Safety of Animal Fats for Biodiesel Production: A Critical Review of Literature

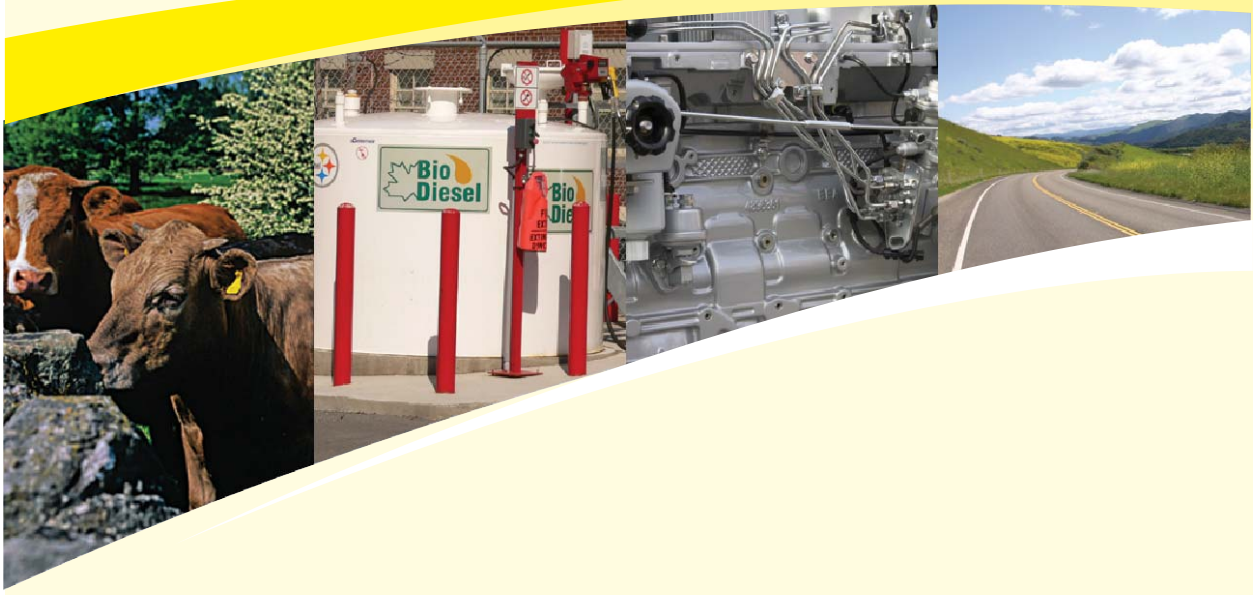

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Table of Contents

List of Figures and Tables

EXECUTIVE SUMMARY

An in-depth review of available literature was conducted on the safety of using animal fats for biodiesel. The review indicated little or no known risk to human and animal health and to the environment relative to inherent microbial, organic or inorganic agents in animal fats destined for biodiesel production.

Animal by-products are generated from the inedible tissues derived from meat, poultry and fish production. This material is thermally processed by the rendering industry to generate a number of industrial materials including use of the fat portion to produce biodiesel. As the biodiesel industry continues to develop, questions have emerged about the safety of animal versus vegetable fats for biodiesel production and utilization. The following report is the result of a detailed literature search into the potential microbial, organic, and inorganic contaminants that may be present in animal fats and the potential for human or environmental safety issues associated with each. The potential safety risks associated with prions are discussed in a separate report, "Biodiesel from Specified Risk Material Tallow: An Appraisal of TSE Risks and their Reduction".

In certain instances, very little was reported about the potential contaminating moiety and its fate in biodiesel production and usage. Establishing an absolute zero risk assessment is impossible on any fat utilized for biodiesel production. Among the potential microbial contaminants, bacteria, viruses, fungi, yeast, parasites, and microbial toxins were considered. In each instance, the nature of the production process and usage of biodiesel via combustion reduce the possibility that microbial contaminants would be a cause for concern to humans, animals, or the environment. Potential organic moieties contaminating the fat should meet a similar fate. Current evidence suggests that metals and metalloids within animal fats will not cause significant safety issues in the production and use of rendered fat-based biodiesel since metallic contamination of animal fats is low.

According to currently available literature, a very low risk of hazard exists from use of animal fats and oils for the production of biodiesel. A number of safeguards mandate the quality of animal fats and oils used in foods, feeds and industrial products. The chemical synthesis of biodiesel as well as the ultimate combustion of the product enhances these safeguards to prevent any potential harm to human, animal or environmental health.

1 INTRODUCTION

1.1 Use of Fats for the Production of Biodiesel

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Inedible animal tissues can account for up to 45% or more of the slaughtered food animal. Organs, integument, ligaments, tendons, blood vessels, feathers, bone, and other materials are collected as inedible co-products and submitted to rendering facilities. In the United States alone, approximately 54 to 55 million pounds of raw rendering materials are generated annually. Raw animal co-products contain approximately 50% water, 25% protein and 25% fat (Pearl, 2004). In recent years, it has been realized that the fat portion of the rendered products may be used in the production of biodiesel. Although an emerging market, biodiesel generated from animal and plant fats and oils has potential as an environmentally friendly renewable fuel to replace a portion of petroleum based fuels currently used for Earth's energy needs (Srivastava and Prasad, 2000).

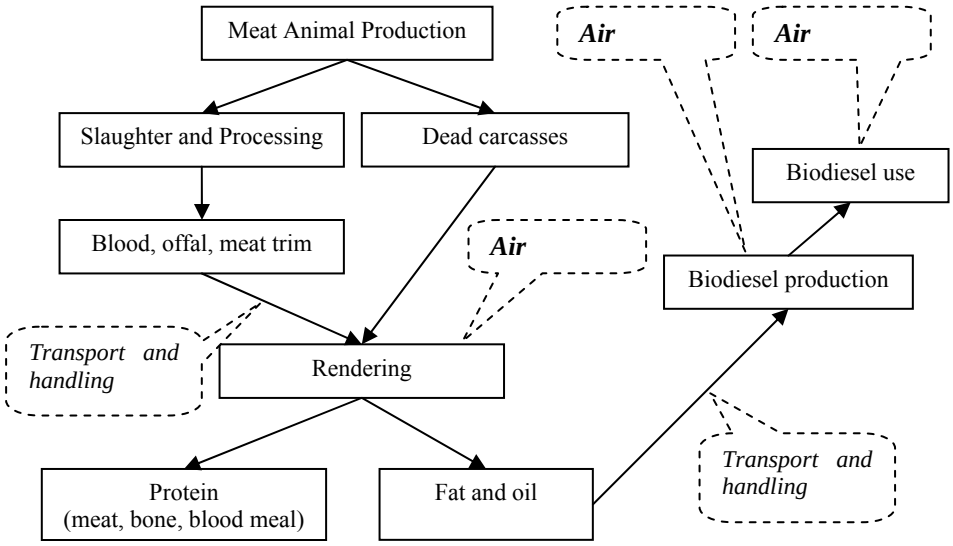
The production of biodiesel involves transesterification of fatty acids derived from a variety of vegetable oils or animal fats. Although most commonly publicized as a product of vegetable fats, biodiesel also can be formed from animal fats. A number of different methods are available for commercial production of biodiesel. In one of these methods, triglycerides are transesterified in the presence of excess methanol, an alkaline catalyst, with atmospheric pressure and reaction temperatures of approximately 60 to 70ºC. Glycerin and excess methanol are recovered and biodiesel is further refined. The German "Henkel" process begins with a feedstock of unrefined oil, excess methanol and a catalyst. The process operates at a temperature of 240ºC and 9000kPa. Again, the excess methanol and the generated glycerin are separated from the mixture and the biodiesel is further refined. The "Lurgi" process requires pre-processed feedstock materials that have been deacidified and degummed. This process is operated at normal pressure in a two stage reactor with refined oil, methanol and catalyst. (Srivastava and Prasad, 2000).

As biofuels emerge, a number of questions will be asked concerning the safety of the product, the safety of using the product as a fuel and the safety of generating that fuel. One of the primary safety issues will be if any contaminants are present in the starting materials. Whether from plant or animal sources, the fats used have potential for chemical and biological contamination. In plant-derived oils, there are potentially agricultural chemical residues from both pesticides and fertilizers, fungal secondary metabolites, and a variety of other environmentally derived contaminants. In animal fats, the potential exists for biological, organic and inorganic contaminants.

The safety of using animal fats for biodiesel includes evaluating a series of unit operations during which the transition from raw material to the refined product leads to

the burning of the refined product. These unit operations include raw co-product transport/handling/storage and processing steps to refine the raw fat into an efficiently burning fuel. Thus, in the review of the safety of using animal fats in the production of biodiesel, research should address the safety concerns related to all of the processing steps and not just the burning of the refined product for energy (see Figure 1.1).

Figure 1.1 Possible Critical Safety Points in the Transport, Handling and Production of Biodiesel from Animal Co-products



The use of animal co-products for biodiesel raw material via rendering has critical points during the process that must be controlled for safety; however, compared to other available methods of handling animal co-products, the rendering route seems the most safe and efficient. Incineration, burial and composting are other options used to dispose of animal co-products. Incineration most effectively destroys infectious agents (Ritter and Chinside, 1995); however, release of airborne contaminants and energy efficiency is still a concern. Burial and landfill disposal requires isolated land to hold organic material, does not effectively eliminate infectious agents and is subject to leaching of hazardous materials into groundwater (Salminen and Rintala, 2002). Composting and anaerobic digestion are effective in eliminating infectious agents and reducing waste volume but require large bioreactors of windrows which may cause concern for air emissions (Tritt and Schuchardt, 1992).

The following review of literature was designed to investigate any possible chemical and biological moieties that have even a mere chance to be present in animal fats and to study the potential consequences that any of these moieties could have on human and environmental health through the production and use of biodiesel made from these animal fats. Currently, the public views the greatest concern to any bovine-based product as bovine spongiform encephalopathy (BSE); however, upon identification of the causative agent and the subsequent implementation of methods for its control, the risks from this moiety are low. Strict regulations are in place concerning bovine tissues

and the associated by-products; however, indications are that some of the more extreme restrictions may be eased as soon as scientists learn more about the causative agent and its etiology. In early June 2005, the European Commission, in response to a recommendation from the European Food Safety Authority's scientific panel, considered relaxing the rules for specified risk materials (SRM) such that all cattle below 21 months of age would be excluded from SRM removal at slaughter (Hisey, 2005).

The issue of transmissible spongiform encephalopathies (TSE) such as bovine spongiform encephalopathy (BSE) has been addressed in a separate review. The following review seeks to survey available literature to determine if there are any other potential hazards that may occur in animal fats which would raise safety concerns during the manufacture, transportation, storage, and use of biodiesels made from animal fats.

2 MICROBIAL ASPECTS OF USING ANIMAL FATS IN THE PRODUCTION OF BIODIESEL

2.1 Bacteria

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2.1.1 Introduction

The European Commission for Health and Consumer Protection Directorate (1999) published an opinion paper from their Scientific Steering Committee (SSC) on the risks of infectious agents and toxic substances entering the human food or animal feed chain via condemned materials or fallen animal stock. They reported that approximately 50% of the over 1700 known microbial pathogens are zoonotic, which means that they can be transferred from animals to humans. In addition to infectious agents, humans could be exposed to a variety of chemical substances present in food products of animal origin. Furthermore, these agents or substances could be modified during the processing or rendering of animal products, making them more of a risk to human health. The committee concluded from published research studies that many animal species are vulnerable to transmissible spongiform encephalopathy (TSE) when fed infectious material originating from the same species. However, handling of such material has not been found to transfer TSEs. The European Commission for Health and Consumer Protection Directorate (1999) SSC recommended that recycling of condemned material should not result in either direct (via cosmetics, pharmaceuticals, medicinal products or devices) or indirect (via food animal consumption) human contact. The SSC also concluded that the current European rendering industry processing standard of 133° C/20 minutes/3 bars or a validated equivalent is sufficient to reduce the risk to acceptable levels.

2.1.2 Microbiological Aspects

Microbial quality of animal co-products is a major concern since slaughter by-products are contaminated with high numbers of microorganisms including bacteria, viruses, virus-like particles, fungi, yeast, and associated microbial toxins (Urlings et al., 1992). Slaughter by-products constitute a potential risk to animal and human health, unless handled and treated properly. Transportation of these products by road or rail is a concern in terms of environmental pollution and the spread of microorganisms.

Since animal co-products originate from mostly food animals, recalls due to contamination of any portion of the animal or its subsequent food products may provide information about the types of hazards entering the animal co-product stream. In 1999, 3.38 billion pounds of meat and poultry were imported, which accounted for less than 10% of the 40 billion pounds of meat and poultry produced in the US. Recalls accounted for less than 0.1% of the total poultry production each year. Recalls are categorized as by class (I, II, or III, with I being the most serious), hazard type (biological, chemical, physical), and plant size (large (> 500 employees), small (10-500 employees), and very small (<10 employees or less than $2.5 million in annual sales)). Food recalls are most often an action taken voluntarily by food manufacturers or

distributors after they determine independently or after being informed by a governmental agency of the possibility there is a negative health concern for consumers. The purpose of a recall is to remove potentially adulterated or misbranded meat, poultry or egg products from the consumer distribution stream. The voluntary nature of recalls differentiates them from the detention or seizure of product by the FSIS or FDA. The FSIS receives information about food safety problems from various sources including consumers, food manufacturers, or HACCP-driven sampling protocols. The FSIS assesses the hazard and identifies products to be recalled, then oversees the recall process through its completion. The information on recalled products is maintained and updated on the FSIS website at: http://www.fsis.usda.gov/oa/recalls/rec_intr.htm (USDA, 2005). From 1997 through 2002 there has been at least one meat/poultry recall greater than 10 million pounds, 5 involved *Listeria monocytogenes* and 2 involved *Escherichia coli* O157:H7. Over the nine year period from 1994 through 2002, 74% of the recalls were of the Class I category, 74% involved processed meat, 26% raw meat, 23% involved poultry meat, 40% red meat and 69% involved biological hazards (Teratanonat, 2004). *Listeria monocytogenes* and *Escherichia coli* O157:H7 were the most frequent biological recall agents despite *Salmonella* spp. and *Campylobacter jejuni/coli* causing many more food borne illnesses (USDA, 2005).

Table 2.1 Reasons for Meat and Poultry Recalls in the US. (USDA, 2005)

	Number of recalls						
Year	Listeria	E. coli	Salmonella	Other bacteria	Chemical/ physical	Undeclared ingredients	Under processed
1994	17	3	0	3	16	1	7
1995	11	5	2	2	13	1	7
1996	6	2	1	1	5	3	6
1997	3	6	1	5	8	4	0
1998	7	13	2	2	11	4	5
1999	30	10	6	0	3	8	4
2000	36	20	4	0	5	9	2
2001	25	26	2	0	11	24	6
2002	40	24	4	0	4	36	4

Since the size of the recall can vary from a few pounds to several millions, just focusing on the number of recalls can be misleading. The recalls, in pounds, for 0-10,000; 10,000-100,000; 100,000-1,000,000, and greater than 1,000,000 accounted for 55, 24, 17, and 4% of the total recalls, respectively, over the nine-year period.

2.1.3 Bacteriological Hazards of Co-Products

Meat and poultry co-products may contain several hundred different species of micro-organisms in feather, feet and intestinal contents (Chen, 1992). Incorporation of intestines results in animal co-products with a high number of microorganisms, including pathogenic bacterial species such as *Campylobacter, Escherichia* and *Salmonella*, some of which have antibiotic resistance (Haapapuro et al., 1997). A variety of meat sources must be considered when assessing the microbiological load. For example, Troutt and Osburn (1997) reported that approximately 17% of U.S. ground meat originated with culled dairy cows which could alter the microbiological

risk of related animal co-products. Major concerns are to reduce the viability of pathogenic bacteria and to prevent the production of bacterial toxins including staphylococcal and botulinum toxin.

2.1.4 Effect of Rendering on Bacteria

Troutt et al. (2001) sampled raw and rendered material from 17 rendering plants for *Clostridium perfringens*, *Campylobacter jejuni* and spp., *Listeria monocytogenes* and spp. and *Salmonella* spp. These bacteria were found in 20-85% of the raw samples; however, no samples were found to be positive for any of these bacteria after they had been rendered. The heat treatment used for rendering animal co-products will destroy nearly all vegetative cells and, in most cases, spores as well. The final moisture content of rendered material (meal and fat) is 4-10% and too low to support bacterial growth (FPRF, 1999). Spore inactivation under specific rendering conditions has been reported to require reaching a final process temperature of 96°C for *B. cereus* and 115°C for *Clostridium sporogenes* (MRC, 1997). Little published information on inactivation of microbial toxins in rendered products was found.

2.1.5 Salmonella and Other Disease-Producing Organisms in Animal Co-Products

Loken et al. (1968) examined samples of protein feed supplements produced by rendering plants for salmonella, total aerobic bacterial counts, coliform counts, and enterococci. Salmonellae were frequently isolated from samples including 6% of samples with total counts of less than 1,000 per gram and 14% of the samples with coliform counts of less than 1 per gram. Serotypes of *Escherichia coli* associated with disease in domestic animals and poultry were also isolated from supplements. Total bacterial counts and coliform counts are useful in providing information regarding general plant sanitation and in removing potential problem areas. The elimination of salmonella contamination of animal feed will help control salmonella in domestic animals and poultry.

In 1989, the incidence of salmonellae in animal feed was 49% (Jay, 2000), while at USDA-inspected facilities the rate in feed was 20-25% and for pelleted feed only 6% (Graber, 1991). Jones et al. (1991) reported a 60% salmonellae contamination rate for breeder and broiler houses with feed being the ultimate contamination source. *Salmonella s*enftenberg, *S. m*ontevideo and *S. c*erro were the primary serovars found in animal feed while *S. enteriditis* was not found in rendered products (Jay, 2000). Grau and Brownie (1968) reported that 45% of the rumen of healthy cattle was positive for *Salmonella* spp. after slaughter.

Monitoring of plant environments and products for salmonella contamination on a regular basis, coupled with careful attention to equipment design and sanitation, can also promote salmonella–free products. The U.S. rendering industry, through its Animal Protein Producers Industry (APPI), conducts salmonella testing on rendered products for assisting in the control of the microorganism (APPI, 2004).

Animal feed is the beginning of the food safety chain in the "farm-to-fork" model. There has been a major transformation and intensification of agriculture in the United States and elsewhere during past 50 years. This has lead to increasing reliance on a wide range of manufactured feed products as food for animals destined for human consumption. Rendering plants process animals, meat trimmings, and other slaughter

by-products into animal feed ingredients. Rendering plants, similar to many food processing plants, have a “wet side”, where carcasses and raw materials are received and prepared for processing, and a “dry side” where rendered products are processed into meal. It is important to note that after rendering, the dry rendered material is salmonella-free but can become re-contaminated at several points. These critical points can include cooling, drying, milling, screening, sorting, mixing, stirring and bagging. There is evidence that animal feed is contaminated with food borne bacterial pathogens (Crump et al., 2002). Since the raw animal co-products enter the rendering plant and emerge as protein for feed or fat, it is possible the fat could be contaminated with similar bacteria as the protein feed fraction. In the United States, the FDA periodically conducts surveys of feed ingredients and feed. In 1993, the FDA tested for the presence of *S. eneterica* in samples from 78 rendering plants that produced animal protein-based animal feed. The organism was detected 56% of the 101 animal protein-based samples. Several incidents have been reported in which human illness was traced back to contaminated animal feed. For instance, in 1958 an outbreak of infection with food borne *S. eneterica* serotype Hadar in Israel was linked to consumption of chicken liver. An investigation of the source poultry farm found that bone meal, which was fed to the chickens, was contaminated with the same serotype of *Salmonella*. A milk borne outbreak of infection due to *S. eneterica* serotype Heidelberg in England in 1963 resulted in 77 human illnesses and was traced to a cow with bovine mastitis due to the same organism. Investigation revealed that meat and bone meal fed to the cow was contaminated with the same organism. Although tracing contamination to its ultimate source is difficult, several large outbreaks have been traced back to contaminated animal feed.

Hazard Analysis and Critical Control Point (HACCP) programs have been implemented worldwide in developing countries for food production and food handling processes. HACCP was first developed by the Pillsbury Company to ensure the safety of foods for the U.S. space program and was used as a company-wide food safety assurance program. Consideration for widespread adoption by the food industry began in the mid 1980s. In the U.S., HACCP has been credited with reducing food borne illness while lessening the “policing” nature of governmental oversight and placing the responsibility and control more on the food manufacturing and food service industry. However the link between reducing food borne illness rates and HACCP plan implementation may not be substantiated. Sumner et al. (2004) investigated the relationship between HACCP and food borne illnesses in Australia by examining statistics from time periods in Australia before and after implementation of mandatory industry-wide meat and poultry food safety (HACCP) programs. For about the past decade, reported salmonellosis in Australia averaged 6000 cases annually, ranging from 4600 (1992) to 7700 (1998). Using a formula to account for underreporting, the governmental revised estimates were between 240,000 and 650,000 annual salmonella-related cases. Since warm-blooded animals harbor *Salmonella* in their intestines, meat, poultry and eggs were thought to be the primary source of this pathogen for humans. Therefore, food safety regulators logically believed that tighter microbiological control on meat, poultry and eggs would reduce the incidence of human salmonellosis. This belief was supported by a few large human outbreaks linked to poultry, meat and eggs in the 1980s. Using all available data from time periods before and after HACCP implementation, the authors used two approaches to determine if new food safety regulations had an impact on illness: (1) determine the number of all salmonella-related cases, and (2) match serovars isolated from meat and poultry with those linked to

human illness. The results show that there was a slight increase in both total number of cases and incidence rate of salmonellosis after the regulatory changes took full effect in 1997 (see Table 2.2).

Table 2.2 Total Cases and Incidence Rate of Salmonellosis in Australia from 1991 - 2001. (Modified from Sumner et al., 2004)

	Salmonellosis reported	
Year	**Total cases**	**# cases/100,000**
1991	5440	31.9
1993	4731	27.5
1995	5895	34.0
1997	7005	38.2
1999	6834	38.3
2001	7147	35.8

When serovars found in raw meat and poultry were linked with human illnesses, similar numbers were observed both before and after regulatory implementation. This indicated that there was little effect from adding HACCP plans to meat and poultry production on human illness, but it may also be a reflection of how the data were collected. Data collection in meat and poultry may be focusing on finding the source of human outbreaks. While some variation in the *Salmonella* serovars found in the pre and post HACCP implementation periods were found, *S. typhimurium* strains were consistently linked to both poultry meat and human illnesses in both periods. Serotype matching in the US has revealed similar results, indicating a lack of matching between human and raw meat serovars. For example, while *S. kentucky* was commonly found in meat and poultry and was predicted to cause 14% of the cases, it only accounted for 0.1% of all human cases. There is evidence that changes in food safety regulations have improved the microbiological status of poultry at the processing level; however, this report and others question the magnitude of this impact on human food borne salmonellosis. Reduced food borne salmonellosis may be more impacted by revised foodservice practices and less to do with food processing HACCPs.

A survey was undertaken by the Animal Disease Eradication Division of the U.S. Department of Agriculture to accumulate information on the occurrence of pathogenic organisms in animal co-products, to evaluate their real or potential disease threat to the nation's poultry and domestic livestock, and to recommend corrective measures where necessary (Morehouse and Wedman, 1961). The primary focus of this study was on *Salmonella* spp., and salmonella was isolated from 718 out of 5,712 samples. There were 59 *Salmonella* serotypes isolated from a wide variety of animal co-products. Serotypes most frequently recovered included *S. montevideo, S. seftenberg, S. typhimurium, S. cubana, S. infantis*, and *S. oranienburg*. Recontamination of animal co-products after rendering was believed the principal factor accounting for the presence of *Salmonella* spp. in the rendered end product; possible sources of this contamination are rodents, wild birds, dogs, and human handlers of products. Seventy-one co-product samples were examined for salmonella from rendering plants producing protein feed supplements (Clise and Swecker, 1965). From these, 94 *Salmonella*

isolations were made involving 27 serotypes. Unprotected storage of the offal following cooking and rendering was one reason for contamination.

In another study, the production of meat and bone meal from 8 rendering plants was examined for the presence of salmonellae (Besink, 1979). Of 164 samples of final product, 114 (69.5%) were contaminated with salmonellae. Of 65 samples, collected at various points from the production line 35 (53.8%) were salmonella-positive. Of the 95 samples collected from the processing environment, 79 (83.1%) were found to be contaminated with salmonellae. Results obtained in this survey suggest that approximately 70% of meat and bone meal was contaminated with salmonellae. One reason for this high level of contamination might be that the environment in the processing area contaminated the rendered material. However, dried blood, often stored in the same area, appeared to be less frequently contaminated. There was also a marked difference in the results between processing plants. A visual assessment of each processing area did not explain these differences. However, the plant in which only 20% of the products were contaminated with *Salmonella* spp. differed from all other plants, in that any product which fell on the floor during processing was discarded and not returned to the processing line.

Timoney (1968) determined that the source of *Salmonella* spp. for entering sterilized rendered material was from the area of the cooker which was found to have the highest environmental levels of *Salmonella* spp. and the most variety of serotypes. Furthermore, the wide variety of closely related serotypes in this area of the rendering plant has led researchers to conclude that this location of rendering plants might be conducive to alterations in serotypes, and rendering plants may play an important role in the propagation of new *Salmonella* spp. serotypes. Plants employing hot solvent extraction in an enclosed process line were found to not have salmonellae in the end product. The contamination of the final product in an open system is likely to originate from the plant environment where *Salmonella* have established themselves on surfaces. Timoney (1968) also found that different *Salmonellae* serotypes where found in the wet and dry sides of the rendering plant. This lead to the conclusion that the salmonellae contaminating the final rendered meal was from “house bugs” that may have originated from raw material but now exist in the rendering plant independently.

Moyle (1966) surveyed Wisconsin rendering plants from July, 1963 to October, 1964 to determine the extent that products from these plants that were used as animal feed ingredients were sources of *Salmonella* infection in domestic animals and birds and to suggest areas where control measures might be most effectively introduced. Approximately 1 day was spent in each of the 18 rendering plants on initial visits to classify plants according to (1) methods used, (2) materials rendered, and (3) sanitary conditions found. Samples were taken twice annually at all of the plants and at monthly intervals in 4 of the plants selected for more intensive studies during the last 6 months. Each different product produced for use as a feed additive in these plants was sampled at different stages of production, storage and handling. This study found a lack of correlation between the incidence of *Salmonella* spp. and plant sanitation, contrary to all previous thinking on this subject. The plants which handled a large percentage of whole dead animals, with a few exceptions, were those which had relatively poor sanitation; yet they more frequently produced salmonella-free material. Much of the dead animal material was received in poor condition, and poorer quality tallow was derived from it. Quality in tallow is measured by acidity which is monitored by titration of free fatty acids. Apparently, decomposition results in an increase in free

fatty acids, and the acidity in the tallow produced from this material was reflected in the animal by product left after the tallow was removed. Gram positive bacteria are adversely affected by certain unsaturated fatty acids. Little work has been conducted on gram negative organisms including *Salmonella* spp. related to possible effects of free fatty acids. This study failed to show as much correlation as was expected between sanitation and the incidence of *Salmonella* spp. in the products of Wisconsin rendering plants. The discovery of a possible antagonistic compound, possibly free fatty acids, related to the putrefactive process should be considered of value in the search for an antimicrobial to assure salmonella-free animal co-products for use in animal feed. Until a method is found to inhibit *Salmonella* spp. growth in rendering plant co-products, the most efficient way of reducing the incidence of these bacteria is through prevention of post-rendering contamination.

2.1.6 Factors Influencing Detection of Salmonellae in Rendered Animal By-Products

Tompkin and Kueper (1973) attempted to understand the factors influencing the detection of salmonellae in naturally contaminated animal co-products. The influence of media and the relationship between total counts and isolation of salmonellae were examined. No practical difference was found between tetrathionate-Brilliant Green-iodine broth (TB) and selinite-cystine broth (SCB) in terms of the number of positive samples. Both TB and SCB failed to detect salmonellae in approximately one out of five of the total samples. This failure to detect salmonellae could be due to inhibition of certain serotypes by the enrichment media, a low level of contamination, or uneven distribution of salmonellae through the product, or all three. In this study, a linear relationship between total plate count and the detection of salmonellae exists only in the total count range of 10^4 through 10^7/g. There was a decrease in the percentage of *Salmonella* spp. positive product having total plate counts greater than 10^7/g. This was probably due to overgrowth of salmonellae by other bacterial species during enrichment or plating, or both. It is less likely that the product was actually less contaminated with salmonellae. The linear relationship of total plate count and *Salmonella* spp. counts is of practical value for in-plant control purposes and for evaluating improvements in manufacturing and sanitation. It is important that the limitations of the total plate count be recognized and it was suggested that total counts be used to supplement salmonellae testing programs.

2.1.7 Fluorescent –Antibody Methods for Detecting Salmonellae in Animal By-products

Smyser and Snoeyenbos (1973) used direct–culture and fluorescent antibody (FA) procedures to examine 550 samples of rendered animal by-products for salmonellae. Tetrathionate brilliant–green (TBG) and selenite brilliant-greensulfapyridine (SBG sulfa) enrichments incubated at 42ºC respectively detected 82% and 73% of the 158 samples found positive by these two direct–culture methods. Three FA procedures were tested in parallel. TBG, SBG sulfa, and gram negative (GN) broth were used for primary enrichment followed by subculturing for 5-6 hr in GN broth from which FA smears were prepared and brilliant–green agar plates were streaked. There were 454 subcultures found to be FA positive from three procedures used, and salmonellae were recovered from 342 subcultures. Of the 150 subcultures which were FA-questionable, salmonellae were recovered from 14; of 1,046 subcultures which were FA negative, the organism was isolated from 17 subcultures. The data indicated that SBG sulfa and

TBG can be substituted successfully for GN broth as the FA primary enrichment. This substitution resulted in greater correlation between FA results and *Salmonella* isolations.

2.1.8 New Decontamination Efforts and Techniques for Elimination of *Salmonella* in Animal Protein Rendering Plants

Salmonella spp. contamination of animal feed ingredients and finished feeds has been well documented. A cooperative study on rendering plant decontamination procedures was developed with the specific objectives to: (1) determine sources of *Salmonella* contamination of the finished product, (2) develop rendering plant decontamination procedures, and (3) evaluate the effectiveness of these procedures in the plant under study Hess et al. (1970). The study was divided into 5 stages: (1) pre-cleanup; (2) cleanup initiated; (3) cleanup; (4) fumigation initiated; and (5) fumigation. In the first stage, or pre-cleanup, *Salmonella* isolations and standard plate counts were made to obtain baseline data on contamination in the plant. Seven rounds of sample collection were used to establish baseline data. During this stage 160 samples were collected, with 35.9% being positive for *Salmonella*. Samples of the product (tankage) collected at time of discharge from the extractor cookers were negative for *Salmonella*. Total bacterial counts were in the range of 10^7 to 10^8/g. of sample.

In the second stage, the plant was shut down and given an intensive cleaning of approximately 200 man-hours. In the third stage 465 samples were collected with 13.8% being salmonellae positive. Stage 4 was the application of fumigation and stage 5 included testing results following fumigation. In the fifth stage 1.2% Salmonella positive samples were found out of 394 samples collected. It was concluded that when finished-product areas of the plant were decontaminated, bacteria entering the chemical extraction stage were reduced. Basic cleaning and disinfection procedures with formaldehyde improved all other efforts and the total plate count indicated a general reduction of bacterial flora in the plant.

Lactobacillus fermentation is an alternative to rendering and has been shown to inactivate viruses and bacteria when added as liquid cultures (Talkington et al., 1981a; Talkington et al., 1981b; Wooley et al., 1981; Gilbert et al., 1983). However, fermentation times are often a minimum of nine days, yet have successfully inactivated viruses including Newcastle disease, frog virus, a porcine picornavirus, avian infectious virus, measles virus and bovine diarrhea virus (similar to hog cholera). Bacteria that have been inactivated include *Salmonella* Enteriditis, *S.* Cholerae-suis, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis, Clostridium perfringens, Cornyebacterium pseudotuberculosis* and *Listeria monocytogenes*. Schotts et al. (1984) reported that *Lactobacillus* fermentation of animal wastes containing animals that had died from viral (Newcastle disease) or bacterial (*Salmonella* Enteriditis) infections effectively inactivated both agents after 5 days. While effective under ideal conditions, fermentation to eliminate microbiological contamination of animal co-products is more costly, more time consuming and more difficult to control to validate inactivation compared to thermal process such as rendering.

2.1.9 Animal Co-Products Contaminated with *Salmonella* spp. in the Diets of Lactating Dairy Cows

The contamination of rendered animal co-products by salmonella and the affect on animal and human health have been a concern for a number of years. Bender et al.

(1997) attempted to assess the clinical and microbiological effects of meat and bone meal (MBM) contaminated with *Salmonella* spp. on lactating dairy cows and to determine whether the same serotypes of *Salmonella* spp. present in the MBM could be detected in the rumen contents, milk, or feces of these cows. Two rumen fistulated dairy cows were fed meat and bone meal that had been artificially contaminated with *Salmonella* spp. Over the 2 month study, salmonella were recovered from rumen contents, feces, necropsy specimens of rumen contents, fecal contents, and mesenteric lymph nodes. No excretion of salmonella in milk was detected and no clinical illness was observed in cows. Meat and bone meal that has been contaminated with low concentrations of *Salmonella* is unlikely to result in clinical illness in healthy adult lactating cows (Bender et al., 1987). However, dairy producers should continue to monitor animal co-products feed ingredients to control contamination by salmonella.

2.1.10 Enterobacteriaceae as Indicators of Good Manufacturing Practices in Rendering Plants

Enterobacteriaceae have been used as indicators to monitor the improvement in sanitation by good manufacturing practices in many food processing plants in the Netherlands. However, in only a few plants has it been possible to relate the results of measures taken to reduce the numbers of Enterobacteriaceae or their frequency of isolation to the reduction of salmonella in environmental samples and end products. Finished products and samples from the environment of the production line in rendering plants were checked for Enterobacteriaceae and salmonellae (Van Schothorst and Oosterom, 1984). Improvements in hygiene and measures taken to limit multiplication of microorganisms in the dry area of production lines resulted in reduction of both numbers of Enterobacteriaceae in environmental samples and frequency of their occurrence in finished products. Simultaneously, there was an equivalent reduction of salmonellae positives in environmental samples and finished products. Consequently, the determination of Enterobacteriaceae can be used as an effective tool to assess the improvements in good manufacturing practices.

2.1.11 Characterization of Antibiotic-Resistant Bacteria in Rendered Animal Products

Harrison and Lederberg (1998) estimated that treatment costs for antibiotic resistant human infections ranged from 4 to 5 million dollars annually. Some research has indicated an association between on-farm practice of therapeutic doses of antimicrobials and the selection of antimicrobial-resistant bacteria. In general, microorganisms can gain resistance to antimicrobial by changing membrane permeability to the antimicrobial, producing enzymes to degrade the antimicrobial, modifying the specific site an antimicrobial might require for attachment, or by creating a target site to mimic the “real” site causing the antimicrobial to bypass the real target. Multiple antimicrobial resistance is acquired via mobile genetic elements called integrons. There are several other ways microorganisms gain antimicrobial resistance including the presence of an efflux pump.

Olah et al. (2004) compared the types of antimicrobial (antibiotic) resistance observed in *Salmonella* spp. and *Campylobacter* spp. from turkeys collected from 2 different processing plants in the Midwest US. Ninety-four turkeys that were *Salmonella* and *Campylobacter* spp. positive were selected and antimicrobial resistance determined. Antimicrobial resistance was found to be common for both organisms; however,

resistance varied in turkeys collected from the two different processing plants from which carcasses were sampled. Possible reasons for the variation might the differences in production practices used at farms supplying the birds and/or the processing plants themselves. The highest incidence of *Salmonella* spp. resistance occurred to tetracycline and sulfamethoxazole. In one of the plants, 100% of the isolates (*Salmonella* and *Campylobacter*) displayed resistance to at least one antibiotic but *Campylobacter* isolates were resistant to a greater number of antimicrobials than *Salmonella* isolates (Olah et al., 2004). Molecular analysis of the mechanisms for resistance showed that *Salmonella* isolates appeared to use an integron gene while *Campylobacter* was more likely to possess an efflux pump.

The emergence of antibiotic-resistant Salmonella has been reported in the US and Canada. *Salmonella* spp. antibiotic resistance is acquired by mutation and transfer of the mutant genes after exposure to antibiotics which can occur during production. One of the most virulent, disease-causing strains is *Salmonella typhimurium* DT104 that emerged in Europe in the 1990s and then later in the US and Canada. Of the 40,000 different *Salmonella* spp. isolates reported in the US annually, 3,400 were *S. typhimurium* antibiotic resistant strains. It has been estimated that 68,000 to 340,000 cases of infection occur annually in the US due to *S. typhimurium* resistant strains. In a study conducted in Ontario, 390 total *Salmonella* spp. isolates were recovered from hog (71), beef (24) and chicken (295) carcasses collected during 1900-2001 in provincially-inspected plants in Ontario (Larkin et al., 2004). Resistance was found to streptomycin (36%), tetracycline (35%), ampicillin (19%), sulfamethoazole (18%), cephalothin (12%), chloraphenicol (18%), and gentamicin (6%). Ampicillin and tetracycline are antibiotics sometimes used as growth promoters in the US and Canada. Gentamicin resistance was highest in poultry probably due to the use of this antibiotic in broiler eggs by injection. Gentamicin resistance for bacteria isolated from humans and poultry in Europe is very rare where this antibiotic is not used.

Until the 1970s chloraphenicol was the drug of choice for treating human salmonellosis but due to the emergence of resistant strains lost favor and has not been used in Canada in animal production now for 20 years. Chloraphenicol resistance was found in beef (25%), hog (12.7%), and chicken (2.4%). Approximately 12% of poultry and 19% of hog isolates were tetracycline resistant, while multi-antibiotic resistance was found in 29% of beef, 42% of hog, and 33-42% of chicken isolates (Larkin et al., 2004). Larkin et al. (2004) found significant numbers of antibiotic resistant *Salmonella* spp. in food animals, and none of the isolates were found resistant to amikacin, ciproflaxin, ceftriaxone, or naladixic acid. However, there was evidence that the antibiotic used in animals results in the selection for, or an increase in, antibiotic-resistant *Salmonella* spp.

Mayrhofer et al. (2004) examined the antibiotic resistance of pathogenic strains of *Campylobacter, Salmonella, Yersinia, Escherichia coli* and *Listeria* species in 922 samples of pork, beef, chicken, and turkey meats. Prevalence of these pathogens found by these researchers is shown in Table 2.3.

Table 2.3 Prevalence of Pathogenic Strains of Bacteria in Selected Meats (adapted from Mayrhofer et al., 2004)

Pathogenic types of genera	Meat Type			
	pork	beef	chicken	turkey
	Prevalence in samples (%)			
Campylobacter	<5	2.4	51	53
Yersinia	16.7	10	0	0
Salmonella	1.4	0	16.4	0.4
Escherichia coli	10	7.5	2.3	1.9
Listeria	22	12	26	14

*Campylobacte*r isolates had an antibiotic resistance rate of 40% for quinolones, 16.4% to tetracycline and 9.7% to streptomycin and ampicillin. Overall, the *Salmonella* spp. resistance rate was 57.7%, with resistance to specific antimicrobials of 42% for naladixic acid, 33% for tetracycline, 27% for streptomycin and 17% for both ampicillin and chloramphenicol. Only one antibiotic resistant strain of *Yersinia* was found and this strain was a non-pathogenic type. Fifty-four of the *E. coli* strains isolated from meat were antibiotic resistant with 8 of 12 from pork, all 3 from chicken, 1 from turkey and 2 from beef. No antibiotic resistant types of *Listeria* spp. were found.

A potential source for antibiotic-resistant bacteria on farms that do not have a history of recent antibiotic use may be the rendered animal co-products used as feed ingredients. To assess the relationship between antibiotic-resistant bacteria in rendered animal products, one hundred sixty-five rendered animal protein products from cattle, poultry, and fish were aseptically collected from poultry mills (Hofacre et al., 2001). Fifty five percent of the poultry meal samples had detectable levels of gram negative bacteria ranging from 40 to 10,440 colony forming units/g of sample. Poultry meal and meat and bone meal had the greatest number of samples with bacteria resistant to five or more antibiotics. Eighty-five percent of feed samples contained bacteria resistant to amoxicillin, ampicillin, clavulanic acid, or cephalothin, whereas few samples contained bacteria resistant to ciprofloxacin, kanamycin, or trimethoprim/silfamethoxazole. *Acinetobacter calcoaceticus, Citrobacter freundii*, and *Enterobacter cloacae* were the most commonly isolated antibiotic resistant bacteria. Salmonellae were isolated from 14% of the meat and bone meal samples, out of which only one of the isolates, *S. livingstone* was resistant to five or more antibiotics.

2.1.12 Post Process Product Monitoring of Rendering Plant Sterilization Conditions by ELISA

The prevention of epidemic diseases and bovine spongiform encephalopathies (BSE) depends, in part, on the availability of reliable techniques to determine if animal co-products have been sufficiently heat treated. According to the technical annex of European Commission Decision 96/449/EC, the recommended sterilization temperature, pressure, and time are 133ºC, 3 bar, and 20 min, respectively. In addition, particle size of the animal waste should not exceed 50 mm. In general, sterilization conditions are checked by temperature and pressure measuring devices installed in rendering plants. The enzyme linked immunosorbent assay (ELISA) test used in this study was actually intended for identification of pork in cooked food, but it can also be

used to check for appropriate heat treatment of animal meal (von Holst et al., 2001). The principle behind this method is that the specific antigen-antibody interaction indicating the presence of pork did not occur if a pork sample was treated at 133ºC or higher. The objective of this study was to set a limit for reading the ELISA method (R-value) on the material heated in a commercial rendering plant. Experiments were conducted in a laboratory autoclave and in a commercial rendering plant to set a limit for the R-value of an ELISA test to prove that animal meal had been sufficiently heat treated. Results of these experiments confirmed that insufficiently heat-treated animal meal has an R-value > 2. Results of the trials also demonstrated that the main parameters of sterilization, temperature, and duration strongly influence the R-value, thus indicating that deviation from the target sterilization conditions is verifiable by the ELISA method.

2.1.13 *Listeria* spp.

Listeria monocytogenes is the main genus of concern for *Listeria* spp. in food and meat products, although 5 other genus and 17 serovars across the genus exist (Jay, 2000). While the temperature for maximum growth rate for listeriae is 45°C, it can grow at a minimum of 1-3°C. *Listeria* spp. are found widely throughout the environment but have become particularly persistent in food processing environments. *Listeria* spp. are destroyed by conventional pasteurization and have D values of 1-5 seconds at 71.7°C in milk (Bradshaw et al., 1987) and 16.4 seconds at 65°C on bologna (McCormick et al., 2003) with z values in the 4.5 to 8°C range. Troutt et al. (2001) reported that 76.3 and 8.2% of raw animal co-products entering the rendering stream contained *Listeria* spp. and *Listeria monocytogenes*, respectively. No samples from the same plants post rendering were found positive for *Listeria* spp. in the Troutt et al (2001) study. The concern for rendered animal products is the same as for processed meats, that being post-processing contamination. Ready-to-eat meat processors of products such as bologna and frankfurters are struggling with elimination of *L. monocytogenes* from products destined for human consumption; therefore, renderers will potentially be addressing this same issue in rendered products.

2.1.14 *Yersinia enterocolitica* and *Campylobacter jejuni*

Yersinia enterocolitica is widely distributed in nature, including soil and water, being found mainly in fresh water and foods (Jay, 2000). The *Yersinia* genus has 11 species including *Y. pestis* which is the cause of the plague. *Yersinia enterocolitica* produces a toxin that remains active after exposure to 100°C for 20 minutes. Pork is a common source among meats; for example Funk et al., (1998) reported that out of 95 swine lots tested, 92.2% had at least 1 pig testing positive for *Y. enterocolitica*. This study was strongly substantiated by a cooperative study between Iowa State and North Carolina State Universities that found 2.1% of the pigs tested in NC were positive for *Y. enterocolitica* (Wesley et al., 2005).

Campylobacter jejuni is not an environmental organism but is associated with warm-blooded animals, especially poultry and to a lesser extent pork. A multi-state IA/NC study reported 90-93.8% of pig carcasses were positive for *C. jejuni* (Wesley et al., 2005). *Campylobacter jejuni* was found by Stern et al. (1985) in a high percentage of processed meat samples: 29.7% in chicken, 4.2% in pork, 3.6% in ground meat and 5.1% in red meats. *Campylobacter jejuni* displays sensitivity to freezing, with fresh meats having a higher positive sample number than frozen meats (Stern et al., 1984).

Both *Y. enterocolitica* and *C. jejuni* are highly sensitive to heat and are destroyed by conventional milk pasteurization temperatures.

2.1.15 *Escherichia coli*

Escherichia coli includes a diverse group of serotypes, many of which are non-pathogenic but with pathogenic strains that cause foodborne illness in various ways. The pathogenic strains can be enteroinvasive, that is infect the intestinal tract to disrupt the function of intestinal cells, or enterotoxigenic, where the cells produce a toxin. *Escherichia coli* O157:H7 produces a toxin that attacks the kidney (verocytotoxin). The heat sensitivity of *E. coli* is similar to that of *Salmonella* spp. with D values in the range of 20 to 40 seconds at 60°C in meat (Ahmed et al., 1995).

2.1.16 *Bacillus cereus*

Bacillus cereus is a spore forming bacterium widely distributed in soil, water and in food. Various *B. cereus* strains produce different toxins that can cause human illness. Konuma et al. (1988) reported B. cereus presence in 6.6% of raw meats, 18.3% of meat products and 39.1% of meat product additives. Some of the *B. Cereus* toxins remain active after heating at 121°C for 30 minutes.

2.1.17 Anthrax

Most cases of anthrax result from animals ingesting spores present on feedstuffs, but cases may also occur in pigs, dogs and cats following ingestion of meat from animals dying of anthrax. It is unlikely that meat from animals dying from anthrax would find its way into the animal co-products stream. However, meat meal, blood meal or bone meal intended for livestock food could serve as a vehicle for introducing anthrax. A major outbreak of anthrax in the United States was attributed to the feeding of contaminated bone meal to swine, from which the disease then spread to other livestock. Due to the rendering process and handling of rendered fat, the presence of anthrax in finished rendered fat would be remote.

Human anthrax cases have resulted from handling animal products such as “wool sorters disease” caused by inhalation of anthrax spores (Stiles, 2000). Contraction of anthrax by humans is most common through breaks in the skin in contact with infected materials.

2.1.18 Botulism

Large numbers of microorganisms will prevent *Clostridium botulinum* from growing and producing its toxin (Jay, 2000). Botulinum toxin production requires outgrowth of the spore state since the dormant toxin form is located in the spore coat. The neurotoxin causes muscle paralysis with serious complications and death due to suffocation from paralysis of the muscles responsible for breathing. Foods containing the botulinum toxin are often heat-treated to a point that eliminates competitive microorganisms, yet not to a point that destroys the *C. botulinum* spore form. Also, anaerobic conditions with pH above 4.6 (or 4.0 in the presence of yeast) are needed for spore outgrowth.

Egyed et al. (1978) conducted feeding experiments on calves to substantiate the tentative diagnosis of botulism causing the death of over 1000 head of cattle in field outbreaks. Suspected concentrate (poultry waste) was fed to vaccinated and

unvaccinated calves. Despite a lack of toxin isolation from affected calves and the feed, a diagnosis of botulism was established based on clinical and experimental evidence.

2.1.19 Brucellosis

Bacteria of the genus *Brucella* are the causative agents for brucellosis and *B. melitensis* primarily affects sheep and goats, although *B. abortus* and *B. ovis* occasionally infect sheep. Two other species, *B. melitensis* and *B. abortus* can infect dogs and pigs, while cats are unaffected by brucellae. When an animal becomes infected with *Brucella* spp., the bacteria initially spreads throughout the body after which it tends to concentrate in lymph nodes, liver and bone marrow. Brucellosis can be spread by eating uncooked meat infected with the bacterium. Brucellae can survive for extended periods in frozen meat, having survived in meat and salted meat stored at 0 – 20°C for 65 days (Timoney et al., 1988). However, brucellae is very heat sensitive (Jay, 2000).

2.2 Viruses

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2.2.1 Viral Hazards of By-Products

Pathogenic viruses possibly present in animal co-products are Newcastle disease, Chicken Anemia, African Swine Fever, Swine Vesicular Disease, Hog Cholera, Foot and Mouth Disease, Aujeszky Disease and the agent of bovine spongiform encephalopathy.

Epidemiological, clinical, and laboratory studies indicate that viral and rickettsial diseases can be spread through foods and feeds. To assess the relative importance of foodborne viruses, greater knowledge of the mechanisms of transmission, minimal infective dose for man and other animals, sources of contamination, host ranges, ability of animal viruses to infect humans and the ability of viruses to withstand environmental, chemical, or physical inactivation is needed (Blackwell et al., 1985). There are internationally-traded meat and animal co-products that originate in countries with animal diseases not present in the U.S. The viral diseases of most concern are African Swine Fever, Swine Vesicular Disease, Hog Cholera and Foot-and-Mouth Disease. Swine Vesicular Disease outbreaks have been incriminated as being derived from the feeding of garbage contaminated with virus-infected meat.

2.2.2 Foot and Mouth Disease

Foot and Mouth Disease (FMD) virus is distributed throughout the body of the infected animal and can be found in different concentrations for various periods in tissues, secretions, and excretions. Survival of the virus in the slaughtered animal is dependent on the stage of the disease at the time of death, characteristics of the virus strain, and environmental factors such as temperature and pH. The FMD virus is inactivated within 3 days in skeletal muscle after slaughter due to reduced pH. In contrast, the virus may survive for weeks or months in refrigerated internal organs, bone marrow, lymph and hemal nodes, glands, and residual blood. Many FMD outbreaks have been traced back to feeding waste to pigs. A 1967 outbreak in the UK was attributed to the importation of the virus in sheep bone marrow from South America. Even though the pH changes associated with *rigor mortis* will inactivate the virus in muscle, FMD virus may persist in bones and lymph nodes of infected animals. The only way FMD virus in meat is likely to enter livestock in an importing country is if infected tissues are fed to pigs (MacDiarmid and Thompson, 2004).

Heat can inactivate FMD virus in meat and meat products but the heating conditions can influence the degree of inactivation. For instance, retort heating to an internal temperature of 58.3°C inactivated FMD in minced beef containing virus-infected lymph nodes (Blackwell et al., 1988), while higher internal temperatures up to 79.4°C were required for minced meat in nylon tubes. The American Association of Food Hygiene Veterinarians (1990) reported that core temperatures of 93°C were needed to inactivate the FMD virus in heart muscle. The International Animal Health Code (Office International des Epizooties, 1995) recommends maintaining a core temperature of 70°C or greater for at least 30 minutes (Article 4.3.2.1) and specifies

that after deboning and defatting. Other research suggests more extensive thermal treatment may be needed to insure complete inactivation of FMD (Lasta et al., 1992; Masana et al., 1995; Pagliaro et al., 1996).

2.2.3 Rift Valley Fever

The liver is the primary replication site of Rift Valley Fever and high titers are achieved in most susceptible animals (CDC, 2004). Humans can become infected through handling contaminated tissues of diseased animals during slaughter. However, the animals most likely to be fed potentially contaminated meat scraps (pigs, dogs and cats) are relatively resistant to infection with this virus (MacDiarmid and Thompson, 2004).

2.2.4 Rinderpest

The Rinderpest virus in infected meat is usually inactivated rapidly due to a rigor mortis related drop in pH. Pigs may be infected when fed meat scraps contaminated with Rinderpest virus and, because the signs of Rinderpest are mild in pigs, the disease could be overlooked for some time, increasing the chances of infection spreading to other livestock. Rinderpest viruses are heat sensitive and thus, as a group, are unlikely to be present in rendered animal co-products.

2.3 Parasites

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Animal parasites present a danger in the handling of raw animal products in that they can exist in dormant states that can survive harsh environmental conditions. Much like *Salmonella* spp. survive in the processing environment then contaminate rendered material, a similar scenario could manifest for parasites. No references as to safety issues related to parasites in the processing of animal co-products into biodiesel fuel have been found. There is considerable concern, however, about foodborne parasites that are associated with meat and water. Gamble and Murrell (1998) stated that in animal products, *Toxoplasma* spp. (a protozoan parasite), *Taenia* spp. (a larval cestodes, tapeworm) and *Trichinella* spp. (a larval nematode) are of most importance. These parasites are found in a wide range of warm-blooded hosts and can be transmitted to humans by ingestion. *Cryptosporidium* and *Cyclospora* spp. are two other parasites more often associated with water but can be found in meat. A recently reported parasite found in poultry and swine is *Toxocara canis* (Taira et al., (2004). Most parasites pass through a resistant oocyst stage which is accompanied with the relatively complex life cycle. Compared to bacteria, this can make parasites difficult to kill and detect.

Many species of livestock (sheep, goats, and pigs) were reported to be infected with *Toxoplasma gondii* at varying levels (Dubey, 1994). Swine are believed to be the primary source of human infection from food animals (Dubey, 1986), although cats play an important role in non-food infections. Swine had a 10-20% prevalence rate (Dubey, 1986, 1994) with higher rates in breeding stock compared to pigs destined for food. *Toxoplasma gondii* can cause birth defects such as mental retardation and blindness in congenitally-infected children. Serious and fatal encephalitis can occur in immuno-compromised adults, in fact 3-20% of AIDS patients die from toxoplasmosis (Luft et al., 1993). Direct detection of *Toxoplasma* spp. by visual means is difficult since the cysts in food animal tissues are microscopic, randomly distributed in tissues and in most instances is present in very low levels. Thus, the most reliable method for detecting *Toxoplasma* spp. is by biological assays that require grinding and digestion of meat samples followed by inoculation into mice. After 4 weeks the serum of mice are tested for *Toxoplasma* antibodies. Several immunological tests have been evaluated, such as the Sabin-Feldman dye test, agglutination tests, ELISA tests, and DNA probes.

Taenia spp. are meatborne (beef and pork) and develop to the adult stage in the human intestine. Those species of human concern are *Taenia solium* (pork tapeworm) and *Taenia saginata* (beef tapeworm), with *T. solium* of greater concern, since humans can serve as an intermediate host if eggs are ingested (which is not the case with *T. saginata). Taenia* spp. infection in animals is detected by visual inspection of the cut surface of animal muscle and other tissues since the cysts are visible without magnification. While visual detection has remained an effective method, the development of antigen-based methods such as ELISA and DNA methods such a PCR have improved and are likely to be used more in the future.

Trichinellosis in man is caused by ingesting undercooked meat containing infective larvae of *Trichinella* spp. While many warm-blooded species carry *Trichinella* spp., human infection is most often caused by consumption of pork, bear or horse meat. *Trichinella spiralis* is the parasitic species found most often in pigs and thus is of most concern to public health. Prevention of pork-transmitted trichinellosis through inspection has been successful in Western Europe; however, human infection due to infected horse meat have been more frequent over the past 25 years resulting in 13 outbreaks and affecting at least 3200 people (Boireau et al., 2000; Touratier, 2001). Murrell et al. (2004) demonstrated that the transmission of *Trichinella* to horses could occur through feeding infected pork meat to horses and that horses would readily consume infected meat. Inspection and testing for *Trichinella* spp. in swine is required by many countries and long term inspection programs have proven effective in virtually eliminating *Trichinella* from domestic pork supplies (Pozio, 1998). Risk of *Trichinella* contamination still exists in countries with inspection programs since infection in wild animals persists. Pigs are a regular source of human infection in regions where trichinae inspection is not strictly enforced (Gamble and Murrell, 1998). *Trichinella* detection is conducted by viewing cysts from digested animal tissues that are isolated by filtration.

Toxocara canis has a wide range of parent hosts, including mammals and birds. *T. canis* is a significant source of human toxocarosis with 7-30% of children in developed countries showing exposure based on serological tests (Kayes,1997) and is believed to be the most common human parasite in the U.S. (Hotez, 2002). Human toxocarosis is most often caused by ingestion of eggs from contaminated soil in playgrounds or sand (Barriga, 1998). However, the practice by some cultures of eating raw or undercooked meat increases the risk since food animals act as hosts for the parasite (Done et al., 1960; Galvin, 1964). Some examples of foodborne infection include a 63 year-old man in North America diagnosed with toxocarosis likely caused by eating raw lamb liver (Salem and Schantz, 1992), a husband and wife in Switzerland contracting toxocarosis possibly caused by eating raw pig liver (Sturchler et al., 1990), two 22 year-old men in Japan having toxocarosis after eating raw chicken (Nagakura et al., 1989) and the discovery of a *Toxocara* larvae in the ankle of a 26 year-old Japanese woman who had a history of eating raw beef liver (Aragane et al., 1999). Free-ranging pigs and poultry are especially likely to acquire *Toxocara canis* larvae from soil while searching for food (Okoshi and Usui, 1968; Pahari and Sasmal, 1991). *Toxocara canis* larvae have been shown to live in chicken for up to 3.5 years (Galvin, 1964; Tsvetaeva et al.; 1979; Taira et al., 2003a) and in swine for more than one month (Taira et al., 2003b). Therefore, there is risk for spread and transmission of this parasite through the handling of raw animal tissues. Taira et al. (2004) reported that *Toxocara canis* could be transmitted between animal species and that the larvae and eggs migrated to organs such as the liver.

Cryptosporidium spp. oocysts are found in many food animals; therefore, it is likely they are also present in animal co-products. Human cryptosporidiosis has emerged as an important gastrointestinal disease due to ingestion of contaminated water and food containing *Cryptosporidium parvum* (Millar et al, 2002). There are currently 10 known *Cryptosporidium* species (Fayer et al., 2000); *C. parvum*, however, is the predominant cause of human infection (Kosek et al., 2001). *Cryptosporidium* spp. has been reported in more than 40 countries on six continents with approximately 20% of the adult U.S. population having been exposed to the parasite (Kosek et al., 2001). While the oocysts are destroyed by high thermal processing temperatures (>72°C), they are highly

resistant to environmental stress and chlorine-based sanitizers (Harp et al., 1996; Gamble and Murrell, 1998). *Cryptosporidium* spp. can also survive desiccation and freezing. The infectious dose to cause 50% of the population to become infected (ID_{50}) is as low as 9 oocysts and there is little available treatment (Messner et al., 2001). Parasites are generally heat sensitive, thus are unlikely to survive the rendering process.

2.4 Fungi

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Fungi include molds and yeasts, with the molds being multicellular and yeasts typically single cellular, although some yeasts produce some degree of mycelium. Mycelium are branched filaments normally associated with molds. While yeasts and molds are not normally a concern with meat products, there are a few genera that are regularly associated with meats. From a food safety standpoint, the biggest concern with fungi is the production of mycotoxins by some molds that can cause serious illness and are carefully monitored in grain and legume products.

2.5 Microbial Toxins

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Certain prokaryote and eukaryote microorganisms are capable of producing intracellular and extracellular toxins which may affect humans and animals. Microbial toxins include those produced by bacteria and molds (mycotoxins). Bacterial toxins include Staphylococcal enterotoxins, cholera-like enterotoxins, verocytotoxins and Clostridial toxins.

Staphylococcal toxins are produced by *Staphylococcus aureus* which is found widespread on animal skin and in mucous membranes. There are seven types of this toxin (A, B, C1, C2, C3, D, and E), all classified as entero since they act on the intestinal (entero) system. All of these toxins are heat-stable with the C types being more resistant to heat than others. Toxin inactivation requires exposure to approximately 100°C for 30 minutes (Murano, 2003). The Staphylococcal toxin acts on cells lining the intestine, sending a signal to the brain to induce vomiting, but the toxin is not absorbed by the body.

Cholera-like toxins are produced by *Vibrio cholerae*, certain *Escherichia coli* (enterotoxigenic) serotypes and *Campylobacter jejuni*. Cholera-like toxins contain 5 B subunits and 2 A subunits, with the whole toxin having an intestinal wall binding site. Upon binding to the intestinal cell the A subunits split, releasing one subunit into the cell, followed by a series of steps leading to a decreased ability of the cell to absorb sodium chloride. The decreased ability to absorb NaCl causes a fluid imbalance in the intestine, resulting in diarrhea.

Verocytotoxins are produced by enterohemorrhagic *E. coli* serotypes with the most well known producer being *E. coli* O157:H7. The receptor site for the toxin is cells on the kidney thus, once absorbed into the blood stream, the toxin attaches to this organ. This toxin blocks protein synthesis, which can eventually result in a kidney shutdown and serious health problems, including death.

Clostridial toxins are produced by various species of *Clostridium*; the most well know being *C. perfringens* and *C. botulinum.* These toxins are part of the spore coat, thus sporulation and sometimes outgrowth are required to release the toxin. *C. perfrigens* spores can attach to the intestinal wall then sporulate to release the toxin resulting in increased water permeability by the cells and diarrhea. *Clostridium botulinum* produces the most potent natural toxin known and is a neurotoxin causing muscle paralysis. This toxin is produced when the organism forms a spore. Then, the toxin is released when the spore sporulates and the cells outgrow. Sporulation and growth occur under anaerobic conditions and the toxin is highly resistant to heat treatment.

The mycotoxins are formed by fungal species growing either saprophytically or parasitically on organic matter. The mycotoxins are released into the tissues of the host material or remain attached to the mycelium of the fungus. Mycotoxins are produced by a variety of molds which can be mutagenic, carcinogenic, display specific toxic mechanisms or target specific organs (Jay, 2000). One of the most potent types of mycotoxins is known as the aflatoxins (Blüthgen and Heeschen, 1999). Aflatoxins

derive this designation from the species and genus from which this toxin was first identified (*Aspergillus flavus*, A-fla-toxin). This toxin was identified as early as 1960 as being the causative agent for the death of 100,000 turkeys in England that consumed imported peanut meal from South America and Africa (Jay, 2000). *Fusarium* spp. also produces mycotoxins that can lead to serious illness and death. Toxin production is triggered at near freezing temperatures in some cases. Mycotoxin-producing molds are normally associated with grains and not animal products. However, Rundberget et al. (2004) found mycotoxins from 25 species of *Penicillium* in food wastes destined for recycling into animal feed. Mycotoxin levels in the food waste from recycling plants were much lower than those found in household food waste. These researchers, however, recommended monitoring of recycled food waste due to concern that mycotoxins could enter the food chain via animal feed.

3 POTENTIAL ORGANIC CONTAMINANTS IN ANIMAL FATS

3.1 Organochlorine and Polychlorinated Biphenyls

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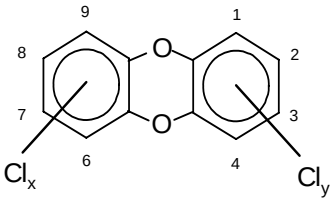
In the production of biodiesel from either animal or vegetable fat, the potential effects of organic contaminants on human and environmental health through the collection, production, transportation and use of the product must be known to ensure safety. A variety of organic compounds in use around the globe for a number of purposes have the potential for contaminating fats and oils; the most important of these compounds are pesticides. The following literature review details the current knowledge on the effect of organochlorines (including dioxins), furans, organophosphates, carbamates, nicotine, 3-methyl indole, 3-ipomeanol, and gossypol in animal fats and proposes potential safety concerns to be addressed.

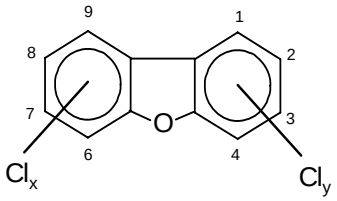
Pesticide development evolved during the early 20th century. After World War II, numerous synthetic organochlorine, organophosphorus and carbamate insecticides were developed along with a variety of herbicides and fungicides. As these compounds were used, it became evident that health risks were associated with chemical residues in foods. Risk assessment and various facets of controlling the public health risks of pesticide residues have resulted from the development and use of many pesticides (Waltner-Toews and McEwen, 1994a; Waltner-Toews and McEwen, 1994b; and Waltner-Toews and McEwen, 1994d).

Today, insecticides and herbicides are utilized in modern agricultural practices to assist in the production of plant crops. Additionally, insecticides are applied to domestic animals for controlling bothersome and disease-spreading insects. When applied topically to an animal, insecticides may be removed or partially removed by volatilization, rubbing off, washing off during rain or in waterways, or by atmospheric decomposition. If the insecticide penetrates the skin or is ingested by licking the topically applied insecticide or consuming contaminated grains or forages, the insecticide is subjected to metabolic attack, which may alter or destroy the insecticide. In modern production, plants are routinely treated with insecticides, herbicides, nematocides, and/or fungicides. The longevity of the pesticide chemical helps to determine the residual content in the harvested plant product (Kuhr and Dorough, 1976).

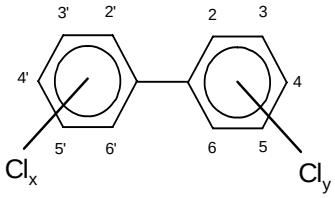
Three classes of organohalide chemicals that have similar biological and toxicological properties may often be termed “dioxins.” The general structures of the polychlorinated dibenzo-*p*-dioxins (PCDD), the dibenzofurans (PCDF) and the polychlorinated biphenyls (PCB) are (Fries, 1995a):



polychlorinated dibenzo-*p*-dioxins (PCDD)



polychlorinated dibenzofurans (PCDF)



polychlorinated biphenyls (PCB)

Dioxins and furans are described as the most toxic class of human generated chemicals. Upon ingestion, these compounds bioaccumulate in fatty tissues and are very difficult to eliminate. Potential health effects of exposure to dioxins and furans are liver enlargement, liver lesions, immunotoxicology, wasting syndromes, thymic and splenic atrophy, tissue specific hypo- and hyperplastic responses, carcinogenesis, and possibly death. Health effects can occur for years after initial exposure (Whitaker and Willett, 1999). Many organochlorine pesticides, polychlorinated biphenyls, dioxins and polybrominated diphenyl ethers act as endrocrine disrupting compounds (Rhind, 2002). Persistent organic pollutants may act as pseudohormones and elicit a variety of maladies such as birth defects, breast cancer, early sexual development, lowered sperm counts, behavioral changes, and reduced intelligence (Manirakiza et al., 2002). Worldwide, incidents of dioxin and polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans have occurred. Campagna et al. (2002) reported impaired sperm function and development and suppressed development in swine.

Although there are 210 different congeners of polychlorinated dibenzoparadioxins and furans, 17 of these compounds (10 furans and 7 dioxins) are of interest as food contaminants (Blüthgen and Heeschen, 1999). The most toxic of the dioxins is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is most commonly referred to as “dioxin” (Roeder et al., 1998). Because of similarities in structure and biological properties, polychlorinated-dibenzo-*p*-dioxins, polychlorinated dibenzofurans and polychlorinated biphenyls have been labeled as “dioxins” (Feil and Ellis, 1998).

Dioxins and furans may be formed by non-anthropogenic means; almost every combustion process including woodland fires results in release of minute levels of polychlorinated dibenzoparadioxins and furans. However, two of the largest sources of dioxin and furan pollution are municipal waste incinerators and steel sintering plants. The contamination builds up in the environment and upon ingestion of contaminated forages, the animal or human bioaccumulates the compounds (Whitaker and Willett, 1999).

Organochlorine compounds have been banned in most industrialized countries. In 1973, DDT was banned in the United States and sale of dieldrin was discontinued in 1987 (Smith and Tramontin, 1995). In the state of Arizona in the United States, a moratorium was placed on agricultural use of DDT in 1969. Analyses of cattle carcass fat residues indicated a decline in residues following the moratorium. A test method was developed to correlate blood pesticide residue levels with carcass fat residue levels (Ware et al., 1975). In Australia, the following organochlorine compounds are banned: DDT/DDD/DDE, aldrin, dieldrin, endrin, chlordane/oxychlordane, HCB, lindane, heptachlor/heptachlor epoxide, methoxychlor, and BHC (a and b) (Zigterman and Crook, 2005).

Maximum Residue Limits (MRLs) have been established in most countries as the legally defined limits permissible for these chemicals in meat and other foods (Zigterman and Crook, 2005). The half-life in soil of these organochlorine compounds ranges from 3 to 15 years for DDT/DDD/DDE, 2 to 8 years for Aldrin and Dieldrin, 2 to 5 years for Endrin, 5 to 12 years for chlordane/oxychlordane, 5 to 12 years for heptachlor/heptachlor epoxide, 7 to 15 years for hexachlorbenzene (HCB), 1 to 3 years for lindane, and 2 to 3 years for BHC (a and b) (Zigterman and Crook, 2005). If soils are heavily contaminated, estimates for reduction to safe residue levels can take many decades. An example given by Zigterman and Crook (2005), states that if an initial concentration is 800 mg/kg, then it can take up to 112 years to reduce the concentration of some of these organochlorine compounds to below the MRL of 0.1 mg/kg. In Australia, the MRLs in fat of meat are: 5.0 mg/kg for DDT/DDD/DDE, 0.2 mg/kg for aldrin and dieldrin, 0.2 mg/kg for chlordane/oxychlordane, 0.2 mg/kg for heptachlor/heptachlor epoxide, 1 mg/kg for HCB, 2.0 mg/kg for lindane, 3.0 mg/kg for methoxychlor, and 0.3 mg/kg for BHC (a and b) (Zigterman and Crook, 2005).

Organochlorines are persistent compounds. One of the most persistent, DDT, was banned in the United States in 1973. In 1971, a study was conducted on a field heavily contaminated with dichlorodiphenyltrichloroethane (DDT) to determine the effects of deep plowing, flooding and organic matter applications on DDT concentrations. Twenty-three years later, in 1994, the same site was re-visited to determine residual soil concentrations of DDT. Results indicated that all DDT isomers had decreased and 1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene (DDE) was the major remaining residue. At the surface, 10-28% of the original residues remained from 23 years prior. However, deep plowing methods employed in the 1971 study apparently deposited the DDT deeper into the soil and protected it from degradation. Significant levels of residues were measured in the atmosphere above the field, indicating that volatization was occurring. The continued volatization of the residues from the field surface created concern for long range DDT contamination of humans, animals, forages or feedstuffs (Spencer et al., 1996).

Dioxins and furans are strongly lipophilic and deposit in the fat. In general, there is a 10-20% carry over rate of dioxins on forages to dioxin in dairy milkfat although some

specific congeners can have carry over rates as high as 60%. Although one of the most efficient methods of excreting dioxins and furans is via milk production (Whitaker and Willett, 1999), the excretion half-life of dioxins in milkfat is approximately 4 months (Blüthgen and Heeschen, 1999). In a study conducted in The Netherlands, Olling et al. (1991) administered eight labeled dioxins and furans to four lactating cows; organochlorine compounds were dissolved in olive oil and the mixture was given in a single dose. Concentrations of congeners were monitored in the milk and body fat for the subsequent 93 days. Most congeners had mean elimination half-lives of 27 to 49 days (Olling et al., 1991). In a continuing study, Derks et al. (1994) developed a pharmokinetic model for polychlorinated dibenzo-p-dioxins using 2,3,7,8-TCDD in cows. The uptake into tissues was deemed limited by blood flow with the notable exception of adipose tissue (Derks et al., 1994).

Organochlorines also are excreted via feces. Unabsorbed material is eliminated within 48 hours of dose. Additionally, compounds that have been metabolized by the liver are excreted via the bile into the digestive tract and are eliminated. However, this mode of elimination allows for only very small amounts to be excreted. Most livestock exposure is caused by accidental contamination of a feed source such as storage in a wooden structure built from pentachlorophenol-treated wood or via forages contaminated by emissions from combustion (Whitaker and Willett, 1999; Feil et al., 2000; Fries et al., 2002). Additionally, intake of contaminated soils may be implicated in animal contamination (Dixon et al., 2000).

Pentachlorophenol (PCP) was a compound used extensively in the United States for a variety of industrial uses including as a bacteriocide and fungicide on paper, paperboard, adhesives, leather, latex paints, rope, ink and most commonly as a wood preservative. Technical grade PCP also contained impurities of dioxins and furans. Studies indicated that ingestion of PCP by cows leads to residues of polychlorodibenzo-*p*-dioxin and pentachlorophenol residues in milk and blood (Firestone et al., 1979). In the United States, eggs produced in backyards near Oroville, CA had increased polychlorinated dibenzo-*p*-dioxin and polychlorinated dibenzofuran levels. Nearby, a former pentachlorophenol (PCP) wood treatment facility and four former PCP waste incinerators were attributed as the cause of the environmental contamination which led to contamination of the eggs (Harnly et al., 2000).

Numerous methods have been developed for analyzing rendered animal fats for organochlorine pesticide residues, including supercritical fluid extractions, and gas chromatography methods (Ashraf-Khorassani and Taylor, 1996). Heath and Black (1980) worked on improvements to methods for the assisted distillation clean up of pesticide residues in animal fats. Methods for simultaneously extracting pesticides from animal fats for analysis were described by Neidert and Sashenbrecker (1984). Sherma and Boldnieks (1990) investigated quantitative thin layer chromatographic (TLC) methods for determining pentachlorophenol pesticide residues in tallow. Goodspeed and Chestnut (1991) used a gel permeation chromatographic procedure to improve repeatability in organohalide analyses. Chen et al. (2003) studied methods of optimizing solvent extraction for determining chlorinated pesticides in animal feed. Dionex, a manufacturer of analytical instruments, developed an accelerated solvent extraction (ASE®) method for rapidly determining organochlorine pesticides in animal feeds (Dionex, 2004).

Organochlorine pesticides and polychlorinated biphenyls are detected in almost every living organism (Covaci et al., 2004). Dioxins and furans are considered ubiquitous

contaminants in the environment. The recognized primary human exposure route is via ingestion of animal fats (Guruge et al., 2003). The U.S. Environmental Protection Agency (EPA) estimated that over 90% of an individual's exposure to dioxin-like compounds was due to ingestion of foods and especially beef, poultry, pork, eggs, fish, milk, and dairy products (Winters et al., 1996). In a U.S. Food and Drug Administration (FDA) sponsored study of 17 dioxin congeners in commercial dairy and fish/shellfish products (butter, cheese, crustacea, catfish, other fish, ice cream, milk, mollusks, shrimp, and yogurt), it was found that all products, with the exception of catfish, had very low and often unquantifiable levels of dioxins/furans. Catfish had the highest levels of dioxin as expressed in terms of 2, 3, 7, 8- TCDD toxicity (TEQ). The TEQs are used to indicate relative toxicity of all dioxin and furan congeners in a food in relation to the toxicity of 2, 3, 7, 8- TCDD. Mean exposure for average catfish consumption of uncontaminated fish was 21 picograms of dioxin per person per day. However, if contaminated fish were consumed, people were exposed to approximately 234 picograms of dioxin per person per day. It was believed that the high levels of dioxin in the catfish were from use of ball clay as a feed additive and not from rendered animal sources. The ball clay originated from mines that had higher than expected levels of dioxins/furans; the source of the elevated dioxin/furan levels in the clay mine was not known (Ferrario et al., 2002; Jensen and Bolger, 2001).

Farm-raised salmon production increased by a factor of 40 over the past two decades. Between 1987 and 1999, United States salmon consumption rates increased by 23% and European Union rates increased by 14%. More than half of the salmon is now farm-raised in the United States, Chile, Canada and Northern Europe. In a study conducted by Hites et al. (2004), 700 farmed and wild salmon were collected from locations around the globe. On average, farm-raised salmon from North America and Europe had higher significantly higher levels of 14 organochlorine contaminants than wild salmon. The levels of PCB, dioxin, toxaphene, and dieldrin were lowest in farm-raised salmon from Chile and Washington State (U.S.) and highest in farm-raised fish from Scotland and the Faroe Islands. Contaminants were significantly higher in farm-raised salmon from Europe than in farm-raised salmon from North and South America. Upon analysis of fish feeds, concentrations of contaminants were higher in European fishmeals and fish oils than in comparable North or South American fish feeds. Fish feed used in this study was obtained primarily from small pelagic fish and not from rendered animal by-products (Hites et al., 2004a).

In a 1997 survey of dioxin-like compounds in U.S. poultry fat, the U.S. Department of Agriculture (USDA) and the U.S. Environmental Protection Agency (EPA) measured the rate of occurrence and concentration of chlorinated dibenzo-p-dioxins (CDDs), and chlorinated dibenzofurans in poultry fat. Two out of 80 birds had elevated concentrations of all congeners and were considered to be statistical outliers. The remaining 78 birds tested were comparable to previously reported levels for poultry, beef and pork fat and ranged from 0.40 to 0.99 picograms per gram lipid (Ferrario et al., 1997).

From 1997 to 1999, several cases of dioxin contamination occurred in foods of animal origin in Europe. The source of the contamination was dioxin contaminated citrus pulp pellets, fat containing polychlorinated biphenyls (PCBs), and kaolinitic clay, which had been used as an anti-caking agent in the animal feed. In a study conducted in Switzerland on the levels of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F), poultry, eggs and pork were the most affected food products, whereas no

cow's milk or bovine meat had increased levels of dioxins or furans (Schmid et al., 2002). In Belgium, dioxins and furans were detected in chickens from the mid to end of 1999 (Luthhardt and Schulte, 2001).

In a study conducted by Kelly et al. (2001), ten cattle were dosed daily with five dioxin/furan congeners over a four week period. Four animals were not treated and were designated as control animals. Upon slaughtering at 5, 18, and 31 weeks after initial dose, the concentration of congeners in animal tissues was measured. At five weeks after dosing, all congeners had increased in concentration in animal tissues. After 18 weeks, concentrations were reduced and by 31 weeks the levels had reduced slightly further. The half-lives of the congeners were calculated to be 13 to 21 weeks; however, the concentrations of congeners differed depending upon the tissue sampled. At 5 weeks, approximately 50% of the dosed levels remained in the subcutaneous fat and perirenal fat. However, at 5 weeks, the concentrations on a fat basis in the muscle tissue and liver were 5 to 10 times higher than in subcutaneous and perirenal fat deposits. At 18 to 31 weeks, the levels in muscle tissue and liver were approximately double the levels found in the fat deposits (Kelly et al., 2001).

A similar study was conducted by Feil et al. (2000) where calves were fed polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans for 120 days at levels slightly higher than would be expected in forages in regions near waste incinerators. In the study, the calves retained 30-50% of the dosed dioxins and furans. Congeners were isolated from back fat, perirenal fat, and ribeye fat (Feil et al., 2000).

In The Netherlands, a small area known as Lickebaert was contaminated with dioxins due to a waste incineration plant. Milk cows in this area excreted levels of dioxins and furans higher than the allowable tolerance level. Studies indicated that dioxin could have an excreted half-life of up to 87 days when contaminated feeds were fed to dry cows prior to calving. Hexachlorinated congeners tended to have the longest elimination half-life (Tuinstra et al., 1992).

Other reports of contamination were associated with feed or spraying animals with insecticidal sprays. In 1992, 8 of 90 mixed-breed feeder calves suddenly developed unusual neurological symptoms. Aldrin was detected in the feed from an undisclosed source and implicated as the causative agent. Aldrin and dieldrin are biotransformed in the liver thereby making tissue tracing difficult (Furusawa and Morita, 2001). Ten calves died from this incident and studies on the body fat of the animals revealed that the herd was clear of aldrin/dieldrin 18 months after exposure (Casteel et al., 1993). In July 1994, two Holstein heifers died after accidentally consuming pesticide that had been stored in a barn in a poorly secured container for more than 20 years. Upon analysis, it was determined that the bottle of pesticide contained endosulfan, dieldrin, DDT and DDE (Smith and Tramontin, 1995). In 1997, Kelch and Kerr reported an incident of two cattle dying from using an illegal lice spray containing endosulfan. In another case occurring in Turkey, endosulfan contamination resulted in two of eleven treated cattle dying within 2 days with tremendous suffering (Mor and Ozmen, 2003).

In Austria, DDT, DDE, dieldrin, aldrin, heptachlor and other organochlorine pesticides were detected in only extremely small amounts in kidney fat of pigs and cattle. The levels detected were considerably below the Austrian maximum allowed residue levels and since these pesticides are banned in Austria, further reductions in residue levels over time were predicted (Kofer and Fuchs, 1994). In Spain, a surveillance program for

PCDDs, PCDFs and PCBs indicated that beef, milk, egg, chicken, pork and olive oil samples all were below the required limits (Eljarrat et al., 2002b).

In neonatal calves, uptake of organochlorine compounds was studied using different congeners in cow colostrums. One hour old control calves absorbed 20-37% of the ingested IgG from the colostrums, whereas 65 hour old calves absorbed only 2% of the IgG leading the researchers to conclude that the calf gut absorption mechanisms had changed over the 65 hour period. However, in the experimental calves fed organochlorines, plasma concentrations of the compounds did not change over the same 65 hour period. Researchers concluded that uptake mechanisms for organochlorine compounds are not affected by changes in the gut in neonatal calves (Keller et al., 2001).

In an earlier study concerning the distribution of chlorinated pesticides in animal feeds, Pierson et al. (1982) evaluated grains, grasses, animal co-products and other feed components for contamination with lindane, β-BHC, heptachlor, aldrin, heptachlor epoxide, p,p'-DDE, o,p'-DDT, p,p'-DDD, and p,p'-DDT. Data was collected over a 7 year period from more than 6500 samples. Corn and soybean meals had the lowest contamination, whereas oats and alfalfa had a higher incidence of heptachlor epoxide and dieldrin than expected. Wheat middlings and animal by-products had the highest levels; however, all feed components had low levels of organochlorine pesticides. All feed components and finished feeds were appreciably below the US FDA action limits for pesticide residues. Additionally, a comparison of data from 1972 to 1975 to data from 1976 to 1980 indicated that chlorinated pesticide levels in animal feeds and feed components were decreasing (Pierson et al., 1982).

Analysis of fish and cattle fat from the Sene-Gambian region revealed levels comparable to other African studies and were comparable to levels found in other developing countries. Organochlorine moieties were detected in fish but only very low levels of the same compounds were detected in shrimp and cattle fat. Concerns about bioaccumulation were raised and levels of organochlorine compounds were measured in human serum. In these areas of the world, the World Health Organization (WHO) recommends the use of dichlorodiphenyltrichloroethane (DDT) and lindane for control of tropical vector-borne diseases. It was suggested that if these compounds were to be banned, clandestine use would likely continue in developing countries (Manirakiza et al., 2002).

Australian researchers have noted that, in sheep, organochlorine contamination has been associated with consumption of contaminated soils. These soils had most commonly been former horticultural areas or point sources from termite treatments. The levels of organochlorine compounds in sheep were related to level of intake, amount of fat on the animal and stage of life (growing, lactating, losing weight). Residues also can appear in wool. In Australia, the maximum residue limit (MRL) for dieldrin is 0.2 mg per kg of fat (ppm) and for DDT is 5 mg per kg of fat. Research indicates that sheep reduce levels of inherent organochlorine compounds more rapidly than cattle when removed to a clean grazing area (Dixon et al., 2000a; Dixon et al., 2000b).

Willett et al. (1993) noted that cattle grazed on land previously used as orchards had detectable levels of 1,1-dichloro-2,2-bis(p-chlorphenyl) ethylene (DDE). The researchers concluded, however, that levels in the animals were higher than could be accounted for by ingestion of contaminated soil. The study continued by investigating

the grass as the source of the contamination but concluded that surface residues on the grass were insignificant. However, grass internal tissue DDE levels were associated with soil concentrations. Air and soil temperature, relative humidity, solar radiation or amount of dry biomass harvested did not affect DDE concentrations. Precipitation increased volatization from the soil and allowed greater deposition on grass (Willett et al., 1993). Application of sludge to crops allows lipophilic organohalogen compounds to be transported into the consuming animal (Fries, 1996a). In many parts of the world, soils contain appreciable concentrations of dioxins and furans. Studies continue on the bioavailability of these compounds from soils (Ruby et al., 2004).

In a report by Spence, et al. (1998), steers that had accidentally consumed chlorfluazuron (CFZ) contaminated cotton were observed and tested for residues. The CFZ levels in the cattle very slowly declined but even by 340 days, significant CFZ levels remained. It was concluded that any non-lacting cattle contaminated with chlorfluazuron would likely remain contaminated indefinitely (Spence et al., 1998). Cattle farmers were urged to use caution in choosing alternative feeds when drought conditions make hay and other feeds scarce (Anonymous, 2003c).

Using chickens as a model for foraging animals, Stephens et al. (1995) determined that chickens foraging on soil contaminated with low levels of PCDD/PCDF had an intake rate of approximately 2.5 ng per kg per day. It was noted that the bioavailability of the congeners was dependent on the level of chlorination; bioavailability was 80% for tetrachlorinated congeners and less than 10% for octachlorinated congeners. Approximately 5-30% of the intake was excreted in the eggs, 7-54% was deposited in the adipose tissue and less than 1% was measured in the liver. However, on a fat basis, the highest concentrations were measured in the liver. This study concluded that animals foraging on soils contaminated with even low levels of PCDD/PCDF bioaccumulated unsafe levels of these compounds (Stephens et al., 1995).

Fries (1996) used a model to predict the concentrations of dioxins in growing pigs. In this study, as constant levels of contaminants were supplied in feed, the animals deposited maximum concentrations in the body fat until 30 kg weight; thereafter, the concentration declined until 100 kg which was the typical slaughter weight. Results of modeling indicated that attempts to produce leaner animals may result in greater lipophilic residue transmission to humans (Fries, 1996). Using gas chromatography-mass spectroscopy, Schecter and Päpke (1998) analyzed raw potatoes and potatoes fried in lard for 2,3,7,8-TCDD and a number of other dioxin and dibenzofuran congeners. In general, cooking caused increased levels for the compounds in potatoes, most likely due to transfer of lard to the potatoes (Schecter and Päpke, 1998).

Not surprisingly, polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and polychlorinated biphenyls have been detected in high trophic level animals such as scavenging birds. All of these chlorinated compounds were detected in black and turkey vultures at the Savannah River Site in South Carolina (Kumar et al., 2003).

Since their industrial debut in the 1930s, the polychlorinated biphenyls (PCBs) have become ubiquitous in the environment. Risk assessment of the PCB congeners is very difficult since over 2 million tons of these compounds were used in the United States as mixtures of 60 to 90 different congeners. Industrial products included Aroclor in the U.S. and Great Britain, Fenclor in Italy, Pyralene in France, Keneclor in Japan, Clophen in Germany, and Solvol in the USSR (Kim et al., 2003).

Although much research has been conducted on levels of organochlorine compounds in foods, animal tissues, livestock feeds, and the environment, these studies do not indicate the effect of organochlorine contamination on the safety of producing biodiesel. Organochlorine compounds are ubiquitous in soil, plants and animals. However, perhaps the most pertinent research for assessing the safety of using animal fats for the manufacture of biodiesel was a study conducted in Spain by Eljarrat et al. (2002). In this study, feed ingredients of animal origin were analyzed for PCDD and PCDF content. Hemoglobin, animal fat, fish oil, fish meal, meat and bone meal were analyzed and compared to samples of mineral origin such as kaolin, bentonite, magnesite, zeolite, sepiolite, and damoline. The mineral origin substances are used as binders or anti-caking agents in animal feeds. Results of the study indicated that for samples of animal origin, the levels of PCDDs and PCDFs ranged from 0.52 to 9.08 picograms WHO-TEQ per gram of fat. However, for samples of mineral origin, the levels ranged from 0.05 to 460.59 pg WHO-TEQ per gram. Kaolin samples contained the highest levels of dioxin contamination (Eljarrat et al., 2002a).

In all of the studies reviewed, low levels of organochlorine compounds were ubiquitous in nature and were detected in almost all animal fats. In countries where organochlorine use has been banned, the levels of these compounds appear to be continually diminishing in the environment but, in general, all of the organochlorine compounds tend to persist (Mahr, 2004). Additionally, the background human exposure to dioxin-like compounds continues to decline. Approximately 90% of human exposure to dioxins is through ingestion of foods: meats, fish and dairy products account for approximately equal amounts of human dietary exposure. In Europe, daily intake of these compounds decreased by approximately 60% from the 1980s to the mid-1990s. Since the restriction of the use of these compounds, the only acute toxic episodes to organochlorine compounds reported in livestock were associated with accidental exposure. Only a few references were located that discussed organochlorine levels specifically in rendered animal fats (Brooks et al., 2000; Eljarrat et al., 2002a; Grochowalski and Chrzaszcz, 2000; Malisch, 1998; and Rappe et al., 1998). Each of these studies indicated very low levels of organochlorine compounds were present. No literature was located that indicated the fate of organochlorine compounds through the production and use of biodiesel made from animal fats or vegetable fats.

3.2 Organophosphates

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Organophosphates are a class of cholinesterase-inhibiting pesticide which account for more than one third of all commercially available registered pesticides. Most usage in the United States is for agriculture and mosquito control. Although more than 70 organophosphates are available, only about 10 to 15 chemicals comprise 75% of use: azinphos-methyl, chlopyrifos, fonophos, malathion, methyl parathion, phorate, phosalone, and terbufos for agricultural uses and fenthion, malathion, naled and temephos for mosquito control (Marr and Ballantyne, 2004).

Organophosphates act with acute lethal toxicity. Farageelawar and Blaker (1992) reported that carbamates could cause long-term neurophysiological impairments**.** The above listed organophosphates are among the most highly toxic of the organophosphates. The principle mode of toxicity of organophosphates is inhibition of enzyme acetylcholinesterase at nerve synapses. The organophosphate binds to the enzyme and prevents breakdown of the neurotransmitter acetylcholine and leads to an accumulation of the compound in the synapse. The result is overstimulation of the nervous system. In mammals and birds, symptoms of cholinesterase-inhibition toxicity are labored breathing, vomiting, diarrhea, tremors, convulsions, copious bronchial secretions, lethargy and death (Hill, 2003). Short term doses of organophosphate toxicity can cause paralysis of the neck, limbs and respiratory system. Another result of single dose exposure to organophosphates is damage to the myelin sheath of nerves and the spinal cord. This syndrome is known as organophosphate induced delayed neurotoxicity (OPIDN). Most of the known causes of OPIDN have been removed from the market (leptophos and mipafox); however, a few (cyanofenphos, methamidophos, trichlorfon, trichloronate) are still used in some countries around the world. Organophosphates have been implicated in mutagenicity, carcinogenicity and toxicity to the heart and kidneys (Hill, 2003).

In general, organophosphates have low environmental persistence (Mahr, 2004). However, with widespread use, organophosphates have been detected in foods of both animal and plant origin. Organophosphate residues have been isolated from vegetable fats and oils (Lacoste and Raoux, 2003) and soy based infant formulae (Cressey and Vannoort, 2003).

Research has indicated that cooking or steaming can degrade a major percentage of organophosphate contaminants in foods. In a study conducted by Coulibaly and Smith, beef muscle contaminated with 50 ppm organophosphate pesticides was cooked in water to 70ºC or 80ºC. Upon analysis by HPLC, organophosphate retrieval levels ranged from 64.58.5% from the raw meat, from 30.0-87.4% in the cooked meat and from 10.6-107.2% in the water (Coulibaly, K. and Smith, J.S., 1993). Researchers determined that organophosphates had been degraded with heat but that considerable levels remained in the cooked beef and water. Changes in pH also caused degradation of organophosphate and simultaneous increase in pH and cooking yielded the greatest destruction of organophosphate contaminants (Coulibaly, K. and Smith, J.S., 1994). In a second study, Coulibaly and Smith added organophosphate compounds to beef at a

rate of 1 ppm. The researchers adjusted the pH of the meat from 5.5 to 4.5 or 6.5 and cooked to 71ºC or 77ºC. Adjusting pH resulted in increased degradation of organophosphates; however, some were more susceptible to increased pH and some were more susceptible to decreased pH (Coulibaly, K. and J.S. Smith 1993). Organophosphate compounds also may be degraded by soil microorganisms, enzymes and photolysis.

Rendering of animal fats for use in biodiesel manufacture affords a significant heat process. No studies were located specifically studying the effect of rendering on organophosphate contamination in animal fats.

3.3 Carbamates

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Carbamates are one of two major classes of cholinesterase-inhibiting pesticides (Marr and Ballantyne, 2004). First introduced in the 1950s, carbamates have since been used as insecticides, herbicides, fungicides, nematocides, sprout inhibitors, household products and as biocides for industrial applications (International Programme on Chemical Safety, 1986). The general structure of carbamates is:

$$R^1NH\text{-}\,C\,(=O)\,\text{-}\,OR^2$$

with R^1 and R^2 representing alkyl or aryl groups (International Programme on Chemical Safety, 1986; Schuphan and Casida, 1979). Three classes of carbamate pesticides have been produced: 1) insecticides and nematocides; 2) herbicides and sprout inhibitors and 3) fungicides. Approximately 50 different commercial carbamate formulations have been generated. Carbamates prevent the breakdown of the neurotransmitter acetylcholine by inhibiting the enzyme acetylcholinesterase. As a result, acetylcholine accumulates in the nerve synapse and, thus, prevents nerve impulse transmission. Although each carbamate compound has different characteristics, in general, carbamates are chemically stable, have low water solubility and low vapor pressure. In general, the carbamates are not persistent and have fairly short half-lives (Mahr, 2004). Carbamates are rapidly degraded in light due light absorption characteristics (Crosby et al., 1965; Bachman and Patterson, 1999) and are heat labile (Santos Delgado et al., 2001). Microorganisms, plants and animals can metabolize carbamates (Hua and Pfalzer-Thompson, 2001) and moisture levels affect the rate of absorption and biodegradation of certain carbamates (carbofuran) in soils (Shelton and Parkin, 1991). Because carbamates are not stable in aquatic conditions and rapidly degrade, in 1986 it was believed carbamates would not significantly bioaccumulate in food chains (International Programme on Chemical Safety, 1986). Later studies indicate a growing risk to soil and groundwater due to increasing use of carbamates as an alternative to organochlorine pesticides (Bogialli et al., 2004; Hua and Pfalzer-Thompson, 2001; Wei et al., 2001). Some carbamates are suspected mutagens and carcinogens (Sikka and Florczyk, 1978) and some such as aldicarb and carbofuran have high acute toxicity (Bogialli et al., 2004). Studies on long-term human exposure to organophosphates and carbamates indicated that certain people are genetically predisposed to greater sensitivity to anticholinesterase pesticide toxicity (Hernández et al., 2004). Certain carbamates can be lethal. Exposure to carbamates during the 3rd and 4th month of gestation was correlated with elevated risk for fetal death (Bell et al., 2001). Aldicarb was implicated in the rapid death of lactating dairy cattle in an incident in the early 1990s (Kerr et al., 1991). Benomyl, a carbamate used as a fungicide, is a neurotoxin, hepatotoxin, tertatogen, mutagen and carcinogen (Dalvi

and Whittiker, 1995). In yet another study, carbofuran was determined to be non-mutagenic (Food and Agriculture Organization of the United Nations and World Health Organization, 1979). Colosio et al. (1999) conducted a review of literature regarding the effect of pesticide exposure on the immune system. In this review, the only reported immunological alteration from carbamates was in a case of chronic aldicarb consumption by 23 women; slightly altered immune cell numbers were observed (Colosio et al., 1999). Farageelawar and Blaker (1992) reported that carbamates could cause long-term neurophysiological impairments. In an interesting note, Dow et al. (1994) determined that certain carbamate metabolites of mofegeline could irreversibly inhibit monamine oxidase B. This chemotherapeutic agent has been investigated for treatment of Parkinson's disease and was most likely was a forerunner of selegiline which is used in treating human disorders as well as canine cognitive disorder. Another medicinal use for carbamates reported was in a mixture of other drugs for the treatment for organophosphate poisoning (Doctor et al., 1993).

Overall, carbamates tend to be fairly short-lived in nature (2 to 4 weeks) and are metabolized and excreted by animals (Bogialli et al., 2004, Barra Caracciolo et al., 2002). Minerals in soil such as Fe^{II} tend to catalyze the reduction of certain carbamates (Strathmann and Stone, 2003). Clay minerals in the soil can contribute to the hydrolysis of carbamate pesticides (Wei et al., 2001) and microbial transformation has been observed in soils (Das and Mukherjee, 2000). Although pesticides are known to be both retained and mobile in soils (Weber et al., 2004), pesticides also are known to volatilize from sprayed fields (Walden and Haith, 2003) and may account for high levels of carbamate pesticides that have been measured in rainwater. In a study in the Netherlands, nearly 2000 times the maximum permissible concentrations of carbamate pesticides were measured in rainwater from an area with intensive horticultural activities (Hamers et al., 2001). Increasing organic matter in the soil was determined to increase the amount of carbamate adsorption into soil (Bansal, 2004).

One of the challenges of studying pesticide contamination is the creation of methodology for accurately quantitating the substance in different media. Carbamate pesticide run-off in rivers and streams was measured using a new method devised by Wang and Budde (2001). In this study, significant quantities of carbamate pesticides were detected. Bogialli et al. (2004) developed a hot water extraction method for analyzing carbamate residues in bovine milk. After extraction, the residues were quantitated using liquid chromatography-mass spectrometry. Del Carlo et al. (2004) developed an electrochemical enzyme inhibition assay for screening food samples for both carbamate and organophosphate pesticides. Argauer et al. (1995) developed a supercritical fluid extraction protocol for separating carbamates from meats. Subsequent detection using high pressure liquid chromatography with fluorescence detection and gas chromatography with ion trap mass spectrometry was employed.

Examination of live animal and animal products in Sweden revealed no carbamate concentrations above maximum allowable residue limits in sampled tissues (Norlander and Frisell, 2000). Early studies conducted in the 1960s on dairy animals using colorimetric methods of analysis (sensitivity to 0.01 ppm), indicated that feeding animals technical carbaryl for two weeks at 50, 150 and 450 ppm of their daily roughage resulted in no detectable carbamates in milk or meat one hour post-treatment. Additional trials indicated that feeding and repeated spraying of 0.5% carbaryl resulted in no detectable residues in milk or body fat. However, increasing the concentration to 1% resulted in detectable carbaryl in milk for the first 24 hours post application, but no

detectable residues thereafter. After dusting cows with 50% carbaryl, residues were detected only in the first hour after treatment (Kuhr and Dorough, 1976).

In a study by Kuhr and Dorough (1976), feeding carbamate compounds to animals resulted in low levels of carbamate compounds in meat and milk. However, scientists concluded that if carbamate compounds are used according to label instructions, no carbamate compounds should appear in meat, milk or eggs (Kuhr and Dorough, 1976).

Additional studies were conducted on spraying hogs, sheep, goats and cattle with 1% carbaryl, slaughtering after a 1 to 7 day holding period and examining residues in fat, muscle, liver, heart, kidney and brain. Residues were detectable if the animal was treated within 24 hours prior to slaughter. However, with the exception of one goat, no detectable carbaryl or its metabolites 1-naphthol or 1-naphthol conjugates were detected by colorimetric methods in those animals slaughtered at least 24 hours after last application (Kuhr and Dorough, 1976).

Carbamate residues have been noted in fruit and vegetable materials (Marrs, 2000). In another study, soybean samples contained detectable carbamate residues but no residues were detected in either soy oil or meal (Food and Agriculture Organization of the United Nations and World Health Organization, 1983).

These studies indicate the possibility that carbamates may be present in both animal fats and vegetable fats/oils. Further studies are needed on carbamates in fats/oils destined for biodiesel production. However, information provided in the literature indicates that carbamates are heat labile; therefore, heat treatments conducted during the rendering of animal fats should presumably destroy any carbamate residues. No direct research was reported to address this issue and, consequently, conclusions are speculative. Further research is needed to determine the fate of carbamates in animal fats through the rendering process and subsequent biodiesel manufacture.

3.4 Polybrominated Biphenyls (PBBS) and Polybrominated Diphenyl Ethers (PBDES)

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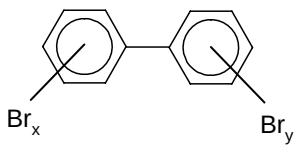
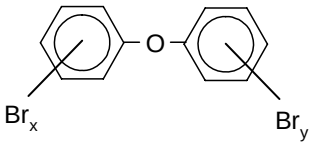
3.4.1 Introduction

Polybrominated biphenyls (PBBs) and polybrominated diphenyl ethers (PBDEs) are compounds of major concern in the environment. The generalized structures of these compounds are as follows (Hakk and Letcher 2003):



Polybrominated biphenyls (PBBs)
x + y = 6-10



Polybrominated diphenyl ethers (PBDEs)
x + y = 3-10

Polybrominated biphenyls have structural similarities to the polychlorinated biphenyls (PCBs) and exhibit similar stabilities, and lipophilic nature. As with the PCBs, PBBs have long biological half-lives and remain persistent in the environment (Gross et. al., 2003). Although more than 200 congeners are possible, only 45 PBB congeners have been synthesized. Of these, a commercial product known as FireMaster BP-6 was the most widely distributed PBB. FireMaster BP-6 was manufactured for use as a flame retardant from 1970 to 1973 and marketed as Firemaster BP-6 and Firemaster FF-1 by the Michigan Chemical Corporation (Willett et al., 1978).

Flame retardants may be categorized into four major groups: inorganic, halogenated organic, organophosphorus and nitrogen-based flame-retardants. Brominated flame retardants (BFRs) constitute the second largest group of flame retardants, and are the largest group of halogenated organic flame-retardants currently in use. Brominated flame retardants are divided into three subgroups: reactive, additive and brominated polymers. Reactive flame-retardants are substances chemically bonded into polymers such as plastics, whereas additive flame-retardants are simply mixed together with other compounds. Bromine may be incorporated into polymers during polymerization of the monomer compound to create brominated polymers (Alaee, 2003).

Polybrominated biphenyls (PBBs) and polybrominated diphenyl ethers (PBDEs) are two additive flame-retardants which have been used in a number of consumer products. Both of these off-white solids have been mixed into plastics through the additive process and, because of lack of chemical bonding, the PBBs and PBDEs could leach

out from the plastics causing severe environmental and toxicological consequences (ATSDR, 2002).

3.4.1.1 Polybrominated Biphenyls (PBBs)

Until a serious incident occurred with the product Firemaster, PBB contamination of the environmental was minor. However, in 1973 the Michigan Chemical Company accidentally replaced magnesium oxide in cattle feed with FireMaster BP-6 and this single incident lead to significant contamination of many Michigan farms. Unfortunately, as the feed passed through the mill, other rations were cross-contaminated; many other farms fed these feedstuffs containing low concentrations of PBBs (Willett, et al. 1978). As a result, more than 23,000 head of cattle, 4,000 swine, 1.5 million chickens and tons of eggs, milk and butter had to be destroyed (Jacobs, et al., 1976). It was later determined that over nine million people were exposed to PBBs through food linked to this one incident (de Wit, 2002).

After the FireMaster BP-6 incident, considerable research was conducted concerning the impact that PBBs would have on the environment and consumers. Polybrominated biphenyls were determined to be extremely persistent with only one pentabromobiphenyl isomer having any significant degree of degradation after 24 weeks of incubation in soils (Jacobs et al., 1976). Leaching studies were conducted using soils amended with 100 ppm of PBBs and leachate levels 20 times that of the normal rainfall for the Michigan region. Results indicated that less than 0.6% of PBB concentrations were reduced. This study indicated PBBs from contaminated manures would not leach through soils and suggested that PBBs would likely become available for re-ingestion via crops and pastures (Filonow et al., 1976). However, additional studies were conducted to determine the ability of plants to extract PBBs from contaminated soils. It was determined that both grasses and vegetable crops produced on PBB contaminated soils had no, or very minor (too low to quantify) uptake of PBBs (Jacobs et al., 1976).

No study was located specifically measuring PBB levels in rendered animal fats. However, the Pesticide Residue Laboratory of the Food Science Department at the New York State College of Agriculture fed PBBs to cattle and sheep to determine the levels of tissue storage and excretion in milk. PBBs were fed at a rate of 50 ppm in both cattle and sheep rations. Cattle excreted an average of 376.7 mg of PBB in milk during the 31-day period or 2.22% of the total dose (16.95 g). Daily milk production did not decrease although daily hay consumption decreased significantly. Following cessation of PBB dosing, the half-life ($t_{1/2}$) for PBB in milk was determined to be 10.47 days (Gutenman and Lisk, 1975). Polybrominated biphenyl levels were determined in the animal tissues and highest concentrations of PBBs were noted in decreasing order in the renal fat, the omental fat, the brisket fat, and liver. Higher concentrations of PBBs were noted in all PBB treated animals than in control animals and, with the exception of thyroid tissues, concentrations of PBB residues were considerably higher in the sheep tissues than in cattle tissues. However, in the experiment, cattle were dosed with capsules containing PBBs whereas sheep were fed rations laced with the PBB dosage. The differences in method of administration of the PBBs could possibly have led to the more efficient intestinal absorption by the sheep (Gutenman and Lisk 1975). This study is of particular interest since it points to the lipophilic nature of PBBs and because many of the noted PBB target tissues are non-food, rendered by-products of the livestock animal. In a similar study, Jackson and Halbert (1974) reported high

concentrations of PBB in milk and fat deposits of cattle foraging on PBB contaminated food.

Polybrominated biphenyls concentration levels were measured in the fat of livestock on several farms where the only known source of PBB residue was from the soil. Since uptake by plants was ruled unlikely by previous studies, results indicating ratios of soil concentrations to animal concentrations of 0.37 for dairy heifers, 0.27 for primaparous dairy cows, 0.10 for multiparous dairy cows, 0.27 for beef cows, 0.39 for beef calves, 0.37 for ewes, and 1.86 for swine led the researchers to conclude that foraging animals may ingest PBBs from contaminated soils (Gutenman and Lisk 1975).

PBBs are stored in fatty tissues throughout the body and can remain in adipose tissues for periods of 13-29 years depending on the level of exposure. Based on studies conducted with animals, researchers concluded that PBBs may interfere with hormone function. It is also known that PBBs are transmitted in breast milk and the researchers wished to determine what the impact PBB-contaminated breast milk would have on the hormonal development of daughters. Researchers estimated the exposure *in utero* from blood serum coupled with exposure via breast milk after birth. Girls who received both *in utero* and breast milk exposure to the chemical initiated menstruation at an average age of 11.6 years as compared to 12.7 years in girls who were not breastfed. It has been hypothesized that PBB binds to the estrogen receptors and affects the thyroid gland causing early pubescence (Blanck et. al., 2000).

In the 1973 FireMaster incident, some residents who consumed contaminated foods complained of nausea, abdominal pain, loss of appetite, joint pain, fatigue and weakness although PBBs could not be clearly established as the definitive cause. However, stronger evidence suggested that PBBs may have caused skin problems, such as acne (ATSDR, 2002). In an attempt to clearly determine the relationship between the individuals' symptoms and PBB exposure, in vitro studies were conducted using liver microsomes from rats. These studies indicated that the congeners in Firemaster BP-6 were not metabolized. However, when Phenobarbital (PB) or PBB-induced microsomes were used, two of the congeners were metabolized and identified as 2,2',4,5,5'-pentabromobiphenyl (BB-101) and 2,2',3,4',5',6-hexabromobiphenyl (BB-149). The rate of metabolism decreased by the level and position of bromination in each compound. The ease in which the congener was metabolized was based on presence of at least one *para* carbon being bromine-unsubstituted and/or the presence of bromine-unsubstituted carbons adjacent to that position (Hakk and Letcher, 2003).

Another experiment conducted by Dannen et. al. (1978) revealed that PBB congeners were not metabolically activated to reactive metabolites by microsomes. This study also indicated that the majority of the PBB binding to macromolecules was to proteins rather than DNA. Since PBB binding affinity to DNA was low, it was concluded that PBBs are not likely have carcinogenic or mutagenic activity (Dannen et al., 1978).

3.4.1.2 Polybrominated Diphenyl Ethers (PBDEs)

Among the flame retardants in use today, the polybrominated diphenyl ethers (PBDEs) have been identified as a major environmental concern due to global distribution. PBDEs have relatively low reactivity, high hydrophobicity and bioaccumulating properties, as observed in humans and wildlife populations (Bocio et al., 2003). The commercially available PBDEs can be divided into three groups based on low, medium, and high bromination rates, or pentaBDE, octaBDE, and decaBDE forms,

respectively. Among the penta and octa forms, 10-20 PBDE congeners are known of each, whereas only one known form of decaBDE is known (Eriksson et al., 2004).

In general, the lipophilic PBDEs have poor water solubility and very low vapor pressures at relatively low temperatures (Eriksson et al., 2004). The octanol-air partitioning coefficient, K_{OA}, was studied as a descriptor of PBDE mobility in the atmosphere. The K_{OA} is the ratio of the solute concentration in air versus octanol when equilibrium exists in the octanol-air system. This ratio describes the absorptive partitioning of semi-volatile compounds between the atmosphere and organic phases in the soil, in vegetation and on aerosols. The results of octanol-air partitioning coefficient study indicated that the K_{OA} exhibited a log-linear relationship with inverse absolute temperatures; PBDE K_{OA} values at 25ºC ranged from 9.3 to 12.0. These values were approximately 1 to 2 orders of magnitude greater than those obtained from the counterpart polychlorinated biphenyls (PCBs). Depending on the congener examined, PBDEs had a 1.2% (PBDE-17) to 85% (PBDE-183) partitioning to aerosols and soils.

In 1992, the global use of PBDEs was 40,000 metric tons and consisted of 30,000 metric tons of decabromodiphenyl ether, 6000 metric tons of octabromodiphenyl ether and 4000 metric tons of pentabromodiphenyl ether. Use of PBDEs in Western Europe accounted for about 30% of the world market. However, by 1998, the European share of the worldwide market for PBDEs decreased to about 11%. These decreases in use were particularly pronounced in Germany, the Netherlands, and Nordic countries. The global demand for PBDEs in 1999 is represented in Table 3.1 (Alaee, 2003).

Table 3.1 Total Global Market Demand for PBDEs in 1999 (all values reported in metric tons) (Alaee, 2003)

	Americas	Europe	Asia	Total
Deca-PBDE	24300	7500	23000	54800
Octa-PBDE	1370	450	2000	3825
Penta-PBDE	8290	210	---*	8500

*There was no market for penta-PBDE in Asia in 1999

Members of the German Association of Chemical Industries voluntarily halted production of PBDEs and PBBs in 1986. In recent years, leading manufacturers who have traditionally used PBDEs and PBBs in their products have accepted policies which prohibit the use of these chemicals in their products (Renner, 2000). The two largest American manufacturers of PBDEs, Albermarle and Great Lakes Chemicals, agreed to phase out two forms of the flame retardants in question by January 1, 2005 (Gagnon, 2004).

Polybrominated diphenyl ethers were found in soil and sludge around U.S. flame-retardants manufacturing plants as early as 1979. In 1981, the first environmental evidence of PBDE contamination of wildlife was reported in pike collected along the Visken River in Sweden. Since then, PBDEs have been detected in fish-eating birds and marine mammals collected from the Baltic Sea, the North Sea and the Arctic Ocean and in numerous other countries around the world (Alaee, 2003). Polybrominated diphenyl ethers were even found in sperm whales in the Atlantic

Ocean which suggests that even the deep ocean is now contaminated with PBDEs (Renner, 2000). Although efforts are underway to limit the use of PBDEs worldwide, a recent study revealed that the levels of PBDEs in North American samples have increased drastically over the last twenty years and seemed to be doubling every two to five years (Betts, 2002). It was reported that levels of PBDEs increased by 300 fold over the past two decades in lake trout from Lake Ontario. Another study revealed a 60-fold increase in the concentration of PBDEs in herring gull eggs from the Great Lakes. Between 1988 and 2000, harbour seals in California had a 65-fold increase in PBDE levels (Alaee 2003). Lake Michigan salmon were recently recognized as having the highest levels of PBDEs ever reported for fish in open waters (Schaefer, 2001). Lake Superior was estimated to contain approximately 2-6 metric tons of PBDEs and the current loading rate was about 80-160 kg/yr^{-1}(Song, Ford et al. 2004). Levels of polychlorinated biphenyl (PCB) were measured in all of these areas and many of the PBDE studies were prompted as a follow-up to the PCB findings (Renner 2000). Mehran Alaee, a research scientist at Canada's National Water Research Institute, in Burlington, Ontario, whose group is responsible for conducting PBDE measurements in North America, indicates that PBDE contamination is indeed global (Alaee, 2003; Renner, 2000).

The environmental fate of PBDEs seems to be similar to that of other structurally similar chemical compounds such as PCBs, for which the main route of human exposure is food. Table 3.2 lists the concentrations of PBDEs in food samples collected in Catalonia, Spain. The highest concentrations of total PBDEs were found in oils and fats, followed by fish and shellfish, meat and meat products and eggs. By contrast, PBDEs were not detected in the groups of fruits, cereals, and tubers. This pattern indicates that similar hydrophobic, low reactivity compounds are found in greatest concentration in foods with high bioaccumulation potential (Bocio et al, 2003).

Table 3.2 PBDE Concentrations (ng/kg wet weight) in Food Samples Collected in Catalonia, Spain (Bocio et al, 2003)

Food Group	Vegetables	Tubers	Pulses	Cereals	Fruits	Fish & Shellfish	Meat	Eggs	Milk	Dairy Products	Fats & Oils
tetraBDE	4.0	0.5	2.3	2.2	0.4	158.3	23.5	17.3	8.0	10.7	169.7
pentaBDE	1.4	0.5	0.6	2.2	0.4	115.9	24.9	25.8	5.2	23.4	157.7
hexaBDE	0.4	0.9	1.1	4.5	0.7	47.4	13.5	11.9	0.5	2.0	139.7
heptaBDE	0.7	1.8	2.2	8.9	1.4	5.4	23.9	4.4	1.1	4.0	77.0
octaBDE	1.4	3.7	17.9	17.9	2.9	6.8	23.4	4.7	2.1	7.9	43.7
SumBDE	**7.9**	**7.4**	**10.7**	**35.7**	**5.8**	**333.9**	**109.2**	**64.5**	**16.9**	**47.9**	**587.7**

PBDEs are definitely a contaminant in the human food supply. In a study of contaminants in foods, flame retardants were identified in several food products in U.S. supermarkets. Arnold Schecter, an environmental sciences professor at the University of Texas School of Public Health in Dallas, observed PBDE contamination in all foods containing animal fats, thereby identifying a major food route of human intake for PBDEs (Gagnon, 2004). The highest levels of PBDEs were detected in fish, followed by meat and then dairy products. In fact, the levels of PBDEs were higher in Dallas, Texas foods than levels quantitated in foods examined in a study conducted in Spain and another study completed in Japan. In the Texas study, a salmon fillet had 3,000 parts per trillion of PBDEs while the highest level in the Spanish food counterpart was recorded at 340 parts per trillion (Schecter et al., 2004).

Another safety consideration, which must be addressed, related to PBDEs is the thermal reaction products of the compounds. Hans-Rudolf Buser, of the Swiss Federal Research Station, conducted an experiment involving the thermolysis of three technical polybrominated diphenyl ether flame retardants. As the compounds were heated in quartz mini-vials at 510-630ºC, a range of potentially hazardous and toxic polybrominated dibenzofurans (PBDFs) and dibenzo-*p*-dioxins (PBDDs) were generated. The mass spectra of the generated thermal reaction products indicated similarities to those of chlorinated analogues (Busser, 1986).

Incineration temperatures have a direct impact on the atmospheric stability of PBDE thermal reaction products. A study completed at the University of North Carolina suggested that consistent 800ºC incinerator conditions caused formation of particulate-bound emissions of PBDDs and PBDFs which, with long half-lives, were capable of travelling great distances. However, combustion temperatures of approximately 450ºC created products that were unstable with atmospheric half-lives of only 1-6 hours. Therefore, combustion temperatures should be carefully considered since conditions are directly correlated to the atmospheric rates of production and decay of incineration-generated air pollutants (Biria and Kamens, 1994). Another important consideration during incineration of PBDEs is prevention of cross-contamination with fuels that contain chlorine and bromine; co-incineration of these compounds can lead to the formation of complex mixtures of polybrominated dibenzo-*p*-dioxins and dibenzofurans. Additionally, researchers discovered that presence of bromine increases the chlorination levels of compounds generated during mixed incineration. The most favored products formed were dibromodichlorodibenzo-p-dioxin and dibromodichlorodibenzofuran (Soderstrom and Markland, 2002).

Numerous articles have been published on polychlorinated dibenzo dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) but little is known about the brominated and mixed chloro/bromo homologs. Due to the similarity in structure to PCDDs and PCDFs and the growing presence of PBDEs, there has been a heightened concern about brominated and mixed chloro/bromo compounds. Available data supports the hypothesis that the brominated congeners have similar biological properties to their chlorinated relatives (Birnbaum, 2003).

Awareness that the PBDEs and the associated thermal reaction products are in the environment and food supply has led to studies on the biological impacts of these compounds. It has already been proven that brominated flame retardants have been linked to cancer, endocrine disruption and brain impairment in laboratory rodents (Gagnon, 2004). These compounds also cause liver and neurodevelopment toxicity and affect thyroid hormone levels (Betts, 2002). A Swedish study measured blood levels of

PBDEs in electronic dismantling plant workers at 70 times higher than levels in the general population. Researchers concluded that PBDEs are bioavailable to humans and have the potential to bioaccumulate (Renner, 2000).

Human adipose tissue samples collected in 1970 and 2000 were analyzed for the presence of PBDDs, PBDFs and PBDEs. The "limit of quantitation" (LOQ) may be described as the lowest concentration at which quantitative results can be reported with a high degree of confidence. Researchers in this study detected levels above the limit of quantitation (LOQ) in all the samples from 1970 and 2000 for congeners of all the compounds (Choi et al., 2003).

Sweden's Karolinsa Institute reported low levels of PBDEs in mother's milk; although levels of polychlorinated biphenyls and other persistent organic pollutants are decreasing worldwide, levels of PBDEs persistent pollutants appear to be increasing (Renner, 2000). Another study conducted by Olaf Papke, a German scientist who collected breast milk samples from women in Austin, Texas and Denver, Colorado, quantitated as high as 200 nanograms of PBDEs per gram of fat (ng/g) and served as the highest levels reported thus far (Betts, 2002).

In human blood, milk and tissues, total PBDE levels have increased exponentially by a factor of approximately 100 during the last 30 years; this equates to a doubling time of approximately 5 years. The levels of contamination seem to be dependent upon the region from which the samples were collected. Current PBDE levels from populations in Europe are approximately 2 ng/g compared to 35 ng/g levels found in individuals in the United States. These trends also are reflected in PBDE levels in wildlife (Hites, 2004). The study of the metabolic fate of these compounds has been limited primarily to in vitro and in vivo experiments conducted with rats and mice. In male and female Wistar rats, most bioaccumulation of PBDEs occurred in the adipose tissue; very little, if any, PBDE congeners remained after four days. In female rats, the half-lives of the PBDE congeners were 25 to 91 days as compared to 19 to 119 days for male rats. Interactions of PBDEs with thyroid hormone homeostasis, estrogen, and Ah receptors have been reported (Vos et al., 2003). Microsomal depletion of PBDEs mechanisms appeared similar to cytochrome P450 (CYP) enzyme mediated metabolism, but several of the metabolites produced from the parent compound had little to no detectable depletion (Hakk and Letcher, 2003). The metabolism of PDBEs has been suggested by in vitro liver microsomal studies, in dosing studies with captive animals, and by the presence of retained OH-PBDE metabolites in blood of a few wildlife species (Hakk and Letcher 2003). Evidence of measurable blood PDBE residues indicated that a variety of animals have the metabolic capacity to degrade PBDE. One difficulty in studying the metabolism of PBDE is formation of pure samples of PBDE related compounds to test against residues found in blood serum (Hakk and Letcher, 2003). Further research awaits to determine PBDE metabolic pathways.

The European Union now enforces restrictions against PBDEs under the European eco-labeling system (Renner, 2000). Risk analysis is being conducted to determine if the unknown health risks of PBDEs outweigh the benefits of the compounds as flame retardants. Complicating the risk analysis is the lack of statistical evidence concerning the number lives saved from fire each year by these compounds.

When considering tallow as an appropriate antecedent to biodiesel, it is important to consider the impact that the lipophilic PBB and PBDE compounds would have on a fuel. PBBs and PBDEs exhibit bioaccumulating properties but have relatively low

reactivity. In addition to examining PBBs and PBDEs, the associated metabolites also must be considered as possible contaminants. Published evidence indicates that PBBs and PBDEs are stored in the fatty tissues of all common food animals at varying concentrations. The fate of these compounds through the production of biodiesel and subsequent use has not been reported. Research is needed to determine the survival of PBB, PBDE and/or the related metabolites during the biodiesel esterification process. If studies indicate that these brominated compounds persist in the biodiesel generated from contaminated fats, additional research will be necessary to determine the impact of storage, handling, transportation, combustion and generation of emissions from using these fuels.

3.5 Biogenic Amines

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3.5.1 Amino Acid Degradation Hazards of Animal Co-products

Biogenic amines are formed via the decarboxylation of amino acids which is preceded by the breakdown of protein into amino acids. Ammonia and acids are also formed by amino acid deamination and both ammonia and biogenic amines can be toxic and are undesirable in animal feed (Urlings et al., 1992). These protein breakdown products are a result of the metabolic activities of microorganisms which are present in high numbers in raw animal co-products. Several strategies were suggested by Urlings et al., (1992) to mediate the production of these products in animal co-products:

- Development and application of Good Manufacturing Practices for the disposal of slaughter by-products, based on the HACCP concept. This will probably result in the separate collection and processing of different animal by-products, such as blood, intestines, intestinal content, feathers, and waste water.
- Chilling of animal by-products during collection to a temperature of 15ºC or below in order to decrease bacterial and enzymatic activity.
- Pasteurization and subsequent rapid chilling.
- Fermentation of by-products. In combination with pasteurization a half-way product can be produced which is less susceptible to recontamination and germination of spores.
- Processing to safety by sterilization and drying, by means of severe heating only, or by radiation combined with a mild heating.

Proper handling and control of raw animal co-products can alleviate or minimize the production amines and ammonia beginning at collection from the slaughter line through storage and of rendered animal co-products.

The decarboxylation of amino acids in meat tissue is promoted by microbial degradative enzymes. For example, lysine and histidine are enzymatically converted to cadaverine and histamine while ornithine, glutamine and arginine are precursors for putrescine. Low levels of amines are naturally present in some foods and feeds. For example, spermine and spermidine are always present in fish and nuts while putrescine is present in oranges and fruit juices. Higher levels of amines are found in fermented foods such as tyramine in ripened cheese, putrescine in wine/sauerkraut/ and agmatine in beer. In some cases the amines formed contribute positively to the unique sensory attributes of certain foods. Amines are also formed in raw animal co-products during holding prior to rendering. The relatively high ambient temperatures during the holding time between slaughter and rendering increases the rate of microbial growth and the subsequent production of biogenic amines. In addition, enzymatic activity not accompanied with bacterial proliferation yielded a significant increase in amines in meat and poultry products (Urlings et al., 1993).

Biogenic amines are a diverse group of compounds that have differing affects on animal and human health. Compounds categorized as biogenic amines include hormone precursors, phospholipids/vitamin components and neurotransmitters. Amines can induce hallucinations (mescaline), promote growth (putrescine, spermidine, spermine), elicit immune responses (histamine, norepinephrine), increase feed intake (serotonin) and alter mood (serotonin, dopamine, norepinephrine). Consumption of amines can trigger a dangerous immune response in mammals. A safety concern unrelated to animal co-products but that illustrates the danger of amines is scombroid poisoning in humans. This is a potentially fatal immune response to consumption of histamine produced by bacteria living in certain marine species. Biogenic amines can be produced in raw animal co-products and transmitted to animal feed. In animal feed, some biogenic amines can have toxic effects on animals such as damage to kidneys, liver or gut mucosa (Bakker, 1994). No research has been conducted to determine if biogenic amines are carried into the rendered oil-soluble fraction destined for biodiesel. However, toxicity studies on biogenic amines in animal diets derived from rendered animal co-products have conflicting results. Poole (1993) found that diets containing high concentrations of biogenic amines were toxic to poultry resulting in enlarged proventriculus, gizzard lining erosion and improper feed digestion. Bakker's (1994) findings supported those of Poole (1993); however, other studies report no toxic effects of amines even at relatively high concentrations (Brugh and Wilson, 1986; Espe et al., 1992; Smith, 1990; Cowey and Cho, 1992; Bermudez and Firman, 1998). Furthermore, several researchers have reported that certain amines are growth promoters that may improve animal health (Al-Batshan et al., 1994; Bardocz et al., 1993; Colnago et al., 1992; Hino et al., 1987; Pegg, 1986; Smith et al., 1996; Smith et al., 2000; Sousadias and Smith, 1995). Smith (1990) reported that adding 0.2-0.4% putrescine to feed yielded significant improved growth of week-old chicks while 0.8 and 1.0% addition levels suppressed growth. Biogenic amines were found to promote intestinal development and absorption while protecting the animal from the effects of unheated legumes. Histamine was not found to be a growth promoter in animals but a toxin while several other amines such as spermine, spermidine and putrescine had positive effects on animal health. Some of the conflicting results may be due to the specific amines tested, the species of animal fed the amines and the purity of the feed itself. For instance, Shifrine et al. (1960) and Smith (1990) found histamine and putrescine in concentrations greater than 1000 and 8000 mg/kg, respectively, were needed to produce toxicity in poultry while Bjeldanes et al. (1978) observed that adding 159 mg/kg histamine with 75 mg/kg cadaverine to diets resulted in 84% mortality in guinea pigs.

Barnes et al. (2001) reported that the major biogenic amines found in animal co-product meals were putrescince, cadaverine and histamine. den Brinker et al. (2003) conducted a comprehensive study of putrescine, cadaverine and histamine content in five different types of animal co-product meals from production facilities in all states in Australia between 1994 and 1997. Fish meal recorded the highest median values for each of the amines compared to poultry, meat, feather, and blood meals (see Table 3.3).

Table 3.3 Ranges of Biogenic Amine Content of Rendered Animal Meals from Production Facilities from all States in Australia from 1994-1997. Median values are shown in parenthesis. (Modified from Barnes, 2001)

		putrescine	cadaverine	histamine
Source	**# of samples**	**(mg/kg)**		
Fish meal	78	7-454 (102)	11-1340 (220)	<5-1620 (570)
Poultry meal	387	7-1340 (82)	<5-1350 (121)	<5-167 (19)
Meat meal	835	<5 – 695 (21)	<5-680 (29)	<5-258 (10)
Feather meal	120	5-267 (31)	<5-159 (42)	<5-90 (5)
Blood meal	25	<5-223 (13)	<5-280 (7)	<5-36 (4)

den Brinker et al. (2003) found a large variation in amine content among all the animal co-product meals, possibly reflecting the variation in how co-products are handled. High amine levels are related to the degree of microbial activity, which increases as raw animal co-products are held at non-refrigerated temperatures. The higher amine concentrations found in fish meal compared to other animal sources may be due to various factors including raw material handling, the presence of higher levels of free amino acids in fish muscle, and the presence of bacteria in fish having high histidine decarboxylase activity.

Biogenic amines are likely to be present in the raw animal co-product material but are not likely to be present in the rendered fat portion destined for biodiesel due to their lack of solubility in lipid material. In addition, biogenic amines present no risk for contact and in fact since some are growth inhibitors, controlled production of some amines may be beneficial in animal feeds.

3.6 Genetically Modified Ingredients and Growth Hormones

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3.6.1 Genetically Modified Ingredients

Oilseeds and grains have been genetically modified (GM) to produce crops with higher yields and increase resistance to plant disease and pests resulting in the use of less and fewer pesticides and herbicides. While there are few published reports on the safety of feeding GM plant material to animals, Hammond et al. (1996) found no adverse effects and no negative nutritional impact of GM soybeans (herbicide tolerance) when fed to rats, chickens, catfish and dairy cattle. Food Safety concerns over GM ingredients in animals have not been supported by scientific data; however, consumer demand for GM-free food have fueled the debate and increased the demand for these types of products. No published research was found on the safety of using fat and oil from animals fed GM plant material for biodiesel.

3.6.2 Growth Hormones

Some synthetic steroids are approved by the FDA for use in animal diets to improve feed efficiency, growth rate and milk production. Most mammalian hormones are soluble in fat thus would be expected to be present in these type tissues in animal co-products. Ryan and Dupont (1975) found 5-10 ppb melengestrol acetate (MGA) in beef fat from cattle feed 0.4 mg MGA. Andresen and Fesser (1996) measured three progestogens (melengestrol acetate, megestrol acetate and chlromadinone acetate) that are common feed supplements in beef fat and found 10 to 1000 ppb. Lesser amounts of MGA were found in liver (1-5 ppb). Krzeminski et al. (1981) used radioactively labeled melengestrol acetate to determine that fatty tissue was the "target tissue" for this orally administered progestogen finding 3.0 to 6.6 ppb of the radioactive marker. C^{14}-labeled progesterone and its metabolites were determined for muscle and adipose tissue in dairy cattle and steers following twice daily injections of 50 µg/kg for 13 to 21 days (Lin et al., 1978). These researchers found 3.4 and 18.1 ng/g of labeled progesterone in muscle and subcutaneous fat, respectively.

3.7 Drugs

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3.7.1 Pentobarbital

During the 1990s, the FDA Center for Veterinary Medicine (CVM) began receiving numerous reports from veterinarians that sodium pentobarbital was losing its effectiveness as an anesthetic (FDA, 2002). Based on these reports the CVM investigated the possibility that dogs were consuming pentobarbital through the food supply and becoming desensitized to pentobarbital. The investigation determined if dog food contained pentobarbital residues and if so did the residues pose any health threat to dogs. Sodium pentobarbital is routinely used as both to anesthetize animals and as an euthanasia agent. Thus, if animals euthanized with sodium pentobarbital entered the rendering stream, pentobarbital residues could be in rendered feed ingredients. To determine if pentobarbital was present in animal feeds, a sensitive test (2 ppb) was utilized to evaluate commercial dog food and some was found to contain pentobarbital. Since pentobarbital is often used to euthanize cats and dogs, it was suggested that these animals could be entering the rendering stream and ending up in dog food. To determine if this was the case, the CVM developed a DNA test for cat and dog protein in dog food. The method was sensitive enough to detect 5 lb of cat or dog protein in 50 tons of finished food (or 50 ppm). All dog food samples that were pentobarbital positive were tested and found not to contain cat and dog protein. Thus it was assumed what little sodium pentobarbital entering the rendering stream was from euthanized cattle or perhaps horses. Finally, the FDA CVM conducted a dose-response evaluation for dogs exposed to sodium pentobarbital in food, combined this with the probability of exposure based on the samples of commercial dog food found to contain the drug, and concluded that there was virtually no risk to dog health from current levels of pentobarbital in dog food (FDA, 2002). No reports on pentobarbital content in rendered animal fat were available, thus additional research on its presence and fate in biodiesel production is warranted.

3.8 Antibiotics and Antimicrobials

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Antibiotic and antimicrobial drugs are important veterinary medicines used to maintain the health and well-being of many animals. However, residues from these drugs can be detrimental in animal food products. Therefore, strict regulations are in place for drug withdrawal prior to slaughter and a number of methods have been developed to measure antibiotic and antimicrobial drug residues in animal products (Delephine et al., 1994; Capece et al., 1999; Myllyniemi et al., 2002). However, instances of misuse of drugs have surfaced. In a study of the aquaculture industry, residues of the banned nitrofuran antibacterials and chloramphenicol, a drug reserved as a last resort against human *Salmonella typhimurium* has been detected in shrimp and prawns (Johnston and Santillo, 2002). In a Swedish study, bovine urine and blood samples were collected from live animals on 50 farms. Additional samples of muscle, liver, kidney, and fat samples were collected from slaughtered cattle. Rainbow trout were collected from fish farms and milk was sampled from milk tanks. In total, approximately 11,000 samples were examined for drug residues, mycotoxins, organochlorines and other residues. Results indicated that only eight slaughtered bovine samples and one sample of milk contained antibiotic residues exceeding the maximum residue limit. However, one of the bovine samples contained chloramphenicol which is prohibited in veterinary use (Nordlander and Frisell, 1999).

Recognizing potential problems with drug residues in meats and meat by-products, risk assessment on the use of antibacterial and antiparasitic drugs in foods of animal origin was conducted in the early to mid-1990s (Waltner-Toews and McEwen, 1994c). In a study of the heat stability of sixteen different antibiotics during high temperature processing, van Egmond et al. (2000) determined that there was some residual antibiotic activity after heat processing of animal and offal tissues. Antibiotics (penicillin, amoxicillin, ampicillin, cloxacillin, oxytetracycline, doxycycline, tylosin, lincomycin, spiramycin, neomycin, dihydro-streptomycin, enrofloxacin, flumequine, colistin, sulfamethazine, and sulfamethoxazole) were added at veterinary therapeutic levels to 90:5:5 mixture of pork meat, pork kidney, and pork liver. Slaughter by-products and condemned carcasses are rendered using high temperature processes to generate meat and bone meals and fats. Potential risks for antibiotic residues may exist when a condemned animal has been undergoing veterinary treatment and is processed without the drug withdrawal times required for food animal slaughter. It was assumed that the high temperature process utilized in rendering would destroy all veterinary antibiotic residues. However, the study by van Egmond (2000) using laboratory scale thermal processing indicated that the following drug activities remained: 80% of lincomycin, 69% of flumequine, 68% of enrofloxacin, 46% of neomycin, 44% of tylosin, 38% of sulfamethazine and 15% of spiramycin. The remaining antibiotics, (penicillin, ampicillin, amoxicillin, cloxacillin, oxytetracycline, doxycycline, colistin, dihydro-streptomycin and sulfamethoxazole) were considered completely degraded by the heat treatment (less than 10% remaining activity) after the heat process (van Egmond et al., 2000).

Kühne et al. (2000) isolated tetracycline residues from the bones of slaughtered animals and Von Donkersgoed et al. (1999) detected oxytetracycline residues in animals up to 28 days post treatment. A later study by Kühne et al. (2001) involved thermal processing trials on tetracycline and chlortetracycline contaminated meat and bone meal. After treatment at 133ºC in an autoclave, approximately 50% of the tetracycline was reduced and 90-100% of the chlortetracycline was destroyed. Additional research projects aimed at determining the thermal survival of veterinary drug residues were conducted by Rose et al. (1996) on oxytetracycline, by Rose et al. (1997a) on the coccidiostatic agent lasalocid, and by Rose et al. (1997b) on the veterinary drug oxytetracycline. In all three studies, drugs were reduced by thermal treatment but not totally eliminated.

3.8.1 Conclusion

No published studies were located concerning the fate of residual antibiotics through the biodiesel manufacture process. Strict drug residue legislation exists in most countries for veterinary medicinal substances and legislative bodies are continuing to implement and enforce more stringent residue regulations. In fact, the strict regulations have lead to problems restricting the use of veterinary pharmacologically active substances and the impact on international trade (European Commission for Health and Consumer Protection Directorate-General, 2004). However, if indeed any antibiotic residues were to survive the esterification process, it would seem that any remaining antibiotic residues would be destroyed upon combustion of the fuel. The effect of these potential contaminating substances on biodiesel production, transportation and use will likely not be an issue; however, definitive research should be conducted.

3.9 Oxidized Cholesterol Derivatives

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Heating animal and plant fats and oils to high temperatures has been shown to result in oxidation of fatty acids and to produce cholesterol oxides. Animal sterols are present in the rendered portion of animal by-products as these are fat-soluble compounds. Consumption of cholesterol oxides have been suspected to have negative health effects including cytotoxicity, atherogenicity, mutagenicity and carcinogenicity (Paniangvait et al., 1995; Schroepfer, 2000). Park and Addis (1986) found four oxidized cholesterol derivatives in tallow heated to 155˚C. Verleyen et al. (2003) found two cholesterol oxide derivatives in crude tallow heated to 95˚C then held for 30 minutes. No studies have examined the possible health risks of chronic exposure via fumes or skin contact with these oxides which might occur in biodiesel production and utilization.

3.10 Nicotine

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Nicotine is one of the oldest known insecticides. More than 300 years ago, extracts from tobacco were described for controlling insects and nicotine sulfate was first commercially available in 1910. Nicotine was named for Jean Nicot who was the French ambassador to Portugal in the mid-16th century who is credited with sending tobacco seeds to Paris. Scientific history records that the chemical nicotine was first purified in 1828, chemically characterized in 1843, and then later synthetically recreated in 1904 (Yamamoto, 1998). In the early 20th century, when such toxic agents as arsenic and lead were being used on food crops, searches for safer alternatives lead researchers to investigate nicotine products (McHargue and Calfee, 1937). In the early 1900s, nicotine became the main crop protection insecticide. In the 1950s, it was used to control cotton pests in Peru (Biengolea, 1954). However, after the creation of parathion, nicotine use began to decline. Worldwide, nicotine use as an insecticide has continued to decrease due to the increased efficacy and lower cost of synthetic insecticides (Jacobson, 1989). As 40% free nicotine, nicotine sulfate is considered a potential public health concern (Yamamoto, 1998). In May, 2002, the U.S. Environmental Protection Service revoked tolerances for nicotine residues on all foods except cucumbers, lettuce and tomatoes. Since nicotine and nicotine-containing compounds are no longer used on foods that are grown within or imported to the United States other than cucumbers, lettuce and tomatoes, there was no need to keep a tolerance. During the comment period, there were no requests were made to continue nicotine use on other foods (EPA, 2002b; EPA, 2002c). Other uses for nicotine have included non-food plant insecticides and dog, cat and rabbit repellents (PAN, 2004). As naturalists seek to find botanical pesticides, interest is being renewed in nicotine-based compounds (Casanova et al., 2002; George et al., 2000). New derivatives of nicotine also are being developed and tested for insect control (Palumbo et al., 2001).

Well known for its biological effects on the human via tobacco products (Clarke et al., 1995), nicotine as an insecticide is most effective against soft-body insects such as aphids and spider mites (Casanova et al., 2002). Nicotine interacts with the nicotinic acetylcholine receptors (nAChRs) at the synapse in the insect central nervous system. In mammals, nicotine acts on the nAChRs at both the ganglia and neuromuscular junctions, initially causing excitation and later paralysis (Yamamoto, 1998). Nicotine is selectively toxic, displaying higher toxicity to insects than to mammals. However, lethal doses of nicotine have been reported at 15 to 100 mg and the compound is listed as “supertoxic” (Smallwood et al., 1997; Tomizawa et al., 2000). The World Health Organization (WHO) classifies nicotine as highly hazardous (Casanova et al., 2002). Nicotine in the form of nicotine sulfate may cause tremors, nausea, incoordination, coma and death in animals (Anonymous, 2003b).

Incidences of nicotine poisoning have occurred after ingestion of contaminated foods. In January, 2003, approximately 1700 lbs of ground beef were recalled in Michigan due to customer illness. Analysis of the product revealed that it was contaminated with 300 mg/kg nicotine. Those who ingested the product experienced an array of symptoms including headache, blurred vision, insomnia, tachypnea or dyspnea, body

numbness, sweating, nausea, vomiting, diarrhea, abdominal pain, dizziness, or a burning sensation to the lips, mouth or throat. After investigation, it was determined that the poisoning was not a natural phenomenon or an accident but rather an intentional poisoning episode by a disgruntled grocery employee. The person was arrested and indicted for poisoning approximately 200 lbs of ground meat with the insecticide Black Leaf 40 which contains nicotine (CDC, 2003).

With greatly decreased use of nicotine as a pesticide, the risk of accidental transfer of this substance to food animals should be very low. Unless livestock ingest the tobacco plant (*Nicotiana tabacum*) or are fed dried tobacco, the path of nicotine contamination to rendered animal fat is minimal. Consequently, unless nicotine insecticide use rebounds, it is unlikely that nicotine would be a safety factor in the production of biodiesel from animal fats.

3.11 Gossypol

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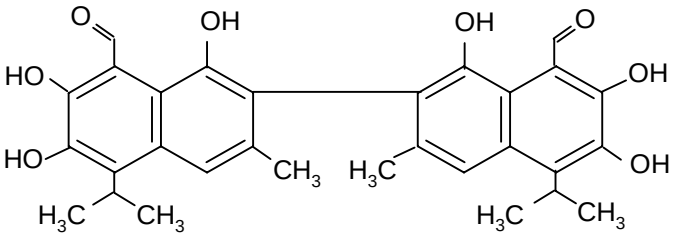
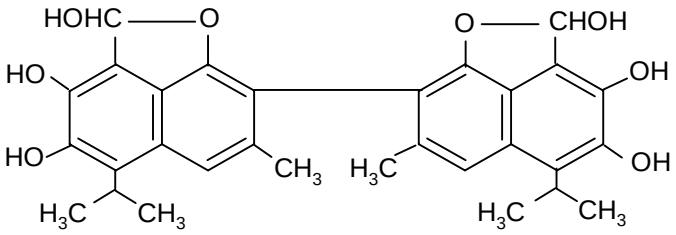
3.11.1 Chemical Structure and Properties

Gossypol is a natural yellow, lipid soluble pigment produced by the cotton plant (*Gossypium* spp.). Chemically known as 2,2'-bis (8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene), gossypol has been studied for a number of uses including male contraceptives, as an anti-neoplastic drug and as an insecticide (Dabrowski et al., 2001; Ciesielska et al., 2002; Dao et al., 2003). Gossypol may exist in three tautomeric forms: 1) an aldehyde-aldehyde, 2) lactol-lactol, or 3) ketol-ketol tautomer. The chemical structures of the three tautomers of gossypol are (Admasu and Chandravanshi, 1984; Ciesielska et al, 2002):

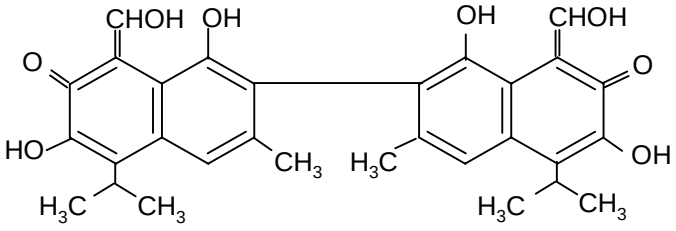
Three Tautomeric Forms of Gossypol



aldehyde-aldehyde tautomer



lactol-lactol tautomer



ketol-ketol tautomer

In alkaline solution, gossypol is rapidly attacked by oxygen and generates intensely colored (deep purple) labile products. The first work on oxidation of gossypol involved ozonation of the compound; however, the earliest studies were aimed at determining structure rather than the by-product identification. In 1964, the labile oxidized by-product was characterized as *o*-binaphthoquinone (Scheiffele and Shirley, 1964). Gossypol forms stable complexes with a number of metal cations including 1:1 complexes with Be^{2+}, Ni^{2+}, Cu^{2+}, Dy^{3+}, Gd^{3+}, Tb^{3+}, and Eu^{3+} with the gossypol in the lactol-lactol form. With Zn^{2+}, the complex formed was the only ketol-ketol tautomer observed. Research indicated that additon of iron salts to cottonseed meal diets reduced gossypol toxicity due to formation of insoluble iron-gossypol complexes (Przybylski et al., 2001). Oxidation of gossypol with ferric chloride in acetic acid resulted in a moiety that possessed steric blocking of the normal oxidative coupling reaction of phenols (Hass and Shirley, 1965). Measurement of enantiomer ratios in cotton seeds indicated that the (+)-gossypol enantiomer is most common in *Gossypium arboreum, G. herbaceum,* and *G. hirsutum* varieties whereas the (-)-gossypol enantiomer is most common in the *G. barbadense* variety.

Gossypol is distributed throughout the cotton plant (*Gossypium* sp.) in pigment glands. Cotton fiber production is widely recognized as the primary valued product of the cotton plant; however, the cottonseed also has great economic importance. Cottonseed oil is used extensively for human food and the meal is used as animal feeds and fertilizers. However, the gossypol content limits the use of cottonseed (Admasu and Chandravanshi, 1984). In the seed, gossypol accounts for approximately 20 to 40 of the pigment glands in the seed (Adams et al., 1960). Little difference in gossypol levels were observed in different varieties of cottonseed from the southwestern U.S. (Robinson et al., 2001).

The term "gossypol" was first coined by Marchlewski from the genus name for the cotton plant (*Gossypium*) and from the chemical nature of the chemical (phenol) (Adams et al., 1960). A number of analytical methods including ether extraction, spectrophotometric and monoclonal antibodies were developed to determine the total gossypol content in cottonseeds and cottonseed meals (Halverson and Smith, 1937; Admasu and Chandravanshi, 1984; Wang and Plhak, 2004). Additionally, high performance liquid chromatography methods were devised to determing free gossypol levels in chicken liver (Botsoglou, 1991).

Acetylation of gossypol was conducted to study diastereomers (Huang et al., 1987). Gossypol is unstable at both 37ºC and room temperature but stability increased with decreased storage temperature. Gossypol exhibits emission properties producing weak fluorescence (Ciesielska et al., 2002).

3.11.2 Toxicity

Gossypol has been known to have a variety of toxic effects in mammals including heart failure, cardiomyopathia, dyspnea, edema in the lungs and other tissues, hepatic necrosis, weakness, inappetence, diarrhea, erythrocyte fragility, and sudden death. All animals are believed to be susceptible to gossypol toxic effects; however, monogastrics, immature ruminants and poultry are most commonly affected. Holstein calves appear to be the most sensitive among the cattle breeds (Anonymous, 2004a; Anonymous, 2004b; Morgan, 2004; Poore and Rogers, 2004). The mechanisms of gossypol toxicity are not completely understood and are under investigation. The mechanism of action of gossypol on the mitochondrial membrane appear to be related

to altering the lipid matrix which results in modifications in protein function in adrenal enzymes (Cuéllar and Ramírez, 1993). In human hepatoma cells, gossypol appears to induce an increase in calcium ions which can be cytotoxic (Cheng et al., 2003). Gossypol induced diarrhea has been related to the induction of chloride secretion in the proximal colon (Kuhn et al., 2002). In fish fed cottonseed meal, gossypol isomers bind specifically to spermatozoa and to blood plasma proteins (Dabrowski et al., 2001; Mena et al., 2001). In feeding trials with tilapia (*Oreochromis* sp.), spleen abnormalities, necrotic areas, and lymphocytic depletion of the white pulp areas were observed in fish fed 50-100% cottonseed meal diets. In fish fed diets containing 75-100% cottonseed meal, erythrocyte abnormalities were observed (Garcia-Abiado et al., 2004). In studies conducted in Finland, gossypol was suspected of causing pancreatitis through activation of polyamine catabolism (Räsänen et al., 2002).

Long known for its toxicity to certain livestock, early attempts to improve cottonseed feed quality included methods of removing the gossypol by extraction (Halverson and Smith, 1933). The reaction of aniline with gossypol forms dianilinogossypol. This stable crystalline product was used as a means of detoxifying cottonseed meal (Shirley and Sheehan, 1956). Later it was determined that broilers fed a high (+) to (-) gossypol enantiomer ratio crushed cottonseed ration performed better than broilers fed a commercial ration with a lower (+) to (-) enantiomer ratio (Bailey et al., 2000). A mechanical extrusion processing method of reducing gossypol levels were investigated by Buser and Abbas (2001). Results indicated a 71-78% reduction in free gossypol levels.

Messiha (1991) determined that gossypol had an effect on the response of liver enzymes in rodents. In mice, gossypol inhibited hepatic alcohol dehydrogenase with different levels noted for male versus female test animals. Interestingly, gossypol caused male rats to have an adversion to ethanol consumption.

Gossypol has adverse in vitro effects on rat liver mitochondria. At low gossypol concentrations, mitochondrial respiration was stimulated; at high gossypol concentrations, mitochondrial respirations was inhibited. Research indicated that gossypol may likely uncouple respiratory chain phosphorylation (Abou-Donia et al., 1988).

Feeding whole cottonseed and cottonseed meal to lactating dairy cattle resulted in increased plasma gossypol concentrations directly proportional to gossypol intake (Mena et al., 2001). High intake of calcium hydroxide or iron salts appears to be protective in cattle (Anonymous, 2004).

3.11.3 Medicinal and Other Uses for Gossypol

Recently, gossypol has become the subject of many investigations on new applications of the complex molecule. However, for decades the complexity and possible compounds that could be synthesized from gossypol have interested scientists. In 1957, Shirley et al. studied the structure and reactions of gossypol and derived two new synthetic compounds: methylapogossypol hexamethyl ether and 2,3-dimethoxy-4-isopropyl-5-allyltoluene.

Rojas et al. (2004) patented the use of gossypol and related terpenes for controlling urban and agricultural pests (U.S. Patent # 6,773,727). In this process, low concentrations of gossypol and other related toxins from cotton were suggested to enhance the efficacy of insecticidal agents against insects such as ants, termites and

cockroaches. Gossypol has been studied as an anti-parasitic agent to destroy African trypanosomes, the causative agent of a number of sleeping sickness diseases (Eid et al., 1988). Gossypol has a bacteriostatic anti-bacterial effect against the fish pathogen *Edwardsiella ictaluri* (Yildirim-Aksoy et al., 2004). Gossypol has been reported to have anti-viral activity. Gossypol inactivated human immunodeficiency virus (HIV) in *in vitro* studies (Polsky et al., 1989).

Gossypol has been studied as a natural male contraceptive and, as early as 1985, a number of gossypol analogues were synthesized as potential experimental male antifertility agents (Meltzer et al., 1985). Both (+)- and (-)- gossypol demonstrated spermicidal activities in human, monkey, rabbit, mouse, rat and hamster studies (Kim et al., 1984). Gossypol reduced sperm motility by reducing mitochondrial activity (Breitbart et al., 1989). Gossypol reversibly damages portions of the seminiferous epithelium with no concurrent effect on hormone production resulting in male infertility (Kuhn et al., 2002). At relatively low dosages, gossypol arrest of spermatogenesis with reversibility in 80% of men (Coutinho, 2002). Gossypol irreversably impaired cell-to-cell communication in both human and rat cells (Hervè et al., 1996). In the female bovine, studies indicated that gossypol did not appear to affect ovarian, follicular or embro characteristics. However, the availability of free gossypol may affect weight gain, embryo viability, and the corpus luteum (Randel et al., 1996).

Additionally, gossypol is active against a number of cancer cell lines and, thus, may have potential as an anti-neoplastic drug. Because the concentration of gossypol necessary to effect these actions may be excessive, Dao and co-workers (2003) generated new derivatives of gossypol as prodrugs of cytotoxic agents. Formation of gossypol derivatives has been studied intensively; in 1989, Vander Jagt et al. patented a variety of gossypol derivates. However, the Dao study created derivatives that masked the aldehyde groups in gossypol and resulted in much lower gossypol and gossypoline toxicity. It was proposed that these gossypol derivatives could be used as prodrugs to target tumor cells that are surrounded by high concentrations of nitric oxide (Dao et al., 2003). As early as 1991, gossypol enantiomers were found to increase the effectiveness of certain drugs when used in combination against cancer cells (Ford et al., 1991). Yurtcu et al. (2003) studied chemicals for treating children with acute myeloid leukemia (AML) and lymphoid leukemia (ALL) and determined that 25 to 50 μM gossypol was needed to induce apoptosis of human lymphocytes without concurrent necrosis via cytotoxic effects. In the study of HT-29 human colon carcinoma cells, gossypol induced cell growth inhibition and death (Zhang et al., 2003).

One of the limiting factors into medicinal uses of gossypol was the need to isolate large quantities of high purity gossypol. Dowd and Pelitire (2001) studied a method of recovering high purity gossypol from cottonseed soapstock. Originally, the cottonseed oil industry utilized crude oil refining to separate the cottonseed oil from the meal, which generated the by-product soapstock. However, newer methods of cottonseed oil recovery involve miscella refining which is refining before solvent stripping. Dowd and Pelitire (2001) suggested that alternative methods of producing research-grade gossypol are needed and likely will involve use of soapstock.

3.11.4 Gossypol Residues in Animal Fats

Gossypol residues can occur in animal organ meats. Tissue residue analysis indicated that gossypol entantiomers accumulated in ovine tissues (Kim et al., 1996). In broilers

fed various types of cottonseed meals, (+)- and (-)-gossypols were measured in plasma, liver, kidney and muscle tissues; levels increased linearly concurrent with increases of free gossypol in the diet. Tissue concentrations were noted in decreasing order for liver, kidney, plasma, and muscle, respectively (Gamboa et al., 2001b). When broilers were fed diets with 0, 7, 14, 21 and 28% cottonseed meal, tissue concentrations of gossypol increased linearly with increasing cottonseed meal fed. At 42 days of feeding, the liver contained the highest concentration of gossypol with a majority of (+)-gossypol enantiomers. Plasma, heart tissue and breast meat had increasingly lower levels of gossypol, respectively (Gamboa et al., 2001b).

Animals for food consumption should be removed from gossypol exposure a minimum of one month prior to slaughter (Anonymous, 2004). In consideration of biodiesel manufacture from rendered animals and animal by-products, if animals were fed whole cottonseed and/or cottonseed meal without one month gossypol withdrawal, gossypol residues may occur in fat. Animals fed gossypol have plasma gossypol concentrations in relation to amount fed (Blackwelder et al, 1998). Since the vast majority of rendered animal fat is derived from food-grade animals by-products from the human food supply, it is very unlikely that significant amounts of gossypol will be in rendered animal fats. However, further studies are needed. Additionally, if gossypol residues were found in rendered animal fats, further study would be needed to determine any potential risks from making biodiesel from these lipids.

3.12 Other Potential Organic Contaminants

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3.12.1 Anthelmenthic Agents and Orally Consumable Insecticides

Anthelmenthic agents are used in animal husbandry to prevent parasites. However, a number of these compounds have known teratogenic or embryotoxic effects in certain species. Therefore, strict maximum residue levels (MRLs) have been established for these agents (Rose, 1999).

The avermectins are macrocyclic lactones, which comprise a class of antihelmenthic drugs including ivermectin, moxidectin and doramectin (Steel, 1993; Craven et al., 2001). Ivermectin is a lipophilic anthelmenthic agent used in a number of animal species (Chiu et al., 1988). Elimination times for Ivermectin® can be extended; one study indicated that residues were still excreted in cow's milk at 29 days post-subcutaneous application (Toutain et al., 1988). Ivermectin was detected in sheep milk products made from milk from treated animals (Cerkvenik et al., 2004a; Cerkvenik et al., 2004b). Liquid chromatography with either mass spectrometry or fluorescence detection is often used to quantitate ivermectin residues (Prabhu et al., 1991; Khunachak et al., 1993; Dusi et al., 1996; Ishii et al., 1998).

Pour on type endectocide drugs are used worldwide in cattle for the control of parasites. Drugs such as moxidectin are known to be lipophilic and stored in the fat (Sallovitz et al., 2003; Sato et al., 2003). In a study by Lifschitz et al. (2000), sheep were treated subcutaneously with moxidectin and sacrificed 21 through 49 days after treatment. Tissues were analyzed for residue and all tissues were below the accepted maximum residue limit at 21 days after treatment and thereafter.

Craven et al. (2001) determined that body composition (fat vs. lean) did not affect the pharmokinetics of ivermectin whereas moxidectin was eliminated quicker in lean animals than in fat animals. However, in general, ivermectin half-life was 8 to 10 days and moxidectin half-life was greater than 40 days. In a later study, Craven et al. (2002) reported that rate of fat deposition influenced the pharmacokinetic disposition of moxidectin but not ivermectin.

Benzimidazole anthelmintics were created for control of veterinary gastrointestinal parasites such as roundworms, lungworms, tapeworms and liver flukes (Virkel et al., 2002) and are the largest chemical family used in the treatment of endoparasitic diseases in domesticated animals (Anonymous, 2003a). The benzimidazole sulfoxide derivaties albendazole sulfoxide, oxfendazole, and fenbendazole are the primary anthelmintics in use. In the rumen, benzimidazole anthelmintics are reduced to albendazole by the ruminal microflora (Virkel et al., 2004). Bidlack (1993) reported the safety of using oxfendazole as an anthelmintic substance for cattle. Studies indicated that hepatocytes are involved in the biotransformation of thiabendazole (Coulet et al., 1998).

In poultry, albendazole residues were detected in broiler tissues 6 hours after treatment but were non-detectable by 96 hours post-treatment. No albendazole residues were isolated from eggs (Cskiko et al., 1995).

In swine treated experimentally with ^{14}C-oxibendazole, residues were highest in tissues at 24 hrs post withdrawal and slowly depleted over time to approximately 1.8 ppm on the seventh day (Gottschall and Wang, 1996). However, in goats and laying hens, thiabendazole was rapidly metabolized. After seven consecutive days of dosing lactating goats were sacrificed 24 hrs later. Nearly the entire amount of the thiabendazole administered was measured in the excreta (69% in urine and 28% in feces) and very little was isolated (less than 3%) from milk and tissues. In laying hens dosed for 10 consecutive days followed 24 hrs later by sacrifice, similar results were noted. Greater than 99% was measured in the excretia and very little was noted in eggs or tissues (Chukwudebe et al., 1994).

In dairy cows, the highest drug residue levels from fenbendazole treatment occurred 24 to 36 hours after drug treatment and declined thereafter (Kappel and Barker, 1996). Studies conducted on pork tissues contaminated with furazolidone indicated that total metabolite concentrations were not reduced by frying, grilling or microwaving the liver, kidney and muscle tissues (McCracken and Kennedy, 1997).

Incidents of cattle mortality have occurred from monensin toxicity occurring from treatment to prevent coccidiosis (Gabor and Downing, 2003). It is not known if any of these animals were rendered and the potential fate of monensin during thermal processing.

Fluazuron is an antiparasitic, insecticide and insect/acarine development-inhibiting agent used in cattle by either oral or dermal dose. After oral dosage, a rapid rise in fluazuron occurs in the bloodstream. The compound becomes distributed to muscle, kidney, liver, lung and brain but especially in the fat. The half-life of fluazuron is very long and may exceed 78 days. Excretion is greatest via feces (Tantiyaswasdikul, 2004). Diflubenzuron boluses have been fed to cattle to reduce fly problems (Fincher, 1991; Miller, 1994).

Rose (1999) conducted studies on methodology for the isolation of nine compounds related to oxfenazole from cattle liver. Kawasaki et al. (1999) and Dreassi et al. (2001) used solid phase extraction methods to measure anthelmintic residues in livestock fats.

A number of medicinal products have been withdrawn from the food animal market due to unacceptable risks to consumers (Federation of Veterinarians of Europe, 1998). Again, since the majority of rendered by-products are derived from food-grade animal residuals, strict veterinary drug enforcement should serve to reduce potential exposure to these drugs through rendered animal products. Medication withdrawal times have been established for each approved drug (Griffin and Groteluesschen, 1997). Additionally, strict drug residue tolerances have been established for meat, poultry and fish products (EPA, 1997; USDA-FSIS, 2001a; USDA-FSIS, 2001b; Australian Government Department of Agriculture, Fisheries and Forestry, 2003). Additionally, pesticide tolerances have been established for a number of chemical moieties, restricting the levels that may be found in animal tissues (EPA, 2002a).

No information was obtained concerning the fate of anthelmintic residues or other carbamates in fats during biodiesel manufacture. However, studies have indicated that carbamates and dithiocarbamates rapidly decompose upon heating. Therefore, the high

cook temperatures employed in rendering should destroy any carbamate compounds in animal fats prior to esterification into biodiesel (Hill, 2003).

4 POTENTIAL INORGANIC CONTAMINANTS

4.1 The Risk of Metals and Metalloids in Biodiesel Production and Use

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4.1.1 Introduction

Chemists identify 88 elements as "metals" and seven elements as "metalloids" on the periodic table. Metals are characterized as malleable, ductile, and good conductors of heat and electricity. Metals have high density, a high melting point and a characteristic luster. Quick to lose electrons, metals are distinctive elements. The metalloids are unique elements that are defined as having characteristics of a metal and a non-metal. Located on the right side of the periodic table, next to the metals, the seven metalloids are boron, silicon, germanium, arsenic, antimony, tellurium and polonium.

Awareness and regulation of metal and metalloid pollution have increased appreciably within the last 50 years. The consequences of releases of these elements into the environment have been reported globally with such examples as arsenic poisoning of groundwater, decreases in mink and otter populations in mercury-contaminated areas, and increase in lead pollution due to the use of leaded gasoline. The adverse effects of metallic pollution to both animal and human health have been well studied and, as such, producers of new fuels must identify and remove such elements to insure that their product(s) does not increase pollution. In this section, the scope of potential metal and metalloid contamination within animal-based biodiesel will be explored along with any possible effects metallic contaminants may have on the safe production and use of these biofuels.

4.1.2 In the Environment

Metals and metalloids are a natural part of the environment. Found within mineral ores, these elements migrate between the soil, air, water, and living organisms in a cycle of volatization, deposition, and solubilization. However, since the beginning of the industrial age, anthropogenic activity has significantly increased the mobility and accumulation of metals and metalloid in nature. Studies of North American glacial ice (Schuster et al., 2002) and European lake sediment (Von Gunten et al., 1997) indicated steady increases in environmental metal concentrations occurred over the last two centuries. Using correlations between metal pollution and human population density, several researchers have suggested that greater than 50% of the total environmental concentration of these elements may be traced to human activities (Bacon et al., 1996; Nowack et al., 2001; Schuster et al., 2002). Studies by Callender et al. (2000) on the Apalachicola-Chattahoochee-Flint river basin in the southeastern United States detected significant increases of lead and zinc levels in and downstream of urban

centers. Declines in contamination levels have been observed within the last decade which most researchers attribute to increased environmental awareness and regulation (Von Gunten et al., 1997; Callender and Rice, 2000; Schuster et al., 2002; Cheevaporn et al., 2004).

Upon entering into soil or water, the chemistry of the metals and metalloids becomes more complicated. Mobility in these media is controlled by element speciation, which is in turn correlated with temperature, pH, and the chemical and biological content of the media. In soil, metal and metalloids are generally absorbed onto minerals or organic matter (Wiener et al., 2003; Zawislanski et al., 2003).

Metals and metalloids remain in the environment after release in various states, forms and locations based upon several factors including pH, uptake by organisms, chemical composition of the media, and hydrophobicity of the compounds formed. Particle size plays an important role in where the metal particles are deposited and how long they can remain in the atmosphere (Environment Canada, 2005). Larger particles are deposited more quickly than smaller particles which may be carried by water droplets and deposited with precipitation.

Approximately 95% of atmospheric mercury is metallic (Hg^0) which is released by the burning of waste in incinerators, the combustion of fossil fuels, or volatilization from surface soil or water (Wiener et al., 2003; Murray et al., 2004). Metallic mercury has a residence life of one year, and so is able to travel globally through winds. Mercuric (Hg^{+2}) particles have a more localized effect, generally traveling only tens of kilometers from the release point before deposition onto the soil or water. Within the atmosphere, metallic mercury is slowly oxidized to the mercuric state and deposited through either wet or dry means.

Inorganic mercury (Hg^{+2}) in water is converted into the more toxic and bioavailable form of methylmercury primarily by sulfate-reducing bacteria (King et al., 2002). Increased concentrations of methylmercury in Minnesota (USA) watersheds have been correlated to increases in microbial growth (Balogh et al., 2003). This methylation process is affected by pH, temperature, and the dissolved oxygen content (DOM) of the water (Keating et al., 1997; Haitzer et al., 2003). Dissolved oxygen binds with inorganic mercury, decreasing methylmercury concentrations by preventing methylation.

In a survey of wild versus farm-raised salmon, no significant differences were observed in methyl mercury levels (Hites et al., 2004). Since farm-raised salmon are often fed rendered by-products, this data indicates that rendered animal fat is not likely a significant source of methyl mercury.

Particulate lead is prominent within the atmosphere, accounting for 90% or greater of the total atmospheric lead concentration in urban environments (Purdue et al., 1973). However, organic leads, such as tetraalkyllead, are still found in significant concentrations (Purdue et al., 1973; Pecheyran et al., 2000). Residence time of particulate lead correlates with particle size. Particles larger than ten microns are deposited close to the emission source. Hashisho and El-Fadel (2004) noticed considerably higher lead soil concentrations in heavily trafficked areas of Lebanon where leaded gasoline is still used, suggesting that lead particles from auto exhaust settle close to their source. Smaller particles can have a global effect as evidenced by lead levels in the snows of Antarctica which Barbante and co-workers (1998)

hypothesize originate in South America and Australia. Erel and co-workers (2002) measured $^{206}Pb/^{207}Pb$ atmospheric ratios within Jerusalem and found that approximately 50% of the airborne lead originated in foreign countries.

While deposition of atmospheric metals and metalloids contributes significantly to metal and metalloid contamination of soil and water, direct contamination routes also occur. Various studies have indicated that soils contained elevated metallic levels subsequent to use of fungicides (Wiener et al., 2003), fertilizers (Arora et al., 1997; Conde et al., 1997), sewage sludge (Granto et al., 1995; Patte et al., 2003), and contaminated irrigation water (Abedin et al., 2002). Shooting ranges have lead levels as high as 10,000 mg Pb/kg soil due the corrosion of lead bullets and pellets (John, 2002). Metallic lead in discarded bullets and pellets are oxidized into soluble Pb^{+2} which may enter soil and groundwater.

Speciation of selenium within water is dependent on the oxidizing conditions of the water (Conde and Alejos 1997). Selenate (Se^{+6}) can be reduced to selenite (Se^{+4}) and elemental selenium (Se^{0}) but under oxidizing conditions, selenate is dominant. Elemental selenium is insoluble and, therefore, settles into the sediment, reducing the overall selenium concentration (Zhang et al., 1996).

Accumulation of tin (as tributyltin) and mercury (as methyl mercury) is a concern for fish destined for consumption as human food directly or indirectly as an ingredient in feed for food animals. The three main sources of heavy metal contamination for fish products are municipal/industrial wastes, anti-fouling paints and runoff containing herbicides, pesticides, fertilizers and other organic materials (Fairgrieve and Rust, 2003). Tributyltin is a common ingredient in anti-fouling paints used on recreational boat hulls and other marine surfaces. Tributyltin rapidly leaches into the aquatic environment and is found along with its breakdown products in sediment, water and fish in areas with high numbers of recreational boats (Waldock and Miller, 1983). Several researchers demonstrated that when tributyltin was used in paint to coat salmon net-pens, tin accumulated in fish tissues (Short and Thrower, 1986; Balls, 1987; Davies and McKie, 1987). These findings resulted in the passage of laws to restrict the use of tributyltin in Europe and North America. The US EPA and 13 individual states have enacted legislation on the use of tributyltin.

4.1.3 In Living Organisms

As with many chemicals, the concentration of metals and metalloids in living systems determines the biological effect. Several of the metal elements, such as selenium and cadmium, are micronutrients; however, large concentrations of these metals will have a detrimental effect on an organism. In plants, metal and metalloid contamination occur either through deposition directly onto the plant or the uptake of the elements from the soil or water.

The amount of metals or metalloids taken up by plants is dependent upon the plant. Sycamore trees, for example, have such an affinity for lead that Watmough and co-workers (1999) suggested using the ratio of $^{206}Pb/^{207}Pb$ found in sycamore tree rings to monitor pollution. However, metal and metalloid uptake affinity differs by plant species. In one study, crops cultivated in soil fertilized with mercury-contaminated sewage sludge had no detectable increase in plant tissue mercury concentrations (Granto et al., 1995). However, Bache and coworkers (1973) reported that onions cultivated in mercury treated soil had approximately ten times greater mercury content

than control onion crops. Similar results were reported for other metals and metalloids. When grown on high selenium content soils, white clover, buffalo grass, and grama contained low levels of selenium within the plant tissue. Sulfur containing plants, such as broccoli and cabbage, grown under similar conditions accumulated considerably higher selenium levels (Ohlendorf, 2003). Wiersma and co-workers (1986) detected significantly higher cadmium levels in Dutch grown lettuce and spinach plants than in fruit plants such as tomatoes, cucumbers, and apples.

Studies of lead and chromium have indicated that the majority of these metals remain within the root tissues of the plants (Arora and Joshi, 1997; Patte and Pain 2003). Metal and metalloid toxicity affect plant growth and development. Arsenic and chromium suppresses plant growth, as well as affecting root development and yield in crops (Arora and Joshi ,1997; Abedin et al., 2002).

Trace amounts (less than five mg/kg) of metal and metalloids have been detected in cattle with ingestion believed to be the greatest exposure route (Sager et al., 1998; Miranda et al., 2003). Studies in North America and Europe have indicated that the metal concentrations within grazing grass were correlated to cattle muscle and renal concentrations (Hintze et al., 2001; Alonso et al., 2003). Additional studies have detected trace heavy metal content within cattle feed stock (Sager et al., 1997; Nicholson et al., 1999), with the highest concentrations for zinc and copper due to the addition of mineral supplements.

Studies within cattle determined that the metals accumulated mainly within the liver and kidney (Vreman et al., 1986; Miranda et al., 2003), although increased concentrations also have been observed by researchers in milk, blood, and muscle tissue (Blanford et al., 1997; Hintze et al., 2001). A significant percentage of ingested heavy metals, however, are excreted through the feces (Nicholson et al., 1999). Blanford et al. (1997) detected a 3,000% increase in fecal lead excretion in cows fed lead acetate. However, Hintze et al. (2002) suggested that cattle are able to adapt to high dietary selenium levels and, thus, are less likely to retain excess amounts.

4.1.4 In Foods and Fats

Metals and metalloids are present in food both as natural minerals (i.e. iron, selenium) and as contaminates (i.e. arsenic, mercury). Dougherty et al. (2000) place total dietary exposure for the United States population at 0.2 µg/kg a day for cadmium and arsenic and between 0.04 and 0.08 µg/kg a day for mercury. The Total Diet Study, conducted by the US Food and Drug Administration and partially summarized for animal products in Table 4.1, ascertained that most American food contains metal and metalloids. Fish (tuna, salmon, and fish sticks) contain the highest concentrations of contaminants, particularly arsenic and mercury. Trace levels were detected in beef, pork, and chicken foods.

Table 4.1 Metals and Metalloids Detected in US Foods from 1991–2002 (USFDA 2004)

	Concentration (mg/kg)								
	As	Cd	Cu	Fe	Pb	Hg	Ni	Se	Zn
Whole milk	0	0	0	0	0.001	0	0.005	0.021	3.6
Cheddar cheese	0	0	0.3	1.9	0.001		0.003	0.215	37.5
Ground beef, pan cooked	0.001	0	0.8	24.8	0.001		0.026	0.201	57.2
Beef chuck roast, baked	0.001	0	0.9	27.7	0.002		0.006	0.259	81.9
Beef liver, fried	0.007	0.063	121	62.7	0.026	0.001	0.001	0.647	54.8
Tuna, canned in oil	0.929	0.021	0.4	8.9	0.001	0.163	0.021	0.711	5.8
Fish sticks	0.831	0.011	0.6	9.4	0.001	0.004	0.105	0.162	5.8
Salmon, baked	0.557	0	0.4	3.3	0	0.03	0.003	0.278	4.4
Ham, baked	0	0.003	0.6	8.1	0.001		0.016	0.289	20.6
Pork roast, baked	0	0	0.8	9.5	0.001		0.002	0.337	28.9
Chicken, fried	0.02	0.001	0.7	12.2	0.001	0	0.016	0.262	19.3
Chicken breast, roasted	0.016	0	0.3	5.2	0.001	0	0.008	0.272	9.0

Studies also indicate that trace levels of metals and metalloids are found in animal fats. Studies by Szlyk and Szydlowska-Czerniak (2004) and by Naraski (1985) detected small levels (less than 400 μg/kg) of cadmium, lead, copper, arsenic, and selenium in

butter. Kohiyama (1991) measured nickel, copper, and iron concentrations in lard of 0.27 mg/kg or less. Metals and metalloids also were detected in rendered fats at levels of less than 50 µg/kg during a study of German rendered products by Bahadir and co-workers (2004).

4.1.5 In Emissions

Trace levels of metallic crude oil contaminants are present in petroleum diesel (Heathcote et al., 2000) and these metals are emitted primarily as part of the particulate matter in a diesel engine's exhaust (McDonald et al., 2004). A considerable amount of information regarding the behavior of metal and metalloid contaminants in diesel engines can be inferred through the study of incinerator and coal power plant emissions. The metal and metalloid content of these emissions are dependent upon the volatility of the analyte and composition of the burning material. Mercury, being highly volatile, is mainly emitted in metallic form (Hg^0) within the flue gas, though it can be reacted to mercuric chloride and captured within scrubbers (Galbreath et al., 1996; Senior et al., 2000; Wu et al., 2004; Xu et al., 2004). Less volatile elements, such as arsenic, zinc, lead, and selenium, volatize during combustion but condense downstream in the exhaust system and are emitted as particles (Wang et al., 2003; Chen et al., 2004; Guo et al., 2004).

4.1.6 Conclusions

Current evidence suggests that metals and metalloids within animal fats will not cause significant safety issues in the production and use of rendered fat-based biodiesel. Metallic contamination of animal fats is low, generally less than 1 mg/kg. Mercury, an element of particular concern, was not detected. Any metals or metalloids present within the fuel would most likely be emitted as a gas or as particles in levels similar to or lower than those observed with petroleum diesel use.

Little scientific literature exists on the metal and metalloid composition of rendered fats. Studies are needed to quantitate metal and metalloid contamination levels in rendered animal fats, but also to determine the fate of these potential contaminants during biodiesel synthesis. Heavy metals accumulate in the food chain and are a concern for long-lived animals. However, since most animal tissue destined for rendering is from animals of 3 years or less in age, there is little opportunity for mercury to accumulate in food animals. The source of most rendered animal products is from food animals, which are typically grown to market size and harvested at a relatively young age. The presence of heavy metals in rendered animal fat destined for use as biodiesel has not been investigated but is not expected to be present in high enough concentrations to pose a direct human health threat.

5 RESEARCH NEEDS AND CONCLUSIONS

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Biodiesel produced from animal fats poses a negligible risk to human and animal health. Although the list of possible biological and chemical contaminants in animal fats used for biodiesel seems ominous, an in-depth review of available literature provides very little evidence of public health and environmental exposure risks when animal fats are used as a feedstock to produce biodiesel fuel. An absolute zero-risk assessment cannot be made specific to animal fat use or any other fat used as a biodiesel production feedstock source. However, numerous references contained within this text substantiated that there was minimal risk even from more direct human and animal exposures to potential contaminants in rendered animal fats than could likely ever occur with the use of this fat as a biodiesel feedstock.

There are a number of safeguards regulating both fats and oils used in food, feed and industrial applications that provide monitoring for any potential hazards - including those that could impact use of animal fats as biofuel feedstocks. These safeguards include the international maximum residue limits (MRLs) established for meat, poultry and fish foods which are the primary tissue sources from which rendered animal fats and oils are derived (Australian Government Department of Agriculture, Fisheries and Forestry, 2003). The United States Food and Drug Administration (FDA) and the Canadian Food Inspection Agency (CFIA) have regulations and established guidelines referencing biological and chemical contamination for food and feed uses. The U.S. Environmental Protection Agency (EPA) has regulations and guidelines for water and air emissions that apply to animal slaughter, processing, and rendering facilities. These monitoring procedures are directed at more specific human and animal exposure risks (i.e. oral) than afforded via usage as a biofuel.

This review investigated possible chemical and biological safety concerns of using animal fats in the production of biodiesel and any potential hazards that may occur in animal fats exclusive of transmissible spongiform encephalopathies (TSEs). The review focused on potential safety concerns that may occur during the manufacture, transportation, storage and combustion of biodiesel made from animal fats. No linkages were identified between use of animal fats for biodiesel and any biological or chemical hazard. No scientific, refereed studies were reported concerning any contaminating residue due to use of animal fats in the biodiesel manufacturing process and use. The production and combustion of biodiesel involves numerous process reactions and it is concluded that those conditions will also be conducive to chemical, thermal and physical degradation of biological and organic chemical agents.

The currently available literature indicates that a very low risk of hazards exist in animal fats for the production of biodiesel. Furthermore, the chemical and physical nature of the production processes for biodiesel and its combustion, respectively,

should enhance the safeguards already in place. Further research may be needed should any potentially hazardous contaminants be identified in feedstocks utilized as biofuels.

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