly, TBARS increased at a greater rate initially compared to later in the heating period. The AnV increased dramatically in SO (22-fold), but there was actually an initial decline (21%) followed by no changes AnV with RO.

These preliminary results are consistent with the limited published data (Fitch Haumann, 1993) which show that the relative production of peroxidation products varies with temperature and time. These results also suggest that the selection of the most appropriate peroxidation measure depends on peroxidation conditions (time and temperature) and lipid composition. For example the increase in AnV was relatively constant and dramatic for SO lipids, but AnV actually declined in oil heated at higher temperatures. Our data suggest that PUFA content is a consistent indicator of extent of peroxidation because it declined in corn oil exposed to both high and low temperature. We now need to determine which of these peroxidation indicators can be used to predict animal growth responses.

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