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Determination of Oxidized Lipids in Commonly Consumed Foods and Their Binding Affinity for PPAR γ

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ACCEPTANCE

This thesis, DETERMINATION OF OXIDIZED LIPIDS IN COMMONLY CONSUMED FOODS AND THEIR BINDING AFFINITY FOR PPAR γ , by Joanna Skinner, was prepared under the direction of the Master's Thesis Advisory Committee. It is accepted by the committee members in partial fulfillment of the requirements for the Master of Science in the Byrdine F. Lewis School of Nursing and Health Professions, Georgia State University.

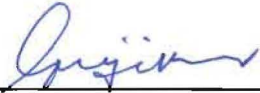
The Master's Thesis Advisory Committee, as representatives of the faculty, certify that this thesis has met all standards of excellence and scholarship as determined by the faculty.



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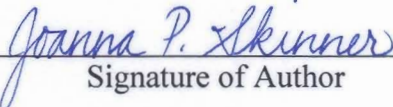
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ABSTRACT

DETERMINATION OF OXIDIZED LIPIDS IN COMMONLY CONSUMED FOODS AND THEIR BINDING AFFINITY FOR PPAR γ

by

Joanna P. Skinner

Background: Foods rich in polyunsaturated fatty acids (PUFA) are susceptible to oxidation through heating or storage. Oxidized lipids are known to act as ligands for a transcription factor (PPAR-gamma) that affects adipocyte differentiation and insulin sensitivity.

Objective: The purpose of this study was to determine the amounts of oxidation products of a variety of PUFA containing foods over time, and to determine whether extracted fats from these foods act as ligands for PPAR-gamma.

Method: To study the effect of room-temperature storage on oxidation, 5 foods (walnuts, sunflower seeds, ground flax, fish oil capsules, and infant formula) were purchased and stored at room temperature for 1, 2, and 3 months. To determine oxidation levels in fried foods, French fries and chicken nuggets were used. Fat was extracted from each food and the levels of oxidation products were analyzed by spectrophotometry and kits designed to measure oxidation products. Using a fluorescence polarization-based ligand screening assay kit, fat extracted from foods was analyzed for its binding affinity for PPAR-gamma.

Results: Among foods stored at room temperature, the levels of oxidation products did not change significantly with time. Most foods exhibited the highest levels of oxidation

at the purchase date. Infant formula and ground flax demonstrated higher levels of oxidation products than did other foods. In preliminary ligand binding assays, extracted fat from French fries showed the greatest binding affinity for PPAR-gamma; a select few other oils showed slight affinity.

Discussion: Surprisingly, storage time did not affect oxidation levels; the greatest amount of oxidation may occur during pre-purchase storage conditions. The processing of formula and ground flax may be the cause of the relatively higher oxidation levels in those foods. The binding affinity for PPAR-gamma demonstrated by French fries needs further investigation.

Conclusion: Certain oxidized lipids from foods may act as ligands for PPAR-gamma. Further research is required not only to determine which component of these PUFA-containing foods activates PPAR-gamma but also to determine whether that component acts as an agonist or antagonist for PPAR-gamma.

DETERMINATION OF OXIDIZED LIPIDS IN COMMONLY CONSUMED FOODS
AND THEIR BINDING AFFINITY FOR PPAR γ

By Joanna P. Skinner

A Thesis

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CHAPTER I

INTRODUCTION

As obesity becomes more common throughout the world, accompanying comorbidities such as diabetes and cardiovascular disease also occur more frequently. According to the World Health Organization (WHO), 10% of the world's population was obese in 2008, and this figure is expected to rise.¹ Further, the WHO attributes 44% of diabetes cases and 23% of ischemic heart disease to overweight/obesity.¹ The WHO estimates that by 2030, 366 million people throughout the world, including more than 30 million in the United States alone, will have diabetes.² In light of the significant effect excessive weight has on global health, scientists are seeking out and investigating an increasing number of factors that may impact weight and health.

As research into dietary fat ingestion has progressed, scientists have found evidence for recommending consumption of a variety of sources of "healthy fats," such as olive oil and walnuts.³⁻⁵ These sources contain primarily unsaturated fats, which are considered less harmful to the cardiovascular system and are sometimes even reported to be cardioprotective, particularly if they are consumed in place of saturated fats in the diet.⁵⁻⁷ One potential problem with unsaturated fats is that they are prone to oxidation and therefore have decreased shelf life compared to saturated fats.^{5,8} In animals, fats oxidized through the frying or other heat-based processing of food have been associated with increased risk of atherosclerosis.⁸⁻¹⁰ It is unlikely that humans would eat oxidized

unsaturated fats in unheated oils and nuts, because oxidation would cause odoriferous rancidity. It is widely known that processing, including heating, of food containing unsaturated fats oxidizes these fats.^{6,11}

Often separate from oxidized lipid research is examination of physiology related to energy balance, weight, and insulin sensitivity. Some of this research centers on a family of nuclear transcription factors known as peroxisome proliferator-activated receptors (PPAR). Though there are 3 PPAR isoforms, of specific concern for adipocyte differentiation and insulin sensitivity is PPAR γ , a receptor found in cells throughout the body but particularly active in muscle and adipose tissue.^{12,13} Recent research indicates PPAR γ in the liver and hypothalamus may exercise important metabolic control as well.¹³ While certain endogenously oxidized lipids are known to act as ligands for PPAR γ , less is known regarding the effects of exogenously oxidized dietary fats on the receptor. The purpose of this study was two-fold: to observe the relative amounts of primary and secondary oxidation products of PUFA-containing products, both between product types and over time, and, primarily, to determine whether oxidized fats act as ligands for PPAR γ . Given the varying fatty acid composition of foods with large amounts of PUFA, the products' tendencies to activate PPAR γ could be expected to vary. This project might therefore indicate the relative potential of certain PUFA-containing products to influence PPAR γ . Thus, the first hypothesis is that as duration of storage or heating increases, the levels of products of oxidation in a food also increase. Secondly, we hypothesize that as the primary oxidation products of an extracted oil increase, the oil's binding affinity for PPAR γ also will increase.

CHAPTER II

REVIEW OF LITERATURE

Lipids and Oxidation

Fats are a necessary component of the diet, and their functions go far beyond provision of calories. Fatty acids are essential structural components of cell membranes. They participate in cell signaling, regulation of gene expression, and control of inflammatory responses; the function depends on the type of fatty acid.^{7,14} PUFA are unsaturated fatty acids containing at least 2 double bonds. Each double bond represents a possible target for hydrogenation or oxidation. The common dietary PUFA are n-6 and n-3 fatty acids, and the correct balance of these two types of PUFA in the diet is the subject of much discussion.^{15,16} It has been reported that n-3 fatty acids may reduce inflammation, lower serum triglycerides, and raise HDL concentrations; however, other studies have shown n-3 fatty acids to actually raise LDL while lowering HDL concentrations.^{3,7,15-17}

Though PUFA in their unoxidized forms may offer health benefits, their structure leaves them vulnerable to oxidizing agents.^{10,18} Oxidation occurs *in vivo* (such as through β -oxidation or oxidation via lipoxygenases and cyclooxygenases) as well as exogenously in fat-containing foods.¹⁸ Due to exposure to heat, oxygen, or light, oxidation in foods can occur during storage, cooking, or other processing.^{10,18,11,19} Increased availability of fried and other processed foods, along with recommendations to replace foods high in

saturated fats with those containing unsaturated fats, has led to high rates of consumption, potentially exposing people to large quantities of oxidized fatty acids.^{6,11} If oxidized fat has the potential to negatively impact health, it may be notable that people with uncontrolled blood glucose seem especially susceptible to absorbing oxidized fatty acids into their chylomicrons.^{6,10}

PPAR γ

PPARs belong to a family of nuclear transcription factors found throughout the body. Three isoforms exist: PPAR α , PPAR β/δ , and PPAR γ .¹⁹ Gene regulation by PPAR is a complex process that begins when a PPAR, following activation by a ligand in the cytosol, forms a heterodimer with a retinoid X receptor (RXR). The heterodimer binds to a gene at the peroxisome proliferator response element (PPRE) in the nucleus, causing a conformational change that allows for activation or repression of the gene.^{12,20}

PPARs are considered adopted receptors, meaning that there are no known endogenous ligands with strong affinity (meaning they act as ligands even at very small concentrations) for them. Ligands with weak-to-moderate affinity (meaning they may act as ligands if presented in high concentrations), including dietary fatty acids and their metabolites, have been identified.^{21,22} PPAR α is present in the liver, though it is found in the muscles (including heart), kidney, intestines, and immune cells. It is involved in lipid metabolism, particularly beta-oxidation and production of ketones.¹⁹ PPAR β/δ is found in tissue throughout the body, though it is reported to be concentrated in the skin, liver, central nervous system, colon, and small intestine; further research into its role in these tissues is warranted, but it is also known to assist in the regulation of lipid metabolism.^{19,23}

PPAR γ participates in regulation of both lipid and carbohydrate metabolism. It has two isoforms, termed PPAR γ 1 and PPAR γ 2.¹⁹ PPAR γ 2 is found mainly in adipose tissue, while PPAR γ 1 is found throughout the body, including in immune cells, skeletal muscle, and hypothalamus.^{12,21,24} Known ligands for PPAR γ include unsaturated fatty acids, thiazolidinediones (TZDs), oxidation products of linoleic acid (13-hydroxyoctadecadienoic acid, 13-oxooctadecadienoic acid, and 9-hydroxyoctadecadienoic acid), 15-hydroxyeicosatetraenoic acid (an oxidation product of arachidonic acid, otherwise known as ARA), non-steroidal anti-inflammatory (NSAID) medications, and oxidized low-density lipoproteins (LDL).^{19,25,26}

PPAR γ is recognized as a mediator of adiposity and insulin sensitivity. When treated with an antagonist, it limits fat accumulation; conversely (and seemingly counterintuitively), when activated by an agonist, such as rosiglitazone, it improves insulin sensitivity while promoting accretion of fat mass.^{21,24} Consequently, the receptor has attracted attention from researchers studying obesity, type 2 diabetes mellitus, metabolic syndrome, and atherosclerosis. Treatment with TZDs, which are PPAR γ agonists, has repeatedly demonstrated improvement of insulin sensitivity in humans and animals with insulin resistance (IR).^{13,19,21,24,27,28} There are several mechanisms by which PPAR γ may affect insulin sensitivity, and because of the variety of study designs and occasionally contradictory results, it is unclear which mechanism is most potent. It has been suggested that because PPAR γ affects expression of adipokines such as adiponectin and vaspin, its activation leads to improved insulin sensitivity.^{13,21}

Recent research indicates PPAR γ might promote leptin resistance (and consequently, weight gain) when activated frequently, such as with consumption of a

high-fat diet or chronic use of TZDs.^{21,24} While adipose tissue differentiation and mediation of insulin sensitivity are the 2 main roles ascribed to PPAR γ , studies also have linked it to atherosclerosis, inflammation, and immune function.^{19,27,29,30}

Oxidized Lipids

A study comparing the impact on murine leptin expression of a high fat, high cholesterol (atherosclerotic) diet without oxidized lipids to the same diet with oxidized linoleic acid showed the diet containing oxidized fatty acids significantly increased the animals' leptin expression.⁹ Though leptin is commonly associated with appetite regulation, with production increased as animals reach satiety, it may have other functions as well. For example, this study showed a significant positive correlation between leptin levels and development of atherosclerotic lesions in the animals' aortas.⁹ It is not known whether the oxidized fatty acids affected leptin concentration by influencing PPAR γ 's activation, but because PPAR γ plays a significant role in adipose accumulation, and leptin is generated by adipose, the possibility of a connection appears to exist.^{9,13}

Ringseis and colleagues explored the *in vivo* binding of exogenously oxidized dietary fats with enterocyte PPAR γ in pigs to determine whether the fat could have a pro-inflammatory effect [defined as increased expression of nuclear factor-kappa B (NF- κ B)] on the animals.²⁵ For 28 days, the pigs were fed a diet containing either fresh oil or oil heated for 24 hours at 200°C. Analysis of the experimental group's enterocytes showed that the exogenously oxidized oil did activate PPAR γ , though only moderately, and no change in NF- κ B expression was noted, suggesting that oxidized oils are not pro-inflammatory.²⁵

In a two-part study of the effect of oxidized linoleic acid on atherosclerosis in LDL receptor knockout mice, Khan-Merchant *et al* found that enzymatically oxidized linoleic acid increased aortic lesion development, serum cholesterol levels, or both.³¹ The authors noted that because only linoleic acid was used in both parts of their study, it was not possible to determine whether other oxidized fatty acids would be similarly atherogenic. The authors noted that the animals consuming the high oxidized fat diet gained less weight compared to those consuming the unoxidized oil.³¹ Similarly, Chao and colleagues found that in mice and rats, a diet containing heated frying oil led to weight and fat pad mass loss but led to glucose intolerance.³²

Though PPAR γ is known to play a role in adipocyte differentiation and insulin sensitivity, and despite the evidence that exogenously oxidized dietary fats can be absorbed to some degree, no studies have definitively answered the question of whether oxidized lipids act as ligands for PPAR γ in humans. Knowing the answer to this question could be a preliminary step in research leading to improved dietary recommendations for people at risk of or currently dealing with obesity or insulin resistance.

CHAPTER III

METHODS

All foods chosen for this project contain PUFA. PUFA contain more than one double bond; hence, they are more susceptible to oxidation than are saturated or monounsaturated fatty acids.^{7,17} Though it does not appear to fit with the other foods selected for this research, infant formula supplemented with DHA and ARA was one of the items chosen for examination, as DHA and ARA are PUFAs which are considered important for normal cognitive development in young children.³³ Table 1 illustrates the PUFA content relative to total fat content of our chosen foods.

TABLE 1: Fat amounts (including PUFA content) in commonly-consumed foods selected for testing

Food	Mean Amount Fat per 100g Food (g)	Mean Percent PUFA in Food (%)
Walnuts	65.21*	72.3*
Ground Flaxseed	42.16*	68.14*
DHA/ARA-fortified Infant Formula*	24.52	20.96*
Fish Oil Softgels	100	40.00
Sunflower Seeds	51.46*	44.96*
Fried Chicken Nuggets	18.82*	28.26*
French Fries	16.51	38.57*

*Fat content was not available from all companies; therefore, information from the USDA's National Nutrient Database for Standard Reference (Release 24) was included in these averages. Except in the case of fish oil, PUFA content was only available from the USDA.³⁴

Purchasing and Storing the Foods

The initial step in this project was to determine the effect of time on the development of both primary and secondary oxidation products. The intention was to observe whether, with more extensive oxidation, they became better ligands of PPAR γ . Three brands of 5 preselected foods were purchased as close in time as possible on the same day with the purpose of being stored at room temperature. All fried foods were purchased on the same day (a separate day from the other foods) from the same 3 fast food establishments. Samples of walnuts, sunflower seeds, ground flaxseed, infant formula, and fish oil softgels [room temperature foods (RTF)] were placed into airtight plastic tubes and stored under nitrogen at -80°C to prevent further oxidation; no weights were taken at this phase.

Changes in oxidation products were measured at 4 separate time periods. After storage of the first set of samples, RTF were left in their original packaging (sealed, to replicate conditions in a consumer's home) at approximately 22°C for 1 month. At the end of that month, 3 more tubes of each product were placed in the -80°C freezer, with the remainder of the RTF left in the original packaging. This process was repeated at 2 months and 3 months from the purchase date. For the French fries and chicken nuggets, on the day before extraction (detailed below), the foods were purchased, pulverized in a coffee mill (Braun, Kronberg, Germany), and stored under nitrogen at -80°C until immediately prior to extraction. Further oxidation was not intentionally induced.

Extracting the Oil

Prior to extraction, foods were thawed briefly and ground in a coffee mill when not already in ground or oil form. Two portions each of the walnuts, sunflower seeds,

ground flaxseed, and fish oil were weighed out; each portion weighed approximately 1.5 g. Because fat is a much lower percentage of weight of formula and fried foods compared to nuts, seeds, and fish oil, a larger initial amount of food was required for those products. Like the other samples, these were weighed out 1.5 g at a time. With the exception of the chicken nuggets, all oils were extracted from foods using a solvent containing hexane and isopropanol (HIP) in a 3:2 ratio according to the modified Hara and Radin method³⁵. For the chicken, the solvent contained chloroform and methanol in a 2:1 ratio using a modified Folch method³⁶. Food samples were mixed with the appropriate solvent (either HIP or chloroform-methanol) using a Poly Tron homogenizer (Kinematica AG, Lucerne, Switzerland). In order to prevent further oxidation, each portion of sample with its respective solvent was kept on ice or refrigerated while other portions of the same sample were homogenized. When all portions of a given sample (for example, all portions of Room Temperature Brand A walnuts) were homogenized, the homogenate was combined and filtered through grade 1 filter paper (90 mm) (Whatmanplc, Kent, UK) in a Buchner funnel into a filter flask. The filtrate was removed from the flask into a graduated cylinder, and the flask was subsequently washed with additional solvent to remove any remaining filtrate; the solvent was added to the cylinder containing the rest of the filtrate.

The filtrate was transferred via Pasteur pipette to a separatory funnel. Five ml of 6.7% NaSO₄ was used to wash the graduated cylinder, and it was then transferred to the separatory funnel with a pipette. The separatory funnel was shaken to thoroughly mix the contents and left untouched for 10 minutes to allow separation of the fat layer from the water layer. An additional 5 ml of NaSO₄ was added for every 1.5 g portion of sample;

each 5 ml was added separately, and each addition was followed by agitation of the funnel and a 10-minute wait for separation. The fat layer was removed by draining the separatory funnel when possible; when it was too viscous to flow out of the funnel, it was removed from the funnel with a pipette. The fat layer was poured into a previously weighed test tube, which was then placed in a 40°C water bath in a nitrogen evaporator (N-Evap; Organomation Associates, Inc., Berlin, MA) to allow the hexane to evaporate. Following evaporation, the test tube was dried carefully and weighed again to determine the weight of extracted oil. Finally, 100 µl aliquots of each sample were weighed, and all extracted oil was stored under nitrogen at -80°C.

Heating the Soy Oil

Unlike the RTF, oxidation of the soy oil (Welch, Holme& Clark Co., Inc., Newark, NJ) was promoted by heating rather than storage at room temperature. Prior to heating, approximately 200 ml of oil was stored under nitrogen at -80°C. The rest was heated until it reached 195°C. Upon reaching 195°, the temperature maintained for the duration of the heating process, the oil was heated for 3 hours, and approximately 200 ml was removed. The remaining oil was heated for an additional 3 hours, and another 200 ml removed. The last portion was heated for another 3 hours, bringing the total heating time of this portion to 9 hours. After its removal from the heat, each portion of oil was cooled for 5 minutes and then stored under nitrogen at -80°C until analysis. Oxidation products in the soy oil were measured in the oil's fresh and extracted (using the extraction process described above) states.

Measuring Products of Oxidation

The initial products of oxidation are peroxy and hydroxyl fatty acids, molecules that degrade into secondary products such as ketones, aldehydes, and hydrocarbons; it is expected that at the start of oxidation, primary oxidation products will form faster than they degrade.³⁷ That peroxides are so short-lived makes it difficult to quantify the extent of oxidation, particularly without the measurement of other, less transient, oxidation species, such as hydroxy fatty acids.¹⁸ Therefore, to get a more complete sense of the extent of oxidation, levels of both primary and certain secondary products of oxidation were measured in all samples.

Primary Products of Oxidation

Assays for peroxides and conjugated dienes (CD) determine the presence of primary products of oxidation. Assays for detecting CDs are sensitive and require very small quantities of sample, making them appealing when little oil is available for use.¹⁸ To prepare the samples, the sample oil was solubilized in hexane to create a 1% solution; this solution was diluted as necessary by a process of trial and error to achieve spectrophotometric readings in the target absorbance range of 0.2-0.8 at 234 nm. Hexane was used as the blank. Because hydroxy and peroxy fatty acids—primary products of oxidation—have CD structures that demonstrate maximum UV absorbance at 234 nm, the oil samples were read at that wavelength in an Epoch spectrophotometer (BioTek, Winooski, VT, USA). CD levels are expressed as absorbance of a 1% solution at 234 nm ($E_{234}^{1\%}$); however, the calculation necessary for expressing them this way requires knowing the extinction coefficient of the sample, which itself requires knowledge of the specific

fatty acid composition of each sample.¹⁸ Because this information was not available for this project, CD levels were expressed in units of raw absorbance.

The concentration of peroxide values, another common measurement of primary products of oxidation, was determined for each sample using PeroxySafe kit (SafTest, MP Biomedicals, Solon, OH, USA). This kit was used in place of the official American Oil Chemists Society (AOCS) method to simplify the process of measuring peroxide values. Though not all correlation studies agree, some^{38,39} indicate that PeroxySafe kits correlate well with the official AOCS method as well as another common method known as the ferrous oxidation/xylene orange (FOX) method. After running the standard curve, samples were prepared for reading according to the manufacturer's recommendations and then read in an Epoch spectrophotometer at 570 nm and 690 nm. The absorbance of each sample at 690 nm was subtracted from the corresponding result at 570 nm to yield the actual absorbance value. The concentration of peroxides was calculated by putting each of the absorbance values into the linear equation from a calibration curve. Each observed value was multiplied by its corresponding dilution factor to yield the concentration of peroxides (measured in mEq/kg oil). To determine the concentration in a serving of food, the concentration in one kg of oil was multiplied by the kilograms of fat in a single serving.

Secondary Products of Oxidation

Conjugated trienes (CT) are secondary products of oxidation which exhibit maximum absorbance at 268 nm. The absorbance values for these were read at the same time as the CD, allowing for the conservation of sample; all assays were run in triplicate. Levels of CT were expressed as absorbance at 268 nm ($E_{268}^{1\%}$).

Another secondary oxidation product is malondialdehyde. The process of measuring the level of malondialdehyde in the samples was similar to that of determining peroxide values. In this case, the kit used was the SāfTest AldeSafe kit (which is specific for malondialdehyde, unlike other commonly-used tests, and has been shown to be well-correlated with the AOCS official method of determining aldehyde levels⁴⁰), and as with the PeroxySafe assays, all assays were prepared according to the protocol recommended by the manufacturer. Controls and samples were read at both 550 nm and 690 nm. The absorbance was calculated by subtracting the measurement at 690 nm from that at 550 nm. Each absorbance was inserted into the linear equation generated from the calibration curve; the result was multiplied by the dilution factor and expressed in mg/kg oil.

Determining Binding Affinity to PPAR γ

To determine the binding affinity of the extracted fat samples to PPAR γ , a fluorescence polarization-based PPAR γ ligand screening assay kit (Cayman Chemical, Ann Arbor, MI) was used. This kit was designed to allow the user to conveniently screen substances, such as oil samples, to observe whether they contain ligands for a specified protein (in this case, PPAR γ). To prepare the extracted fat samples for screening, four concentrations of each (all time periods included) were prepared: 1%, 0.1%, 0.01%, and 0.001%. The solvent used for this process was dimethyl sulfoxide (DMSO). A 384-well plate was prepared according to the manufacturer's protocol, and all assays were performed in triplicate. In accordance with the manufacturer's protocol, the plates were read at 450 nm in a Victor³ spectrophotometer (Perkin Elmer, Waltham, MA, USA). A standard curve was read concurrently with the samples and percent binding of samples was calculated using the standard curve.

Calibration curves with a normal dynamic range were not obtained on the same days as binding activity of sample oil was observed. Because it was not possible to attempt the assay again before completion of this project, data from separate assays were used to estimate binding activity of the sample oil. It is understood that this assay will need to be attempted again in the future to test the legitimacy of the results. Further, after the first assay failed to show binding affinity of the fresh (unextracted) soy oil for PPAR γ , the oil was extracted in an attempt to hydrolyze more of the triglycerides in the oil and determine if doing so affected the oil's binding affinity.

Data Analysis

All data were analyzed with SPSS, version 18.0 (IBM Corporation). The Kruskal-Wallis and Kolmogorov-Smirnov Z tests were used to compare means among and between time periods and products for non-normal data. For normal data, ANOVA was used to compare means. Post-hoc tests included the Tukey honest significant differences (HSD) for samples with homogenous variance, and Dunnett's T3 for samples with heterogeneous variance. For the comparison of RTF at purchase date to each other and to fried foods, peroxide data were log-transformed.

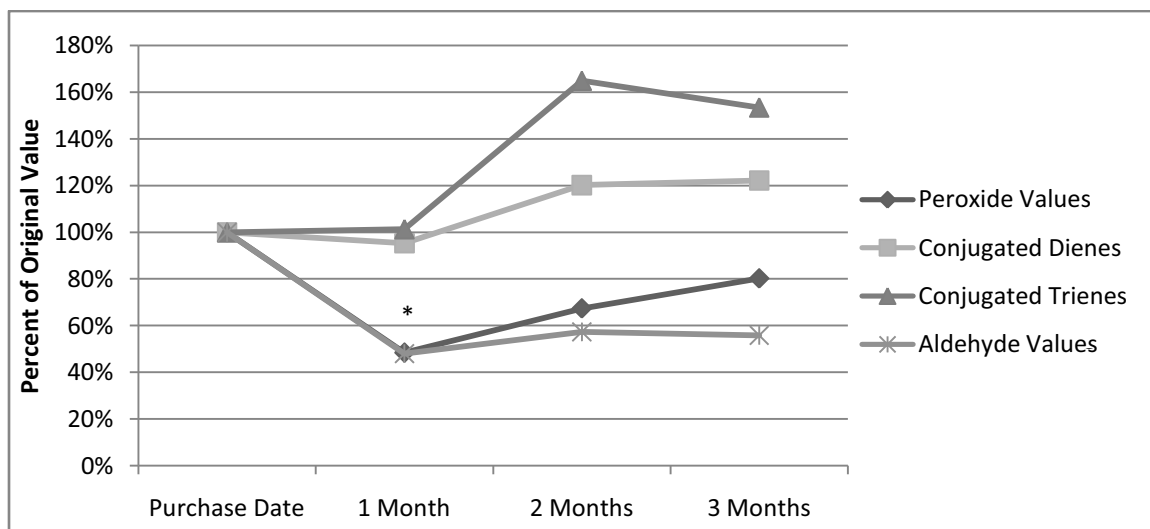
CHAPTER IV

RESULTS

Amounts of Oxidation Products in All Foods

Figure 1 illustrates the effect of time on the level of each type of oxidation product. Mean oxidation amounts were aggregated for all RTFs at each time point to illustrate the change in primary and secondary products over time. Among the RTF, aggregated peroxide and aldehyde values were greatest on the date of purchase. The levels for peroxides and aldehydes were significantly higher ($p=0.013$ and $p=0.038$, respectively) at the purchase date than after 1 month of storage; there were no other significant differences between time periods.

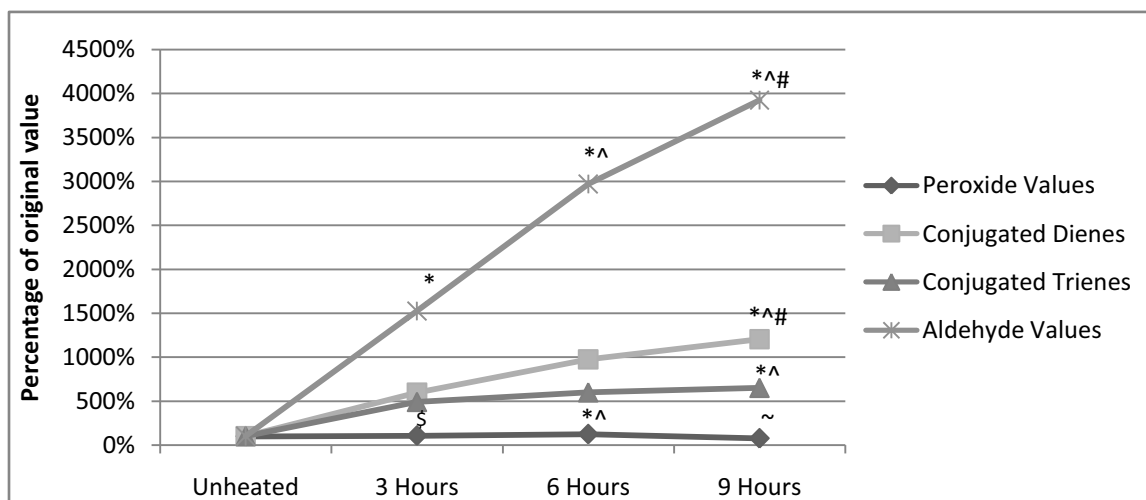
FIGURE 1: The effect of time on the oxidation of Room Temperature Foods



* Peroxide values and aldehyde values significantly lower at 1 month compared to purchase date ($p \leq 0.05$)

By contrast, there was a distinct pattern of increasing products of oxidation in the soy oil (Figure 2). The soy oil used in this project was aged on a different timeline (hours instead of months) and exposed to a different method of oxidation (heating) than the other products, so it was not compared directly to the room temperature foods. As heating time increased, there was a corresponding, non-significant increase in the level of all products of oxidation except peroxide values. The peroxide levels increased from the unheated state until the 6-hour mark; by the final measurement at 9 hours, the level was below the starting point. There were significant differences between oxidation levels at each time period in both peroxides and aldehydes. Percent changes in oxidation levels are illustrated in Figure 2.

FIGURE 2: The effect of heating duration on each product of oxidation in fresh soy oil



* Significantly higher than unheated stage ($p \leq 0.05$)

^ Significantly higher than 3 hour stage ($p \leq 0.05$)

Significantly higher than 6 hour stage ($p \leq 0.05$)

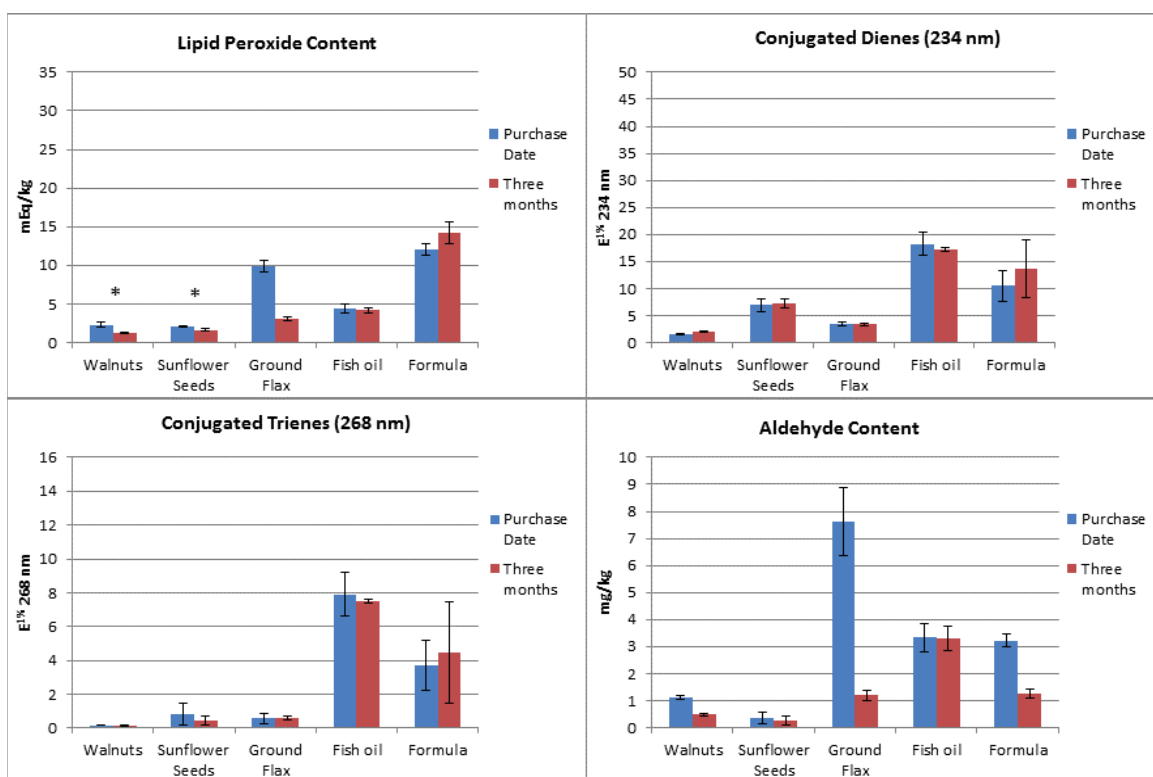
\$ Significantly lower than unheated stage ($p \leq 0.05$)

~ Significantly lower than each preceding stage ($p \leq 0.05$)

As shown in Figure 3, there was not a consistent pattern in the levels of oxidation products—regardless of type—between foods. In both walnuts and sunflower seeds, there was a significant difference ($p=0.01$ and $p=0.034$, respectively) in the level of

peroxides and aldehydes between the date of purchase and 3 months of room temperature storage. There were no significant differences in oxidation products between any two foods at any one time period.

FIGURE 3: Comparison of levels of oxidation products in room temperature foods at purchase date and 3 months



Significant differences are denoted by *. Small sample sizes and non-normality of data may have influenced appearance of significance between purchase date and three months of storage. Fried foods and soy oil are not represented because prior to the assays, they were not systematically oxidized at room temperature as the above foods were.

For the purposes of this project, changes in the levels of oxidation products of chicken nuggets and French fries (“fried foods”) could not be measured. As a result, mean levels of oxidation for each brand of food were compared to each other and to the brands of the other fried food; they also were compared to the oxidation levels of RTF at purchase date, since the fried foods’ oxidation levels were also measured essentially on

the date of purchase. A comparison of levels of oxidation products in French fries and chicken nuggets can be found in Figure 4.

FIGURE 4: Oxidation product levels for each type of fried food analyzed

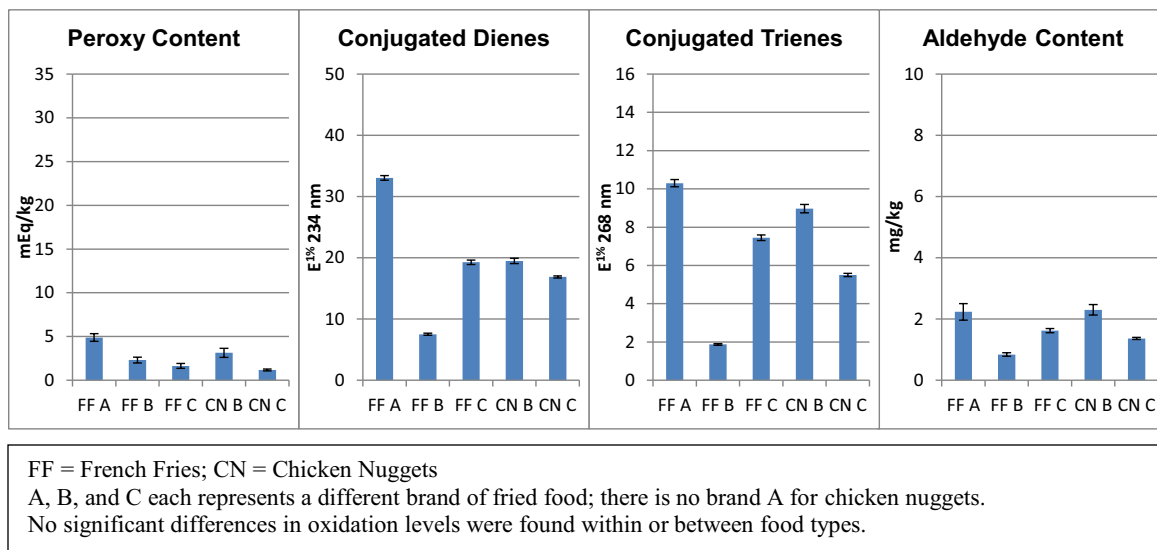
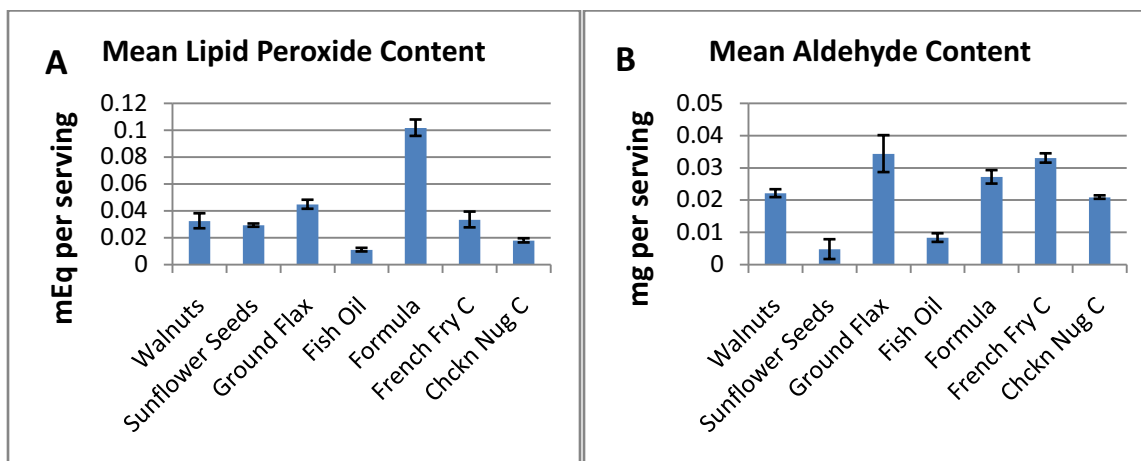


Figure 5 shows a comparison of peroxide and aldehyde levels in servings of fried foods to those in servings of RTF on date of purchase. Levels of peroxides in formula were significantly higher than all other foods represented here ($p \leq 0.01$). Peroxide levels in fish oil were significantly lower ($p \leq 0.01$) than those in all other foods. Beyond this, there were significant differences in peroxide values between most (but not all) other foods as well. No significant differences in aldehyde levels were found between foods. However, though it did not demonstrate higher peroxide levels relative to oil from other foods, oil extracted from fried foods did contain higher aldehyde levels.

FIGURE 5: Levels of peroxide values (A) and aldehydes (B) per serving of each food on purchase date



One brand of each fried food was selected for comparison to the RTF.

A – Levels of peroxides in formula were significantly higher than in all foods represented here ($p \leq 0.01$). Peroxide levels in fish oil were significantly lower ($p \leq 0.01$) than all other foods.

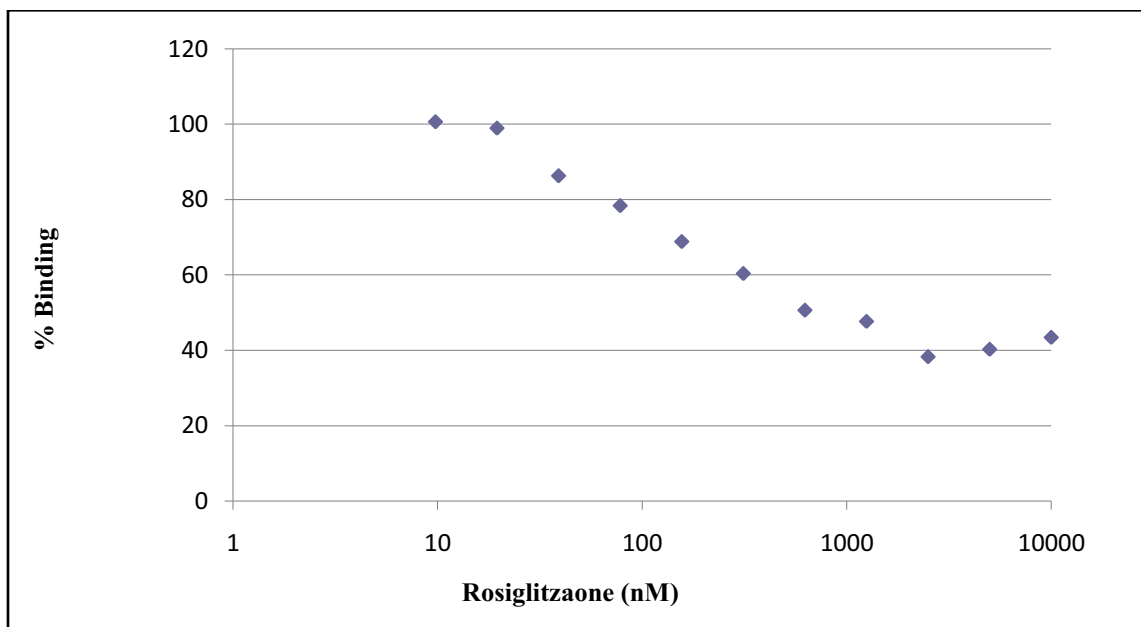
B – No significant differences in aldehyde levels were found between foods.

PPAR γ Ligand Screening Results

Several samples were screened for potential PPAR γ binding activity using a ligand screening assay kit. In this type of assay, a small fluorescent probe is bound to PPAR γ before the sample is introduced; if a component of the sample solution is a stronger ligand for the receptor than is the probe, the probe will be displaced. The percent binding determined through the use of this kit refers to the amount of probe that remains bound to PPAR γ at the time of reading, and thus the lower the percent binding, the higher the binding of a component of the sample. Because these assays were preliminary in nature, only a few samples were screened each time. The assay was later re-tried, again with a variety of foods, and the French fry oil showed the greatest binding affinity.

A few extracted oils from the first assay—particularly from the French fries (again, though not as strong an affinity as was seen in the second assay), the unheated soy oil (0.001% concentration), soy oil heated 6 hours (0.001% concentration, soy oil heated 9 hours (both 0.01% and 0.001%), and walnut oils (all concentrations)—also showed a slight binding affinity compared to the other oils. Figure 5 and Tables 3 and 4 show the calibration curve and the binding affinity of samples, respectively.

Selected products whose extracted oil was measured in the second assay are included in Table 4 for purposes of comparison. Oil extracted from French fries and unheated extracted oil (0.1% concentration) exhibited stronger binding affinity for PPAR γ than did the oils extracted from other foods on the same day. The binding strength of the French fry oil lessened as the oil concentration decreased with dilutions. Infant formula was included because of its relatively high levels of oxidation products compared to other foods.

FIGURE6: PPAR γ ligand binding assay calibration curve demonstrating percent binding

Normal calibration curve from assay on 10/25/2011. Rosiglitazone was used as the control.

TABLE2: Percent binding of extracted oil from foods and soy oil as determined by PPAR γ ligand binding assay

Food Type	Concentration:	% Binding		
		0.1%	0.01%	0.001%
Fresh Soy Oil – Unheated		95.78	99.42	88.83
Fresh Soy Oil – Heated 3 Hours		96.88	98.37	86.78
Fresh Soy Oil – Heated 6 Hours		96.39	91.51	94.48
Fresh Soy Oil – Heated 9 Hours		95.10	86.12	87.55
French Fry C		88.97	82.27	86.42
Walnut B – Purchase Date		90.43	87.27	84.33
Walnut B – Stored 3 Months		90.19	87.63	83.13

Foods from assay on 10/25/11; of all the foods tested, only these showed binding. Minimal binding was demonstrated.

TABLE3: Percent binding of extracted oil from selected foods and soy oil as determined by PPAR γ ligand binding assay

Food Type	Concentration:	% Binding			
		1%	0.1%	0.01%	0.001%
Extracted Soy Oil – Unheated		98.56	63.62	99.55	104.46
French Fry C		24.71	45.62	67.31	69.46
Formula – Purchase Date		98.35	97.06	100.7	103.9
Formula – 3 Months		103.46	104.42	101.78	96.81

Foods from PPAR γ assay on 12/13/11. Only the unheated soy oil and French fries demonstrated binding. Formula is included here because it showed the greatest amount of lipid oxidation of all foods.

CHAPTER V

DISCUSSION AND CONCLUSION

With regard to the oxidized oils, it was hypothesized that as room temperature storage or heating time increased, levels of all products of oxidation would increase. Further, it was hypothesized that the greater the level of primary products of oxidation, the greater the amount of binding affinity an extracted fat would demonstrate for PPAR γ .

While the soy oil showed the expected “classical” (containing the lowest level of oxidation products at the start and increasing over time) trend for all products but peroxides, the products stored at room temperature did not consistently demonstrate such a pattern. Peroxides are rapidly converted to more stable compounds, and this may happen at least as quickly as they form⁴¹; over time, this can cause a decrease in peroxide levels.¹⁸ This is a possible explanation for the trend in the soy oil as well as the RTF (with the exception of the infant formula). Because CDs represent both hydroxy fatty acids as well as peroxy fatty acids, and hydroxy fatty acids degrade to secondary oxidation products more slowly than do peroxy fatty acids, CD levels increase more steadily than peroxides alone.

Another factor affecting oxidation in a food is the amount of antioxidants present in the food. Many of the products analyzed for this project are known to contain vitamin E (α -tocopherol), a common antioxidant. Multivariate regression was used to find the Pearson correlation between vitamin E content and the level of each oxidation product at

purchase date. One significant association was found: controlling for PUFA concentration, vitamin E content had a -0.937 correlation ($p=0.037$) with peroxide levels at purchase date. Thus, greater vitamin E content might, as expected, be associated with lower oxidation levels.

One obstacle encountered in the measurement of peroxide values in particular was difficulty in obtaining a consistent calibration curve when using the PeroxySafe kits. Foo *et al* compared several SāfTest kits for the measurement of oxidation products in frying oil to the comparable American Oil Chemists' Society (AOCS) methods of measuring the same products of oxidation.⁴⁰ The authors noted that the PeroxySafe kit was prone to substantial variation in results and was not well-correlated with the AOCS method; though contrary to some studies^{38,39}, this finding is supported by others.⁴² By contrast, the AldeSafe kit, which was found in the course of this project to be stable and reliable, was found by Foo and colleagues to have the same characteristics; they also noted its high correlation with the official AOCS method of measuring aldehyde levels in heated oil.⁴⁰

In any food, there is a mixed fatty acid content; the composition differs considerably between products.³⁴ Though the sample oils could not be examined at the molecular level to determine the FA content of each, there is a reasonable chance that the differing fatty acid content contributed to each sample's susceptibility to oxidation. For example, of the products analyzed for this project, ground flax had the largest amount of 18:3 fatty acids per 100 g of food, both as an absolute number and as a percentage of PUFA.³⁴ This likely explains why flax had higher aldehyde levels than any of the other foods in this project, as malondialdehyde levels are highest in foods with large amounts

of fatty acids containing 3 or more double bonds.⁴³ By definition, CTs can only form from products with ≥ 3 double bonds, so it was no surprise that these also were found in larger amounts in flax than in the other RTF.¹⁸

To add to the analysis of oxidation levels, it would have been interesting to know the pre-purchase storage conditions (particularly including duration, temperature, and light exposure) of the products to be stored at room temperature. That the levels of peroxide and aldehyde content on the date of purchase were so often as high as, if not higher than, the levels at 3 months of storage, suggests that there is considerable opportunity for oxidation between harvesting or manufacturing and purchase date. Given that so many of the products chosen for the room temperature storage portion of this project were packaged in clear or light-colored plastic packaging, it is likely that these products were highly susceptible to temperature and light-related oxidation.

As stated previously, the fried foods presented a special case for analysis, because it was not possible to determine the type of oil, nor the age, number of uses, or frying temperature to which the oil and foods fried in it were subjected. Research indicates that each of these factors plays a role in the extent of oil decomposition.⁴² The type of food being cooked in the oil also may influence the rate of oxidation.⁴¹ Given the many factors and their likely complex interactions, it is not surprising that significantly different levels of oxidation products were not observed within or between foods. Notably, the levels of oxidation products (all types) per 100 g of fried foods did not differ significantly from the levels in 100 g of other foods. However, given that the aldehyde values of these foods were higher relative to the RTF than were their peroxide levels, it is possible that the oil

might have been old or used several times, thus demonstrating that its primary oxidation products had already mostly degraded to secondary products.

Fried foods were not given opportunities to oxidize after purchase; they were frozen immediately after purchase and their oxidation levels measured because fried foods are typically eaten immediately after purchase. This is in contrast to the RTF used in this project, which may be stored for weeks or months at room temperature. As Figures 3 and 5 show, infant formula consistently demonstrated some of the highest levels of oxidation products—particularly peroxides—of all foods. Similarly, fish oil and ground flax also had higher levels of oxidation products relative to other foods. This may be due to the processing used in manufacturing these items. The oxidation of formula is of particular interest because it has been suggested that preterm infants who consume formula supplemented with DHA and ARA have increased adiposity later in childhood compared to children consuming formula without the added fatty acids.⁴⁴

PPAR γ Ligand Screening Assay

Of the oils tested in the preliminary assays, oil extracted from French fries demonstrated the strongest binding affinity for PPAR γ . Though it cannot be truly compared to the binding of the other oils, as they were assayed on a separate day, extracted French fry oil assayed on the second day showed a much stronger affinity than the other oils. This may be due to its specific fatty acid composition, but because the assay used to determine binding is only a ligand screening assay, it was not possible to determine which fatty acid(s), and more specifically, which type of oxidized lipid, within the extracted sample bound most strongly. As there is little evidence for the binding of

secondary oxidation products, it is likely that primary oxidation products did most of the binding.

A potential explanation for the lack of binding demonstrated by most foods is that even if oxidized fatty acids activate PPAR γ , large amounts of them may be required for this purpose.¹⁹ If this is the case, it would indicate that PPAR activation due to dietary lipids would require a person to eat considerable amounts of food containing oxidized PUFA. The fact that formula did not demonstrate this seems to conflict with previously-mentioned assumptions about infant formula based on its relatively high oxidation levels compared to other foods. However, it may be that if oxidized formula has the potential to cause fat accumulation in infants, there could be other associated factors besides its interaction with PPAR γ . Alternatively, it could be that the process of extraction left many triglycerides intact; though it is speculated that whole triglycerides may be able to act as ligands, individual free fatty acids (not attached to the glycerol backbone) are more commonly thought to fill the role. To explore this further in the future, alkali hydrolysis could be used as part of the extraction process to ensure more complete breakdown of triglyceride molecules; the primary drawback to this step is its potential to promote further oxidation.

The influence of PPAR γ on gene transcription varies depending on whether the ligand binding acts as an agonist or antagonist.^{21,24} Further, the binding affinity of a ligand does not always correspond to the level of its functional activity. The activation potential of exogenously oxidized oils, such as those included in this project, therefore will need to be elucidated through future research, such as cell culture-based PPAR γ transactivation assays.

Conclusion

It is important to determine the levels of oxidized lipids in foods, because they might influence adipose tissue metabolism and thus the risk of diabetes and CVD. This project showed that commonly consumed foods contained oxidized lipids on the day of purchase—often in higher amounts than are seen as they age. Processed foods had greater amounts of oxidized lipids compared to unprocessed foods. This underscores the need for further research into the consumption patterns of oxidized lipids and how they influence health, because consumption of processed foods is on the rise globally.

PPAR γ activation mediates multiple metabolic pathways in the immune system, cardiovascular system and the adipose tissue. Drugs that activate PPAR γ are currently used to treat diabetes. However, this treatment is associated with undesirable side effects, including weight gain^{21,24} and edema⁴⁵, among others. Therefore, there is a great deal of interest in identifying food components that act as PPAR γ ligands with therapeutic properties. This project demonstrated for the first time that lipids extracted from certain foods have an affinity for PPAR γ . Further research is required not only to determine which component of these PUFA-containing products activates PPAR γ but also to determine whether that component acts as an agonist or antagonist for PPAR γ . Only then can a reasonable guess about these products' effects on adiposity and insulin sensitivity be made.

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