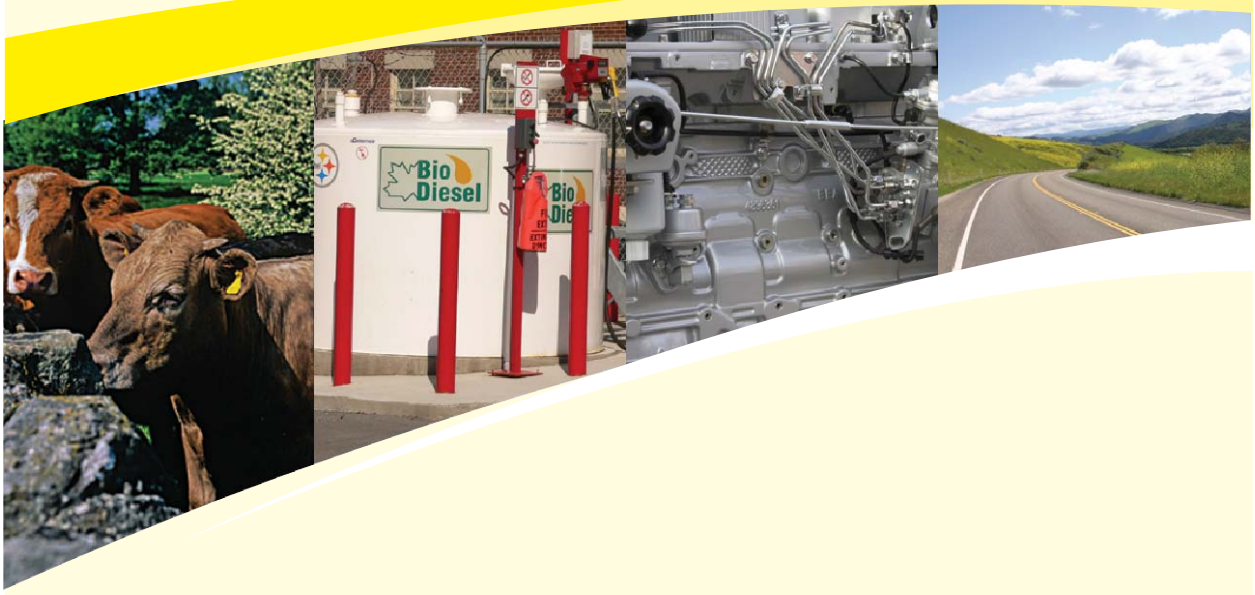
Biodiesel from Specified Risk Material Tallow: An Appraisal of TSE Risks and their Reduction



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Foreword

In the past few years, concern over rising oil prices and the peaking of oil production have increased global interest in pursuing transportation fuels derived from renewable feedstock sources. The use of animal fats and tallow to produce biodiesel fuel is one such pathway actively being pursued, and is gaining attention in Canada, the United States, and parts of Europe as certain ruminant animal risk material is coming under regulatory review and further control. Animal tallow, which has proven to be a dependable source material for a variety of value-added consumer products, will be impacted to some degree due to segmentation of animal fats as regulatory controls are enacted. The use of specified risk material to produce fuel is an important economic and disposal option, as the materials could be an excellent feedstock for value-added production of renewable biodiesel.

The goal of the study resulting in this report was to determine what, if any, animal and public health risks and environmental exposure issues might result from using animal fats for biodiesel fuel, particularly those materials specified at higher risk for transmissible spongiform encephalopathy (TSE). An absolute zero-risk assessment cannot be made and would be impossible to prove at this juncture in time. This study, therefore, examines current research results to determine what potential risks exist, and whether risk reduction processes would result in an immeasurably small (*i.e.*, negligible) risk to public health, animal health and the environment. Specific emphasis was directed at collecting information that will be needed by government decision makers, commercial interests, private investors, and the general public to understand the merits of animal tallow-based biodiesel.

To produce this report, ATFCAN engaged the services of scientists with substantial expertise and experience, from both Europe and North America. A literature search was undertaken to investigate chemical and biological considerations that could result in any potential for adverse health effects. Of particular interest is the processing of tissues from animals potentially infected with bovine spongiform encephalopathy, or "mad cow" disease. This report presents a systematic survey of current knowledge devoted specifically to this topic. It is organised into an introductory background section on TSE, followed by chapters covering the sequential stages of biodiesel production.

The work resulting in this report was commissioned and begun in 2004. This report was largely written in 2004 and 2005. Research findings and developments constantly result in additions to literature and knowledge. It will be prudent, therefore, for government officials, regulators and the renewable fuels industry to continuously assess whether such new information might require the conclusions of this report to be modified in regard to the safety of biodiesel made from animal fats. An example of new information is the announcement made by the Canadian Food Inspection Agency (CFIA) on June 26, 2006, two days before printing this report. The CFIA announced new regulations enacting a ban on the use of specified risk material in any animal feed, pet food or fertilisers in Canada. In addition, one of the disposal options listed by the CFIA for specified risk material is processes that can generate biofuels.

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EXECUTIVE SUMMARY

This document presents a systematic survey of current knowledge about the risk to human and animal health posed by the processing of tissues from animals potentially infected with transmissible spongiform encephalopathy (TSE, or 'prion disease') into biodiesel. It is organised into an introductory background section on TSE, followed by chapters treating the sequential stages of biodiesel production. The principal conclusions are:

Animal tissue sources. The choice of geographic origin, based on published scientific evaluations of the risk of TSE to be present in a given country, can largely reduce or even eliminate the entry of contaminated tissue into the biodiesel feedstock. Further safeguards can be provided by selection of animal species not susceptible to TSE, and of tissues without any detectable infectivity even in susceptible species. None of these measures, however, would be applied to the biodiesel projects under consideration, which have the specific aim of using animals and tissues (including specified risk material, or SRM) considered to have sufficient potential risk to be unacceptable for use in food, feed, fertilisers, or pharmaceuticals (including biologicals and medical devices) and therefore are designated for other approved uses, or destruction.

Tissue rendering to produce tallow. Experiments to test the survival of TSE infectivity in the products of rendering failed to detect any infectivity in the crude tallow fraction, even following processing methods that allowed survival of infectivity in the co-produced meat and bone meal fraction. It is therefore extremely unlikely that pure tallow originating from diseased animals would be infectious; however, lower grade tallow might contain infectious impurities. A requirement that tallow derived from SRM for use in biodiesel contain no detectable protein would reduce this possibility.

Transesterification of tallow to biodiesel. Several steps in the manufacturing process have at least the potential to inactivate TSE infectivity, for example, exposure to strong acids or bases and, depending on the process, exposure to high temperature or purification by chromatography or precipitation. However, the two-phase aqueous-lipidic nature of transesterification introduces an element of uncertainty about the effect of these procedures on infectivity.

Biodiesel use. Combustion temperatures, even at millisecond exposures, might partially inactivate contaminated biodiesel. But experimental data have so far been limited to saline tissue suspensions of infected tissues and several minute exposure times in static rather than dynamic combustion processes. A substantial body of epidemiological and laboratory evidence indicates that TSE is not transmitted by aerosol inhalation.

Overall conclusion. **Biodiesel produced from animals infected with TSE poses a negligible risk to animal and public health.** This conclusion extends even to the use of SRM as a source of tallow, based on experimental evidence showing that rendered tallow from infected animal tissues does not transmit disease to inoculated susceptible animals. Although infectivity reductions during biodiesel manufacturing steps should therefore be redundant, any such reduction would still be desirable as an added measure of safety. At present, the potential for infectivity reduction through biodiesel manufacturing and combustion can only be estimated from analogy to methods known to inactivate infectivity in saline suspensions of infected tissue. Studies of the actual

biodiesel process, using experimentally contaminated input tallow, are recommended as the only means by which a scientifically-based conclusion can be made about the capacity of these processes to reduce or eliminate TSE infectivity.

GLOSSARY OF TSE-RELATED ACRONYMS AND TERMS

Commonly Used Acronyms and Abbreviations

ACDP – Advisory Committee on Dangerous Pathogens (UK)
BSE – Bovine Spongiform Encephalopathy
CFIA – Canadian Food Inspection Agency
CJD – Creutzfeldt-Jakob Disease
CNS – Central nervous system, including the brain, spinal cord and all nerve tissue.
CWD – Chronic Wasting Disease, a TSE disease affecting deer and elk.
DEFRA – Department for Environment, Food and Rural Affairs (UK)
EC – European Commission (Civil Service of the European Communities)
EFSA – European Food Safety Authority, similar to the US FDA
EU – European Union of Member States
FDA – Food and Drug Administration (US)
FSIS – Food Safety and Inspection Service, US. Department of Agriculture
GBR Assessment – Geographical BSE Risk Assessment (EC)
ID_{50} – mean infectious dose (a dose sufficient to produce a 50% probability of infection in the recipient).
i/c – Intra-cerebral (inoculation into brain tissue)
i/p – Intra-peritoneal (inoculation through the abdomen)
LRS – Lymphoreticular system
MBM – Meat and bone meal, the protein by-product of rendering.
OIE – Office International des Epizooties (World Organisation for Animal Health)
***PrP* gene –** The gene encoding the prion protein (called *PRNP* in humans and *prn-i* in mice).
***PrP^{C}* and *PrP*-sen –** Common abbreviations for normal *PrP*.
***PrP*-res**, ***PrP^{Sc}*, *PrP^{TSE}* –** Common abbreviations for abnormal *PrP*.
SAF – Scrapie-associated fibril, first observed in the brains of scrapie infected animals by electron microscopy, and subsequently shown to be specific to, and diagnostic of, all TSE diseases.
SEAC – Spongiform Encephalopathy Advisory Committee (UK)
SRM – Specified risk material(s), animal by-products or waste designated as potentially carrying TSE risk.
SSC – Scientific Steering Committee of the EC
TME – Transmissible Mink Encephalopathy, a TSE that affects farmed mink.
TSE – Transmissible Spongiform Encephalopathy
USDA – United States Department of Agriculture
WHO – World Health Organisation

Scientific Terms

Amyloid: An abnormal (misfolded) form of any of several different proteins that have specific staining characteristics and appear as homogeneous aggregates or plaques under a microscope.
Animal by-products or any material containing such products: Entire bodies; parts of animals; products of animal origin not intended for human consumption (including

hides and skins, ova, embryos and semen); parts of animals fit for human consumption but not intended for human consumption due to commercial reasons and potential TSE infectivity; animals killed for TSE eradication; pet, zoo and circus animals; experimental animals; wild animals suspected of being infected with a disease communicable to any species; SRM; entire bodies or carcasses containing SRM; carcasses containing certain residues; animal material collected during the treatment of waste water, sludge etc; catering waste. (Source: Regulation EC No.1774/2002)

Biodiesel: Diesel oil derived in whole or in part from non-mineral sources and satisfying the engineering and legislative requirements for use in unmodified diesel engines.

Biodiesel (as discussed in this document): Biodiesel produced in whole or in part from animal fat sources. Biodiesel can also be produced from plant oils, but this topic is not discussed in this report.

BioDiesel: A brand name of biodiesel.

Bovine Spongiform Encephalopathy: A degenerative disease affecting the nervous system of cattle, first identified in the 1980s.

Cellular protein: A normal, usually proteinase-sensitive cellular protein that, in the context of TSE, is usually abbreviated as PrP^{C} or *PrP*-sen.

Centrifugation: a process by which a complex liquid material is separated into its components by high-speed rotational force.

Chronic Wasting Disease: A contagious fatal TSE disease of the deer and elk family.

Creutzfeldt-Jakob Disease: A rare neurological disease that usually afflicts humans over 55 years of age. It occurs at a rate of about 1 case per million each year worldwide. It has four main types: sporadic (the most common), familial, iatrogenic, and variant.

Discrete adipose tissue: Animal material passed as fit for human consumption, recognisable as depot fat from certain body sites, such as kidney, knob and channel fat, fat round the heart, the omentum and mesentery, and including fat trimmings from the abattoir, cutting plants and butcher's shops. This fat is *premier jus*—of the highest quality and used mostly for human food.

Dose: The product of the titre per unit mass and the mass inoculated/consumed.

Encephalopathy: A degenerative condition of the brain.

Esterification: The process used to transform fats or oils into biodiesel.

Fat melting: Extraction of fat from discrete adipose tissue, bones and fat trimmings by melting under gentle heat at temperatures below 100 ºC.

Feedstock: The starting material used for biodiesel production.

Greaves: The main protein product of rendering after water is driven off and fat extracted. The immediate precursor of meat and bone meal which is generated by grinding greaves.

Immunihistochemistry: A laboratory method using histological sections of brain tissue that have been reacted with a labeled antibody specific for a given protein. In TSE, an antibody is chosen that reacts with the prion protein, and the coloured product is visualised microscopically (light emitted from a fluorescent label). This is the type of test performed on cattle brain samples in BSE surveillance programmes.

Infectivity: The ability to infect, in this case with TSE. TSE infectivity is almost always associated with the presence of the misfolded protein.

Kuru: A nearly extinct neurodegenerative disease of a few indigenous populations in the Highlands of New Guinea. It was caused by endocannibalistic rituals at funerals, and is the prototype of human TSE.

Misfolded protein: The transformed and usually proteinase-resistant form of a normal cellular protein. In TSE, the misfolded protein is called a 'prion', and carries numerous superscripts such as PrP^{Sc}, *PrP*-res, PrP^{TSE} and PrP^{d}.

Parenteral: A route of exposure/challenge other than the oral route.

Plaque: A patch or small differentiated area consisting of amyloid protein.

Pressure-cooking: Rendering in which the standard parameters used are particle size ≤50 mm, 133 ºC, 3 bar 20 min in a batch or continuous system. Also known as 'sterilisation'.

Prion: The name of the protein that in its misfolded form is thought to be the cause of TSE.

Protease: An enzyme that splits proteins into simpler substances.

Proteinase K: A specific digestive enzyme that degrades normal proteins into smaller polypeptide chains. It is widely used to destroy the normal form of prion protein to leave only its partially proteinase-resistant misfolded form.

Rendering: Cooking animal waste material with or without added fat at temperatures usually in excess of 100 ºC in a batch or continuous process, to separate fat, greaves and water for further treatment and use.

Ruminant: Cattle, sheep, goats, deer, elk, camels, buffalo and antelope: a family of herbivorous animals with four stomach chambers that chew the cud.

Scrapie Associated Fibrils: Aggregations of misfolded *PrP* or protein fibrils, seen with an electron microscope.

Species barrier: The natural barrier that restricts or prevents transmission of TSE between species.

Starting material: Any substance or ingredient used in or during the manufacture of biodiesel, including for rendering and biodiesel manufacture and for blending after production and before sale.

Tallow: Rendered animal fat or grease. Animal fats obtained by melting or cooking and separated from other material by pressing, drawing off or centrifugation and further refined by filtration, heat or chemicals.

Titre: The concentration of infectivity per unit mass, measured by bioassay.

Trans-esterification: a process in which the triglycerides in fatty tissue are converted into free fatty acids and hydrocarbon fuel.

Transmissible Spongiform Encephalopathy: The name for a small group of fatal neurodegenerative diseases caused by misfolding of the prion protein and characterised microscopically by a sponge-like appearance of the brain.

Variant Creutzfeldt-Jakob Disease (vCJD): A human form of TSE, first reported in the UK in 1996, resulting from infection by the agent of BSE.

Western Blot: A laboratory test for the identification of proteins that is widely used to detect the pathological (misfolded) form of the prion protein.

1 INTRODUCTION

Until very recently, fuel for internal combustion engines has nearly all been derived from mineral and fossil sources. But these sources are finite and appear to be dwindling, which poses a critical problem for meeting future fuel needs. Moreover, oil has become a major political and economic issue, since production and prices often fluctuate beyond the control of major user countries. Alternative fuel sources would help mitigate this looming fuel crisis. Renewable sources of fuel could help stabilise the world supply of non-renewable fuels, while reducing the environmental impact of fossil fuel extraction and combustion.

In the past few years, new technologies have been developed for processing non-mineral fats into fuels, including diesel engine fuel. Virgin oils from plants grown specifically for fuel production, and food industry fats, have been used as feedstock for biodiesel production; but virgin animal fats have only rarely been used. Animal fats are a dependable source since they are regular by-products of the meat industry. But concerns about animal by-product safety have become a factor affecting commercial uses for these fats.

The occurrence of bovine spongiform encephalopathy (BSE) in Europe, Asia and North America since the 1980s has resulted in new restrictions on animal processing, which in turn has severely limited the availability of fat designated 'safe' for use in human food, animal feed, cosmetics, and medicines. Consequently, 'safe fat' is now in reduced supply, and high prices limit its use in biodiesel production. Meanwhile, some animal by-products, which are also renewable sources of fat, have been designated as 'specified risk material' as a precaution against spreading BSE and related diseases. Thus, there is now a surplus of these less desirable fats. Given the attractive price and the lack of alternative uses (or even disposal options) for these fat surpluses, other than furnace fuel, they are an excellent potential source of feedstock for biodiesel production.

1.1 Objectives of this Report

The goal of the study resulting in this report was to determine what (if any) animal and public health risks and environmental exposure issues might result from using animal fats for biodiesel fuel manufacture. In particular, if a proportion of the source animals were actually or potentially infected with a transmissible spongiform encephalopathy (TSE) agent, what (if any) risks might there be to the public and to the environment?

The study's primary objective has been to examine low-value fat derived from potentially TSE-infected tissue—specified risk material (SRM)—and evaluate its safety as a renewable source of biodiesel fuel. There are several reasons for focusing only on low-value fat:

- This fat currently has no use other than as fuel. It cannot be returned to the animal food chain or used as a source material in products for human use. Engine fuel production is one of its potential applications.
- If SRM and other hazardous animal waste (*e.g.*, Category 1 Waste, as designated by the European Commission) are not put to use, then they must be disposed of. Currently, incineration, alkaline hydrolysis and deep burial are the only approved

options, each of which is controversial. A new alternative use would solve a large part of the disposal problem.

- Biodiesel is a product of added value from a largely unusable processed animal by-product, and its production is self-financing.

For these reasons, biodiesel production is an attractive use for low-value animal fats.

There has already been considerable expert discussion of the topic. The Scientific Steering Committee (SSC) of the European Commission (EC) has produced more than 234 *opinions* (formal evaluation papers) on TSE-related risk assessments. These opinions are scientifically based, with thorough examinations of relevant risk factors and comprehensive literature reviews. They have been adopted after careful scrutiny by an independent EC TSE ad hoc committee and after independent experts from EU Member States and other countries have made presentations and provided information. These opinions form a valuable source of information freely available on the Internet at http://europa.eu.int/comm/food/fs/sc/ssc/outcome_en.html.

Certainly, TSE is a controversial subject. Moreover, there is still considerable scientific uncertainty about the structure of the agents that cause TSEs, as well as the effects they have on people, animals and the environment. The controversies and limits to current knowledge are factored into this report's discussions.

An absolute zero-risk assessment cannot be made and would be impossible to prove. Therefore, this study examines current research to determine what potential risks exist and whether risk reduction processes would result in an immeasurably small (*i.e.*, negligible) risk to public health, animal health and the environment. It examines all factors affecting risk of TSE transmission from animal fats rendered for biodiesel manufacturing processes to determine realistic risk levels:

- Does it matter which species the fat comes from? And it if does, then how much?
- Does it matter which tissues or organs the fat is derived from?
- Does it matter what the titre (concentration) of TSE infectivity is in the starting material used for rendering?
- Can the rendering process inactivate any TSE agent infectivity present in the starting materials?
- Can the biodiesel production process inactivate any TSE agent infectivity present in refined fats?
- Can combustion in diesel engines inactivate TSE infectivity present in animal fat-based biodiesel fuel?
- How strong is the species barrier between the species of origin of infectivity and the species exposed to it?

This report will therefore examine TSE risk and risk management associated with all major steps in the biodiesel process: (1) the starting materials (SOURCE); (2) the rendering PROCESS; (3) the biodiesel production PROCESS; and (4) biodiesel USE.

Because this paper discusses the potential risks from TSE agents, a Background Paper on TSE provides readers with basic information about TSE: the agents that cause it, the species affected, the epidemiology and pathogenesis, and public concerns about TSE.

The Background Paper concentrates on explaining those features of TSE that are necessary for understanding the nature of TSE, the uncertainties about risks associated with animal-based biodiesel, and the ways that risks can be reduced.

1.2 About Risk Communication

Communicating risk to the public is an integral part of the risk analysis process. Because uncertainty increases speculation and creates mistrust, good risk communication is crucial to consumer and trading partner confidence. It requires involvement of all interested parties: governments, industry, science, consumers and trading partners. Full, open participation promotes transparency and credibility in the decision-making process, and increases public confidence in the final decisions.

Because there are still scientific uncertainties about TSEs, risk communication about fat-based biodiesel production must be handled carefully. Describing the findings of in-progress research as facts that may later be proven incorrect decreases public confidence. At the same time, failure to communicate research information because it has not been proven also increases uncertainty and speculation. For these reasons, a balanced approach is recommended. Risk communication about TSEs in biodiesel production should include the following information:

1. What measures are being taken to reduce the risks, and why?
2. What is known?
3. What is unknown, and why?
4. What is being done to fill the knowledge gaps?
5. What precautionary measures are being taken in the interim?

Communication should also strike an appropriate balance between the 'pessimistic perspective' and the 'optimistic perspective' as outlined here.

1.2.1 The Pessimistic Perspective: A Worst-Case Scenario

A pessimistic view of the risks from animal-based biodiesel would be as follows:

> Although scrapie, a TSE of sheep, has been known since the 1700s, before 1986 no one imagined that animal TSEs might transmit to humans, and certainly no action was taken to prevent it. But since 1986 naturally occurring TSE has been found in several mammalian species previously considered to be TSE-free. Originally confined to the UK, BSE has now been reported in over 20 countries, as far apart as North America, Japan, the Middle East and Europe. It is now clear that at least one animal TSE (BSE) can transmit to humans. Human-to-human transmission of TSE is also a reality, but so far only through direct or indirect contact with internal human organs or tissues.
>
> Most large epidemics of TSE have been caused by one of the least efficient infection routes: the oral route (via food or feed). Cattle, sheep and some captive wild ruminant species have developed TSE from eating cross-contaminated animal protein by-products. Moreover, new TSE problems may already be developing. Some sheep and goats in Europe were fed meat and bone meal (MBM), which was the vehicle for transmitting BSE to cattle, and both species have been shown to be susceptible to experimental oral infection with BSE. A goat in France has been

diagnosed at slaughter to have had a BSE infection, and a goat that died in Scotland in 1990 is presently under suspicion. Moreover, a new phenotype of scrapie known as NOR98 has been reported in Scandinavia and some other European countries. At this stage, it is not clear whether this represents the emergence of a mutant strain of scrapie agent or an unusual response to existing strains. There is also at least one new phenotype of BSE in cattle (in Italy and Finland), which could be due to a new agent strain with unpredictable biological properties and host species range.

Furthermore, TSEs have long incubation periods. Recent results from 'Rapid' testing of brains of clinically healthy sheep in the EU, including those with a TSE-resistant genotype, have shown sub-populations with deposits of pathologically abnormal protein usually associated with TSE. Some of these are believed to be associated with NOR98 scrapie and appear to include infection in so-called resistant genotypes. Final conclusions cannot be made at this point, but these incidents remain a concern.

There are additional reasons to be concerned about TSE:

- TSE agents are highly resistant to inactivation.
- The minimal infectious dose for humans is unknown. In cattle, the amount of tissue that can carry an oral infectious dose is tiny: BSE can be transmitted experimentally by as little as 1 mg of brain tissue.
- Procedures such as blood transfusion, previously regarded as TSE-safe, may potentially transmit TSE between humans. Two patients have been infected in this way with the human form of BSE (variant Creutzfeldt-Jakob disease, or vCJD), and one of these humans was of a genotype previously believed to be resistant to BSE infection.
- TSEs are not believed to transmit by the respiratory route; but recent studies have found infectivity or abnormal protein in olfactory epithelium, nasal mucosa and preferentially in the olfactory lobes of the brain in some infected animals, thus raising concern.
- Some people may already be infected with vCJD without showing signs yet. Studies have found abnormal protein in tonsils, appendix or spleen of humans with and without clinical vCJD.
- Muscle tissue, previously considered free of TSE infectivity, has been shown to be infectious in laboratory models of TSE; and abnormal protein has been reported in ovine muscle in natural scrapie.
- Clinically normal animals can harbour levels of TSE infectivity in their brain tissue, previously considered a sign only of advanced disease.
- There are forms of TSE that suggest it could be contagious. Little is known about chronic wasting disease (CWD), the fatal TSE disease spreading rapidly through North America's deer and elk population, and no study has shown whether this disease transmits to humans.

Finally, despite the high temperatures and chemicals utilised, no rendering process has the capacity to completely inactivate high levels of infectivity in starting

material. There is little knowledge about titres of infectivity in tissues from cattle infected with BSE, or the clearance factors following transesterification of tallow and combustion of biodiesel. Thus, given all the unknowns about TSEs and what is already known about the unpredictability of TSE, a pessimistic perspective concludes that using SRM as feedstock for commercial products should be regarded as risky.

1.2.2 The Optimistic Perspective: Sensible Precautions

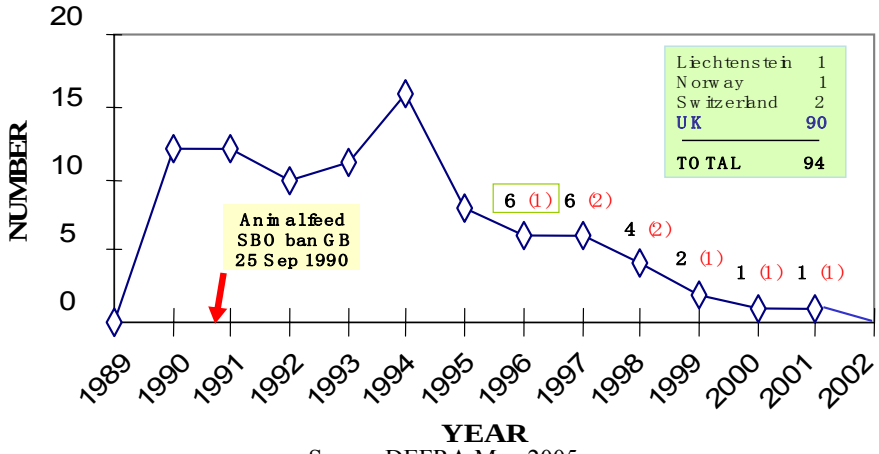
An optimist's perspective would attempt to examine certainties and uncertainties, and look for historical patterns which might indicate future trends consistent with reduced risks:

We know a great deal about TSEs, and humans have lived with TSE-infected animals since the 1700s, when scrapie was first discovered in sheep. Overall, TSEs are rare diseases, even counting BSE (< 200 000 clinical cases out of a population of around 75 000 000 cattle in a period of over 18 years in Europe). What we do know is that despite the unpredictable and unknown aspects of TSE, we have the ability to control, or even perhaps to eliminate, its risks to humans, animals and the environment.

First of all, TSEs can be eliminated with appropriate management. TME, a form of TSE in farmed mink, has not been reported anywhere since 1985 due to stringent management, and feline spongiform encephalopathy (see Figure 1.1) has apparently been eliminated in the UK. Meanwhile, BSE epidemics have declined in response to stringent control measures, even in the worst-affected countries.

Figure 1.1 Incidence of Spongiform Encephalopathy in Domestic Cats

In the UK by Year of Report (Number of cases born after 25 September 1989 when a specified bovine offal ban was introduced). No cases have been reported in 2002 through May 2005.



Source DEFRA May 2005.

Moreover, recent efforts in risk management have proven to be very successful. Steps taken in the EU and Switzerland to ensure that infected bovine material is

eliminated for any purpose other than fuel have prevented new human exposure. In addition, efforts to reduce the risk of human-to-human transmission by iatrogenic means (medical procedures) are already place. Finally, in many countries laws prohibit the feeding of most animal protein to species considered at risk; some, like those in the EU and Switzerland, ensure that the animal starting materials for feed are derived only from materials that are approved for human consumption and use. We can expect these controls to further reduce the number of BSE cases in the future. Furthermore, legislation requiring the removal and complete destruction of central nervous system (CNS) material from carcasses before butchering effectively prevents contamination of any food or feed. It is now known that TSE risks in milk and muscle and most other non-SRM are either absent or several orders of magnitude lower than the infectivity in the brain of an affected animal. Thus, even if an infected animal accidentally entered the food chain, current risk management practices would ensure negligible TSE infectivity to be in consumable products.

Moreover, TSEs do not spread easily. In the UK alone, it has been estimated that between 3 and 4 million BSE-infected cattle had been eaten by humans, and over 50 million infectious oral doses in feed had been consumed by cattle, before effective measures were in place. Yet to date fewer than 150 cases of vCJD have been confirmed in the UK, and the annual number of new cases is declining. The estimated future number of new infections from cattle through food transmission is likely to be very small. While it would never be possible to ensure there is a zero risk to humans from animal TSE agents, the measures now in place in the best-protected areas of the world are based on current science and have blocked all known loopholes. Thus, even if new research identified a risk in some material previously regarded as safe, that risk is likely to be comparatively small in the EU and other countries that have adopted similar prevention measures. Furthermore, those same scientific studies that revealed the new risk could be used immediately to improve existing measures or develop new ones to reduce the risk even further.

With careful studies, continuous risk assessment and fully enforced risk management procedures, a new process like the production of biodiesel fuel from animal fat can be accomplished safely. But the residual risks should be monitored. The general public is unlikely to be exposed to any risk beyond negligible. However, members of directly affected trades, such as those in the fuel and engine repair/disposal industries who might become exposed to material where concentration of protein might accrue, especially where repetitive exposure might occur, should undertake a local TSE risk assessment and if appropriate take any precautionary measures that are indicated.

1.3 Summary

Committees, scientists and regulators using this report to draw conclusions about TSE risks in biodiesel production are encouraged to balance their perspectives between optimism and pessimism. There is a great deal of difference between a potential risk and a real, quantified risk. Moreover, it is tempting to retreat behind the idea of unpredictable future events. The public interest is better served by examining current scientifically based precautions and procedures to reduce the incidence of TSE and to determine their effectiveness. Certainly, there is reason for caution; but there is also opportunity. A balanced perspective will weigh both sides of each issue.

It is also important to keep in mind that SRM already poses a disposal problem around the world. Currently, there is no safe and environmentally sound solution to this problem, especially one that adds value. Biodiesel production using SRM feedstocks can be considered a potentially beneficial approach to resolution of this issue.

The information in this report is based on the best research to date on the subject of TSEs, rendering, and biodiesel production and use. It is intended (a) to help the reviewer reach a balanced perspective on the advantages and risks associated with the production of biodiesel from lower-value animal fats, and (b) to make recommendations for additional research.

2 BACKGROUND PAPER ON TSE

This Background Paper has three main purposes:

- To provide non-expert readers with basic information about TSEs and their various forms;
- To establish the level of current knowledge about TSE issues; and
- To create a foundation for understanding more complex issues involved in using animal fats as a source for starting material used for the manufacture of biodiesel.

For simplicity, references have been placed at the end of the chapter, organised by topic.

2.1 Transmissible Spongiform Encephalopathies (TSE)

TSEs form a group of consistently fatal mammalian diseases affecting the nervous system. They have several characteristics in common. First of all, they are insidious in onset and progressive, mainly affecting older adults. The incubation periods are long: in humans exposed to environmental sources of infection, incubation usually ranges from 5 to 15 years but can exceed 40 years. Second, TSEs are all experimentally transmissible, even though transmission does not always occur in a natural environment. Third, unlike many other pathogens, TSE agents cannot multiply or replicate naturally outside the body. Although a TSE can be suspected on clinical grounds, definitive confirmation requires microscopic examination of the brain, either from biopsy or autopsy. There are no characteristic gross findings anywhere in the body: microscopic spongiform changes accompanied by deposits of PrP^{TSE} in the brain are the only basis for diagnosis. Finally, TSE affecting humans, cattle and cats do not seem to be 'naturally' transmissible, whereas those affecting sheep and deer do spread from animal to animal. (The placenta of infected sheep is infectious but the precise vehicle of transmission is unknown.)

2.1.1 Causal Agents of TSE

All TSEs are caused by infectious agents that have certain general properties. These properties include the lack of a conventional immune response in the host (no antibody response is triggered) and resistance to decontamination by chemical and physical methods known to be effective against bacteria, viruses and fungi. But the individual TSE agents can differ biologically and exist as 'strain types'.

TSEs are also sub-microscopic, possibly molecular, units in which no nucleic acid has so far been found. Genetic material (DNA) has not been detected in TSE agents, and so alternative hypotheses have been proposed to explain why they sometimes show variable biological and molecular properties (strain type), even when isolated from the same host. There are several different hypotheses about the nature of TSE agents, but three have dominated the literature: the prion hypothesis, the virino hypothesis, and the unconventional virus hypothesis.

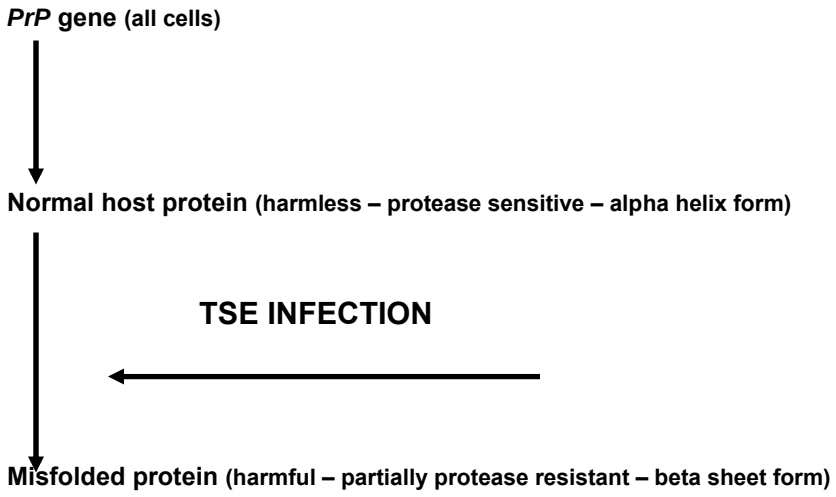
2.1.1.1 The prion hypothesis

Based on studies of scrapie-infected hamster brain showing co-purification of infectivity with protein, the term *prion* was introduced in 1982 as an acronym for 'proteinaceous infectious particle'. Subsequently, the protein was identified and named

the *prion protein*. When it was discovered to be a host protein rather than a foreign protein, the encoding gene was named the *PrP gene* (*PRNP* gene in humans). Further studies described the distinguishing features as an infectious *misfolded* form of an otherwise healthy protein. In this report, 'normal protein' will refer to the normal, proteinase-sensitive, cellular protein (usually abbreviated as PrP^{C} or *PrP-sen*), and 'misfolded protein' will refer to the transformed (amyloid) protein in any of its proteinase-sensitive, intermediate or resistant forms (usually abbreviated as PrP^{Sc} or *PrP-res*, but also embracing all other notations).

The prion hypothesis proposes that the causative agent of TSE is an infectious protein, something that until the 1980s had not been either seriously considered or justified, even though it had been first proposed in 1967. Essentially, the hypothesis is that a normal host membrane protein (a sialoglycoprotein with a predominantly alpha-helical structure and a molecular weight of 33 to 35 kD) is post-translationally misfolded into a predominantly beta-sheet (amyloid) configuration that 'catalyses' an ever-increasing number of the normal molecules to transform into the abnormal misfolded configuration (see Figure 2.1). The new protein form is resistant to the immune system and to protease (a digestive enzyme), which allows it to survive, multiply, enter the body via the gut and to spread to other parts.

Figure 2.1 Relationship between the PrP Gene and Misfolded Protein



The normal protein is thus thought to acquire its infectious property by virtue of misfolding into an amyloidogenic beta-sheet structure that has the ability to continuously convert normal protein molecules to misfolded protein molecules. The mechanism is not completely understood but may involve dimerisation (a sort of chain reaction) or nucleation (aggregation). The primary chemical structure (amino acid sequence) of the protein remains the same in both forms (in the same host); but the two forms of protein are distinguished physically only by a change in the shape. The most convincing evidence to support the prion hypothesis is that genetically engineered mice in which the *PrP* gene has been removed (*i.e.*, they lack the normal protein from which prions develop) do not develop TSE diseases when challenged by inoculation (injection) with TSE-infected material.

Very recently, infectivity has been 'created' by experimentally misfolding a portion of the normal host protein and inoculating it into transgenic mice, which then went on to develop TSE. If this result can be independently verified, it will prove the prion hypothesis. Even so, these laboratory findings will need to be applied to naturally occurring TSE in humans and animals. Thus, for the purpose of this report, the prion hypothesis will continue to be considered as hypothesis, rather than fact.

2.1.1.2 Normal proteins versus misfolded proteins

Interestingly, the precise role of the normal *PrP* protein is uncertain. For mice, this protein may even be redundant, since its absence is not life threatening. However, changes in circadian rhythm and in transmission of neural signals have been reported.

But the misfolding of this protein results in new properties that enable the two forms of protein to be simply identified in the laboratory. No one has ever seen a prion (it may only be a single molecule). But research has shown that the agent is 'sticky', which leads to difficulty in removing it from instruments and equipment exposed to infectious tissues. The protein is also insoluble in most solvents and tends to aggregate easily, so larger accumulations of single molecules can be observed in the form of plaques in some (but not all) TSE-infected brain tissue sections.

Furthermore, prion-agglomeration structures known as *scrapie-associated fibrils* (SAF) can be seen via negative stain electron microscopy (a method used to visualise viruses in infected material) of detergent extracts of infected central nervous system (CNS) material treated with proteinase K, a technique that has been used to confirm the disease. Digestive enzymes called proteases normally split proteins into chains of shorter polypeptides as part of the digestive process. Proteinase K, which is a protease, is frequently used in the laboratory because it completely digests most normally structured proteins, including the normal *PrP*. However, proteinase K does not completely digest the misfolded protein, but rather splits it, losing part of the molecule and leaving behind a truncated piece with a molecular weight of 27 to 30 kD. These truncated pieces aggregate to form microscopically visible plaques.

Prions can also be detected using cross-species antibodies. Although the misfolded protein is not antigenic in the host from which it is derived, it is possible to generate antibodies to it in rabbits (polyclonal antibody) or in particular laboratory rodents (monoclonal antibody). This enables its detection by several different immunological methods, including immunoblotting (Western blotting) and immunohistochemistry. In this way, an infected tissue can be identified without resorting to expensive, time-consuming bioassays. Infectivity is assumed because the presence of the misfolded protein is so intimately associated with TSE infectivity that they are, for practical purposes, inseparable. As a result, the misfolded protein is accepted as the marker of infectivity.

2.1.1.3 Other hypotheses

The other two main TSE hypotheses are the virino and unconventional virus hypotheses (see Figure 2.2). The former declares that the infectious agent is a hybrid of a misfolded protein and an infectious genome comprised of a small nucleic acid. In this hypothesis, 'virinos are host proteins sequestering the agent genome which may code for no product other than copies of itself'. If such nucleic acids actually are present in the misfolded protein, then conventional molecular theory could be used to explain the

wide variation in types of TSE agents and diseases. But no such nucleic acid has been found despite intensive search.

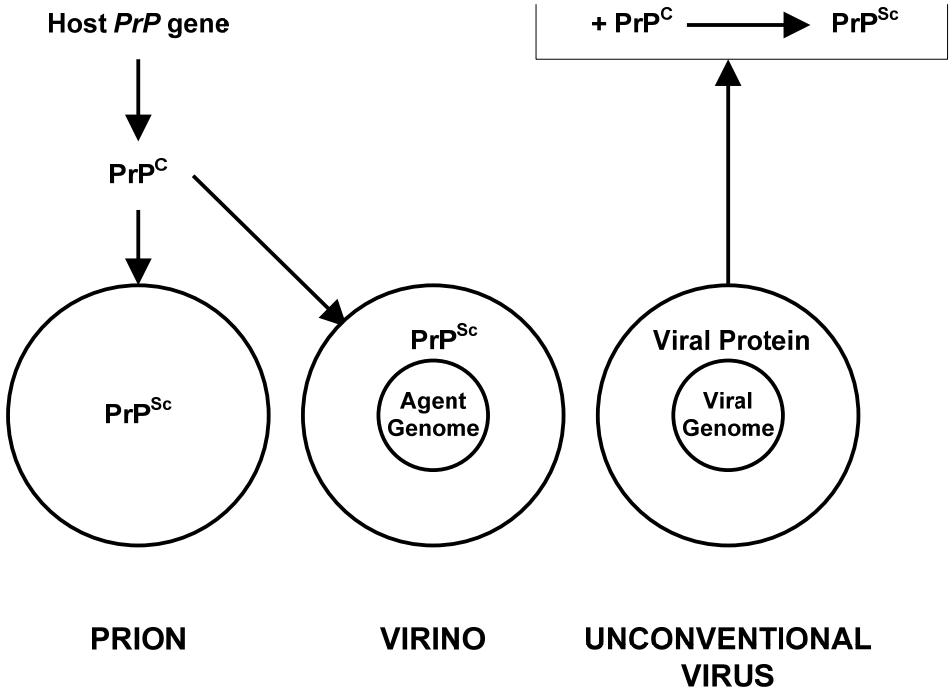
The unconventional virus hypothesis proposes that the agent also includes a viral genome and suggests a more conventional viral structure, but with the unusual properties of resistance to destruction and absent antigenicity. The problem, again, is that no disease-specific nucleic acid has been found.

Figure 2.2 TSE Agent Hypotheses

PrP gene – the gene responsible for generating PrP^{C}

PrP^{C} – normal host protein

PrP^{Sc} – misfolded protein



2.1.2 Species Affected by TSE

Given the importance of abnormal protein in TSE, any species with a *PrP* gene in the central nervous system (CNS) is hypothetically susceptible to TSE. All mammalian species and some others studied to date do express the normal protein in the brain.

However, TSE has so far been found to occur naturally only in a relatively small number of orders of mammals and individual species. These include Primates (humans and possibly lemurs), which can contract both kuru and Creutzfeldt-Jakob Disease (CJD) and variant CJD (vCJD); Bovidae (sheep, goats, cattle and similar captive-wild species), which can contract scrapie (sheep and goats) or bovine spongiform encephalopathy (BSE); Felidae (domestic cats and captive wild cats), which can

contract feline spongiform encephalopathy (FSE); Mustelidae (mink), which contract transmissible mink encephalopathy (TME); and Cervidae (deer and elk), which contract chronic wasting disease (CWD) (see Animals of Origin pg. 39).

2.2 Transmission of TSE

Studies of the transmission of TSE between species focus on three factors:

- The dose of infectivity, which is a product of the mass/volume consumed/inoculated and the infectivity titre/unit mass;
- The route of administration (intracerebral inoculation is most efficient, followed by parenteral routes, then by the oral route);
- The species barrier, which in turn depends on the strain of agent and the donor species effect.

2.2.1 Dose and Amount in TSE Transmission

To study the transmission of TSEs, two measurements are needed: the concentration of infectivity (infectivity *titre*) found in the tissue, and the *amount* of infected tissue administered. Together, these two measurements make up the *dose*.

Note that the terms *dose* and *amount* are often confused. It is untrue to say that 1 g of brain tissue will infect an animal by the oral route. This *amount* of brain tissue will infect only if an infectious *dose* is present. Therefore, in order to measure the dose, we need to know the concentration of the infectivity (the *titre*) per unit mass of brain tissue as well as the amount.

Titre of infectivity is measured by making successive 10-fold dilutions of the neat material and inoculating groups of experimental animals (*e.g.* mice) i/c with each dilution (up to 20 mice at each dilution) and then observing them to identify the death rate in each group. For example, if all mice inoculated with 10^{-1} to 10^{-3} dilutions of TSE-infected material died, but half of the mice inoculated with the 10^{-4} dilution died, and none of the mice inoculated with the 10^{-5} dilution died, then the mouse i/c ID_{50} titre would be 10^{-4}. For another example, in a situation where the oral dose to infect 50% of the animals to which it was administered (the ID_{50}) was 1000 i/c ID_{50}/g, and if 1 g of brain with this titre were administered, then half of the recipients would be expected to acquire the disease and die. Statistical formulas are used to measure the titre when odd numbers of animals die in the limiting dilution groups.

Some agents, like hamster scrapie strain 263K and murine BSE strain 301V, can be grown to high titres (about $10^{8\text{-}10}$ i/c ID_{50}/g of brain). Field isolates from sheep and cattle, however, are usually titrated in rodents, which involves the crossing of a species barrier (see Species Barriers and the Donor Species Effect pg. 21). Consequently, the titres are much lower (about $10^{5\text{-}6}$ mouse ic/ip ID_{50}/g).

In one study, pooled brain material from cattle with confirmed BSE was titrated in mice and gave a titre of $10^{3.3}$ mouse ic/ip ID_{50}/g. When the same brain was titrated in cattle, it was shown that the titre in mice underestimated the titre in cows by a factor of 500, reflecting the existence of a bovine-murine species barrier effect. Furthermore, in several mouse models of TSE, infectivity in the brain becomes detectable halfway through the incubation period, with the infectivity titre increasing on into the clinical

phase of disease. But in experimental BSE in cattle, infectivity becomes detectable much later—only about 3 to 6 months before clinical onset.

2.2.2 Routes of Infection

Experimental evidence indicates that different routes of infection have different efficiencies of transmission. The intra-cerebral (i/c) route is the most efficient, followed by the intravenous (i/v), intraperitoneal (i/p), intra-muscular or subcutaneous (i/m and s/c), and finally, the oral routes. Skin scarification and intra-ocular inoculations are also proven routes of infection. The olfactory/respiratory route of exposure has not occurred in natural disease, even though olfactory epithelia have shown the presence of misfolded protein, and nasal mucosa infectivity has been found in sheep.

2.2.2.1 The i/c and i/v routes

The i/c route is about 10 times more efficient than the i/v route. But recent experiments have shown that the i/v route is often more useful. In one experiment, a transfusion of 400 ml of blood from sheep with natural scrapie or experimental BSE successfully transmitted disease to healthy sheep. In another experiment, i/c inoculation of 0.02 ml infected sheep blood or blood products into mice did not transmit disease. There are two points to consider when interpreting these results. First, the sheep-to-sheep experiment had no species barrier, and in this particular study, genetically susceptible sheep were chosen; thus, disease transmission would have faced few challenges. Secondly, the i/v route enabled a much larger amount of infectious material to be inoculated into the mice, while sacrificing only a ten times greater efficiency for the i/c route. Thus the i/v transmission route is very efficient as relatively large amounts, and thus high doses, can be administered this way.

2.2.2.2 The oral route

The main TSE epidemics recognised since the 1950s have all resulted from oral exposure to tissues from the same species, via infected food or feed. For example, in New Guinea in the pre-1960s, a human TSE called kuru resulted from consumption of kuru-infected tissues through endocannibalistic funeral rites; TME resulted from mink feed contaminated with an animal-derived TSE of uncertain origin; and BSE resulted from consumption of meat and bone meal (MBM) derived from BSE-infected cattle by-products. Measures to control the diseases have therefore concentrated on the food chain: prohibiting human access to human tissues for consumption (kuru); preventing cattle access to mammalian protein in feed (BSE); and removing specified risk material (SRM) from the human diet (vCJD). These measures have so far been very successful.

2.2.2.3 The iatrogenic route

Iatrogenic means ‘caused by medical treatment’. Some iatrogenic forms of CJD and scrapie have been known to occur. Iatrogenic CJD has resulted from CJD-infected neurosurgical instruments and electrodes, human *dura mater* used in neurosurgery, human pituitary-derived hormones, and corneal transplants. One case of vCJD was caused by a blood transfusion from a vCJD-infected donor who was healthy at the time of donation. Another CJD case was found to be infected while dying of other causes. An unknown number of iatrogenic cases may yet be discovered. In sheep, two outbreaks of vaccine-related transmission of scrapie have been reported. In the 1930s,

the UK sheep vaccine against louping ill accidentally transmitted scrapie; and in the 1990s in Italy, a goat and sheep vaccine against *Mycoplasma agalactia* resulted in scrapie transmission. Note that neither of these vaccination incidents resulted from the use of commercially produced vaccines, which are strictly regulated and monitored, unlike independent veterinary investigation centres or research labs. Note also that iatrogenic transmission of BSE by any means has never been reported.

2.2.3 Species Barriers and the Donor Species Effect

Inter-species transfer of TSE diseases is only sometimes possible. The exact nature of species barriers is not yet understood. Since there is currently no way of determining in advance whether an agent is able to transmit to and cause disease in another host, there is an understandable concern that any new TSE disease might, under the right conditions, be able to transmit to humans and remain undiscovered for years or decades because of the long incubation period. This is even more likely if sub-clinical infection occurs (*i.e.*, infection that never leads to disease however long the animal lives).

Inter-species transmission of TSEs is generally more difficult than intra-species transmission. One aspect of the species barrier is small differences in the *PrP* gene sequence in the host and recipient. Under experimental conditions, successful cross-species transfer of TSE can occur, but usually with a lengthened incubation period at first passage. This extended incubation is presumed to be caused by the genetic differences between the incoming *PrP* and the host *PrP*—referred to as the *donor species effect*. But at the second transmission of the disease (within the new host species), the incubation period shortens, presumably because the incoming and recipient *PrP* are now the same. In subsequent transmissions, the incubation period stabilises in a predictable manner. The difference in the incubation periods between the first and second transmissions in these circumstances is a measure of the species barrier for that agent.

2.2.3.1 Gut barrier effect

There are several instances in which experimental transmission is successful by parenteral routes but fails following high-dose oral challenge. Examples are scrapie to mink, US scrapie strains to cattle, and BSE to pigs and to corticotropin-releasing hormone (CRH) mice. This suggests the possibility of a 'gut' barrier to transmission of some strains of agent in some species, but its nature and extent is unknown.

2.2.3.2 Transgenic animals in transmission experiments

To overcome the species barrier effect in experimental studies, transgenic animals have been produced. In these animals, the natural *PrP* gene of the host animal (*e.g.*, a mouse) is replaced by a replica of the gene from the host under study (*e.g.*, a cow). Such animals are called *transgenic animals*. Generally, when challenged with TSE infectivity derived from a cow, these transgenic mice appear to respond like cows but with a shorter incubation period. The species barrier effect is eliminated because the incoming and resident *PrP* are chemically identical.

Several modifications of the method have been developed, such as increasing the copy number of the gene in order to further increase the sensitivity of the transgenic animal. However, because several factors other than the primary *PrP* gene sequence influence transmission, these new transgenic animals are used only in experimental research.

2.2.3.3 Special case: scrapie in sheep

In general, it is more difficult to transmit TSE between species than within species. However, there are many *PrP* genotypes in sheep, some conferring susceptibility and others resistance to natural and experimental TSE transmission. As a result, transmitting scrapie from a susceptible sheep to a resistant sheep may be more difficult than transmitting it to another species, such as a goat or mouse. In fact, field scrapie isolates from sheep do not all transmit successfully to mice: in a study of Icelandic sheep scrapie, 20% failed to transmit at all.

If a disease appears to be confined to a particular species, then there is likely to be concern only for that species. However, since the arrival of BSE, with a wide natural host range including humans, there is considerable interest in the nature and extent of the inter- and intra-species barriers. To date, there is no confirmed report of a genetic resistance occurring in cattle.

Curiously, there is no recorded instance of scrapie from sheep causing any TSE in humans, despite 250 years of history of the disease in Europe and the close contact between humans and these small ruminants and their products (including the placenta that is known to be infected). Thus, species vulnerability to TSE can be very unpredictable.

2.2.4 Transmission of Human TSEs

Human TSEs are rare, and only four forms have been identified: kuru, Gerstmann-Straussler-Scheinker Disease (GSS), Fatal Familial Insomnia (FFI), and Creutzfeldt-Jakob Disease (CJD).

Kuru is an almost-extinct TSE that had been caused by long-standing traditions of consuming human brains at funerals among certain tribes in New Guinea. Since these practices ceased in the late 1950s, cases of kuru in the world have virtually disappeared.

GSS and FFI occur exclusively as inherited familial forms of TSE and have a number of distinctive clinical and neuropathological features that set them apart from each other and from CJD; but they share the same basic pathogenesis as the familial forms of CJD.

CJD is an invariably fatal neurodegenerative disease that is usually categorised into four subsets, according to their routes of transmission and characteristics: familial CJD, caused by mutant *PRNP* genes; iatrogenic CJD, caused by medical procedures; sporadic CJD, of unknown cause; and variant CJD (vCJD), a form of CJD with unique characteristics and considered to be due to infection with the BSE agent or more rarely by a human adapted form of the BSE agent. Of the four, sporadic CJD is by far the most common and seems to be caused by a spontaneous ‘misfolding’ of protein in the brain. Variant CJD, first reported in the UK in 1996, is the form believed to be caused by consuming cattle products infected with the BSE agent.

In the UK, the incidence of vCJD has been declining since its peak of 29 cases in 1999, and only five new cases have occurred in each of the past 2 years. As of November 2005, the total number of cases of vCJD in the UK is 157 of which six patients are alive. Thirteen cases have been identified in France, two in Ireland and one each from Canada, Italy, Japan, Hong Kong, and the USA. Only the French and Italian cases had never resided in the UK. There are now also single cases in Saudi Arabia, the

Netherlands, Spain, and Portugal. All are believed to have been infected by consumption of cattle products infected with the BSE agent before effective controls were in place.

The estimated future number of new UK infections from cattle via food ranges from fewer than 300 to several thousands, a range that reflects the uncertainty of assumptions about silent carriers, genotype influence, and tonsil/appendix screening studies for the presence of misfolded protein. The special importance of carriers, those still-healthy but infected individuals, lies in the fact that they could be a reservoir of infection for other humans by indirect human-to-human transmission through blood transfusion, organ transplantation or inadequately decontaminated surgical instruments. Because of success in reducing new exposures by consumption of infected cattle products, efforts are now directed at eliminating human-to-human transmission. Measures have already been taken to reduce these risks to a minimum.

Researchers at one point considered a genetic susceptibility in the *PRNP* protein in humans who acquired vCJD. Of 134 tested human vCJD cases, all had a similar *PRNP* gene sequence: they were homozygous for methionine (MM) at codon 129 of the PRNP gene, whereas this genotype occurs in only 40% of the human population (10% are homozygous for valine, and the remaining 50% are heterozygotes). However, genetic susceptibility has been shown to be less important to transmission than previously thought, because one of the two recently reported transfusion-related vCJD cases was an M/V heterozygote. Thus, if the heterozygous MV gene offers any protection against TSEs, it cannot be considered to be complete.

2.3 Diagnosis of BSE

2.3.1 Clinical Signs and Clinical Diagnosis

The onset of BSE is insidious and progressive, involving behavioural changes and lack of coordination, as well as loss of condition and milk yield. The clinical signs remained the same throughout the UK epidemic and are essentially the same as those in other countries where BSE has occurred. The dominant signs are neurological: apprehensive behaviour, hyperaesthesia and gait ataxia. The duration of signs can range from 7 days to 14 months, but is typically 1 to 2 months. This duration range helps veterinarians distinguish BSE signs from those of other acute, inflammatory diseases, such as rabies. But since BSE is a notifiable disease in all countries where it occurs, the full duration of the clinical phase is now foreshortened by compulsory slaughter once a clinical diagnosis has been made or the disease is suspected.

In countries with a high incidence of BSE, veterinarians have developed excellent skills in recognising the clinical signs. However, not all suspected cases proved to have had BSE; for example, in the UK during most of the epidemic, only about 85% of reported cases were subsequently confirmed. About half of the unconfirmed group had alternative diagnoses; the other half had no brain lesions, their BSE-like signs being due to metabolic causes. Occasionally, adult cattle brains examined for non-BSE reasons have revealed spongiform encephalopathy, reflecting BSE infections associated with atypical clinical signs or no signs at all.

In countries with a low incidence of BSE, veterinary experience of the disease is usually minimal, and therefore sporadic cases may easily be overlooked, despite training and observation of video recordings of clinical signs. With the advent of new

'Rapid' testing techniques for BSE, the misfolded prion protein has been detected in the brain in cases where clinical signs hadn't been noticed or recognised. Therefore, in countries with no reported BSE, low incidence of BSE, or a lack of 'Rapid' testing facilities, BSE cases might be missed or mistaken for other diseases.

2.3.2 Tests to Confirm TSE

There is currently no satisfactory test to detect pre-clinical BSE in cattle. However, two approaches shown to be useful for detecting pre-clinical scrapie in sheep are being studied as potential methods for detecting pre-clinical BSE as well: the immunohistochemical detection of misfolded protein in the nictitating membrane, and its Western blot detection in plasma using ligands that specifically attach to the misfolded form of protein.

The BSE chapter in the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* describes the kinds of tests approved for the post-mortem confirmation of BSE. These include microscopic examination of the brain, IHC, Western blotting, detection of SAF in brain, and approved 'Rapid' screening tests. Positive 'Rapid' tests must be further confirmed by one of the other tests.

The EC has approved five 'Rapid' screening tests for BSE using CNS material (see Table 2.1). These tests have also been used to screen sheep for scrapie. One of the tests (the TeSeE test) has been able to reveal the presence of misfolded protein in sheep with no clinical signs of scrapie and no positive diagnoses by other tests. Currently, brain tissue from these same animals is being tested to determine whether it can still transmit infection to experimental animals despite its low level.

Table 2.1 'Rapid' Tests Approved for Use in Cattle

[and provisionally approved for use in sheep and goats in the EU using brain material in the context of TSE Regulation 999/2001 of the EC.]

Bio-Rad TeSeE (Formerly Bi-Rad Platelia)	1999	France
Enfer BSE test	1999	Ireland
InPro CDI-5	2003	USA
Prionics Check Western	1999	Switzerland
Prionics Check LIA	2003	Switzerland

2.4 Inactivation of TSE Agents

TSE agents are notoriously difficult to inactivate compared to viruses and bacteria. They tend to aggregate, and because the agents are sticky, they adhere to surfaces. Inactivating macerates (smeared tissue) is more difficult than inactivating homogenates (tissue broken into small particles in fluid). Furthermore, fat tends to protect microorganisms from destruction.

2.4.1 Physical and Chemical Methods of Inactivation

The resistance of TSE agents to inactivation is significant (see Table 2.2). High temperature is frequently used. A large body of literature has documented that wet heat (*i.e.*, autoclaving) is much more efficient than dry heat, provided that the required temperature (≥ 132 °C) is sustained for a period sufficient to penetrate into the center of the load of contaminated material being processed. Even so, a small resistant sub-population occasionally remains after the heat inactivation process, and it resists further inactivation attempts by the same method. Other commonly used inactivation methods, such as boiling and electromagnetic radiation, are of no use against TSE agents. Moreover, low temperatures (freezing or liquid nitrogen) preserve rather than inactivate TSE agents. However, the combination of heat and ultra-high pressure is under investigation and may prove useful for inactivating infectivity in SRM.

Most chemicals used for conventional pathogens have little or no effect. In fact, only two are known reliably to disinfect contaminated materials: concentrated solutions of either lye (NaOH) or bleach (NaOCl). Hot NaOH is particularly effective but constitutes a serious handling hazard and is not recommended for use outside the laboratory.

Table 2.2 Methods of Physical and Chemical TSE Inactivation

Ineffective	Partially effective	Effective
Chemical methods		
Alcohol	Chlorine dioxide	Hypochlorite (1 to 5%)
Ammonia	Gluteraldehyde	NaOH (≥1 N)
ß-propiolactone	Iodophores	Formic acid (100%)
Detergents	Guanidinium thiocyanate	
Ethylene oxide	Sodium dichloroisocyanurate	
Formaldehyde	Sodium metaperiodate	
Hydrochloric acid*	Urea (6 to 8 M)	
Hydrogen peroxide	NaOH (0.1N)	
Peracetic acid		
Permanganate		
Phenolics		
Physical methods		
Boiling (100 °C)	Wet heat (121 °C)	Wet heat (≥132 °C)
Microwave radiation	Dry heat (300 °C)	Dry heat (>600 °C)
UV radiation		
Ionizing radiation		

*Note: HCl is partially effective at 60 ºC; in general higher temperatures may enhance the effectiveness of chemical treatments.

2.4.2 Disposal of Animal Carcasses and Waste

Disposal of animal carcasses and animal waste is an ongoing problem, especially since the onset of BSE. Because SRM must now be removed from the carcass before

butchering, the removed material creates a concentration of potentially infectious material. Consequently, disposal of such material is complicated and controversial.

Carcasses or carcass parts not required for a particular purpose (*e.g.*, consumption or making leather) have typically been disposed of by burial, incineration or rendering. Of these methods, rendering is potentially the most versatile and useful because it is very adaptable. It quickly prevents foul decomposition of perishable starting material, significantly reduces the volume of the starting materials by converting them to stable by-products, and generates materials of value for subsequent use.

More recently, disposal using biological methods (*e.g.*, composting and treating with enzymes), chemical methods (*e.g.*, hydrolysis with hot alkali under pressure), and a combination of these methods have been used. But concerns remain about the environmental impact of some of these methods and the efficiency of inactivation of potential TSE infectivity.

In the EU, there are few disposal options left for animal waste. Unprocessed animal material can no longer be buried even in a licensed landfill. Incineration, a highly effective means of TSE agent destruction, is not a practical option in many areas because of public concerns. Co-incineration, such as using MBM as a fuel in the manufacture of cement, is practical and employed in Europe. An alternative method of reducing the TSE risk in materials is to remove the TSE agents by filtration or electrophoresis. However, these methods are both expensive and time consuming, and they still leave an infected residue for disposal.

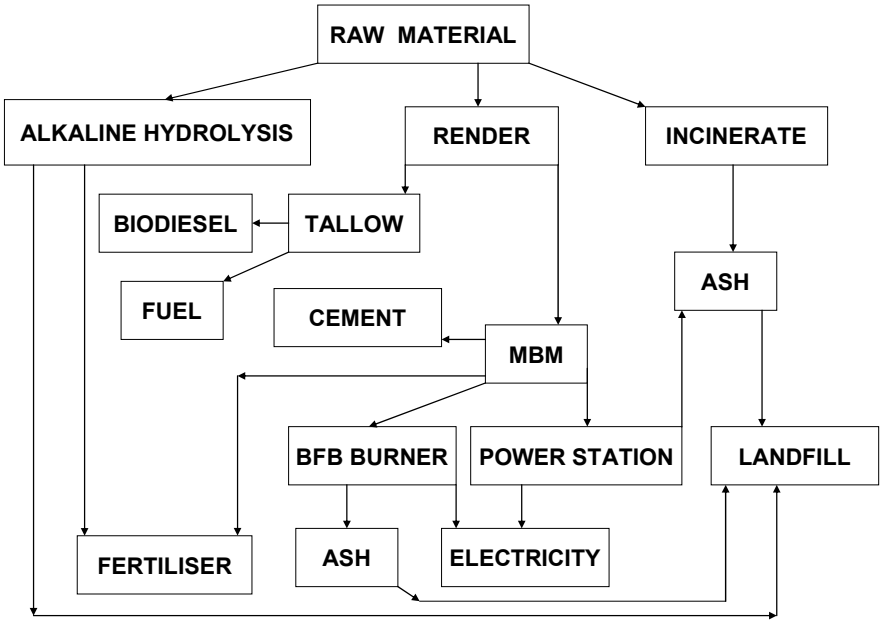
Table 2.3 lists animal by-product disposal systems and their current stage of development. Figure 2.3 shows a network of disposal methods that are currently in use. The Specific EC Regulations should be consulted for details on approved methods of disposal. For the scientific evaluation of the approved biodiesel manufacturing and other methods for processing rendered fats, the opinion of the EFSA Biohazard Panel should be consulted.

Table 2.3 Methods of Animal By-product Disposal and Stage of Development	
In use	Incineration (purpose built)* Co-incineration as fuel for cement manufacture* Rendering + incineration (MBM) + burning tallow as fuel* Rendering by pressure-cooking + burial in licensed landfill* Burial in licensed landfill (processed material, *e.g.*, ash and catering waste from international transport only)*
Recently approved for use (EC 2005)	Alkaline hydrolysis (autoclave in NaOH or KOH)** High pressure hydrolysis biogas** Biodiesel** High pressure, high temperature hydrolysis*** Brookes gasification***
Under research	Proteolytic enzymes (Genentech)
No progress being made	SDS, NaOH, HCl, HCHO, NaOCl, chaotropes
Unlikely to be useful	Radiation, alkylating agents, organic solvents, oxidising agents, salts, other detergents
No data	Composting

*In use for EC Category 1 (high and TSE risk), Category 2 and 3 animal by-products.
** Conditionally approved for use for Category 1, 2 and 3 animal by-products.
***Approved for use for Category 2 and 3 animal by-products.
Animal carcasses (including fallen stock) can no longer be buried without processing in the European Union.

For biodiesel production, the method of interest is rendering, because it is the only one that produces fat with high calorific value suitable for generating the methylesters needed to create biodiesel (see Rendering pg. 77). Filtration as a second step is an acceptable method for cleaning the fat and reducing the risk of TSE agents present in insoluble impurities.

Figure 2.3 Network for Possible Decontamination/Disposal of Animal Waste Material



Note: Not all pathways (*e.g.* fertiliser) are currently authorised for disposal in the EU.

2.4.3 TSE in Animal Fats

Tallow is animal fat derived from animal by-products through rendering (see Rendering pg. 77). Studies have shown that pure tallow does not contain detectable TSE infectivity because any infectivity in the starting material partitions with the protein residue from rendering (greaves). However, it is possible that the impurities in tallow could contain proteinaceous material, and some of this material might contain residual misfolded protein if inactivation is not complete. Research is required to investigate this issue.

Research from the UK does not support the notion that tallow is a vehicle of BSE transmission via feed. Most scientific and government authorities believe that infectious MBM in cattle feed, rather than tallow, causes BSE. In addition, geographical variation in BSE outbreaks is not consistent with transmission through tallow in cattle feed.

To date, no study has shown that fat of any kind forms part of the infectious agent, or that fat is closely associated with the agent or infectivity.

2.5 Summary Points

Although TSE is not a recent phenomenon (scrapie was recognised in the UK as early as the eighteenth century), TSE occurrence in humans is uncommon. Sporadic Creutzfeldt-Jakob Disease, the principal form of human disease, occurs at an annual

frequency of only one case per million population. The recent outbreak of variant CJD, believed to be caused by the consumption of cattle products infected with BSE, is declining in response to control measures put in place over the past 15 years.

The cause of TSE is disputed, but current science favours the "prion" hypothesis. TSE infectivity is associated with a misfolded form of a normal body protein, or ‘prion’. Infection can be initiated via many routes: the intravenous and intra-cerebral routes are the most efficient; other peripheral routes (such as intra-peritoneal or subcutaneous) are less efficient; and the oral route is least efficient. Respiratory or venereal transmissions are not known to occur for any TSE.

In countries where BSE levels are low, the ability of veterinarians to recognise the clinical signs of disease is often lacking. At present, diagnostic proof of TSE requires either microscopic examination of brain tissue or immunohistochemical demonstration of the misfolded protein in brain or peripheral lymphoid tissue.

Disposal options for TSE-infected animal tissue are limited. The TSE agent can be inactivated by some physical and chemical treatments, including exposure to temperatures $\geq$ 132 °C, or to concentrated aqueous solutions of bleach or lye (caustic soda).

Animal fat is considered to be free of infectivity unless contaminated by protein-containing greaves.

2.6 Reading List

PURPOSE

The purpose of this reading list is to provide sources of information for reviewers desiring further detail on selected topics. It is not intended to be exhaustive or to include the most recent publications in this fast moving field. In fact several old references are included as they give a useful insight to the earlier days of TSE that helps to put current knowledge into context. The titles have been selected as a valuable and readable selection, written mostly by experts or specialists with an international reputation in the field. Important national or international agency websites, where some of the most recent information available can be found, complete the list.

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WHO – http://www.who.int/en

3 SOURCE: TSE IN STARTING MATERIALS

In an assessment of the risk of using SRM in biodiesel production, all steps in the biodiesel production process need to be considered. This process starts with sourcing appropriate materials (in this case, materials of animal origin). This chapter addresses three factors affecting the risks: 1) the animal species that is used, because different animal species have different likelihoods of carrying TSE; 2) the tissues that are used, because different tissues may carry a different TSE risk; and 3) the country of origin, because the prevalence of TSEs varies among countries.

3.1 Animals of Origin

Although BSE in cattle is probably the best known TSE, the occurrence of TSE is not restricted to bovines. TSEs affect several different species of animal (Bradley, 1997). Scrapie occurs naturally in sheep, goats and moufflon; transmissible mink encephalopathy (TME) occurs in farmed mink; chronic wasting disease (CWD) occurs in Rocky Mountain elk, mule deer and white tailed deer. BSE has a wider natural host range than any of the above diseases because of recycling of infected carcasses as nutritional supplements. Thus, the BSE strain has been spread to ungulates, domestic and captive wild cats, and humans, in which it has caused a variant form of CJD (vCJD) (Bruce, 1996; Bruce *et al*, 1994, 1997; Collinge *et al*, 1996, 1997; Hill *et al*, 1997).

If we consider experimental infection, as distinct from 'naturally occurring' infection, the number of susceptible species is considerably larger (see Table 3.1). Although this suggests that a potential risk could be associated with almost any mammalian species used as starting materials for rendering, the risk to humans is presently considered to be confined to BSE. This view may need to be modified if agents causing other animal TSEs (*e.g.*, scrapie or chronic wasting disease) are shown to be human pathogens.

Table 3.1 Natural and Experimental Host Range for the BSE Agent					
PRIMATES	**RUMINANTIA**	**FELIDAE**	**MUSTELIDAE**	**RODENTIA**	**OTHER ARTIODACTYLA**
Human Lemur? Rhesus Monkey? Monkeys: Marmoset Macaque Squirrel* Capuchin* Lemur?	Cattle Goat Nyala Gemsbok Greater kudu Arabian oryx Eland Scimitar-horned oryx Ankole *Bison bison* Zebu Sheep Goat	Domestic cat Puma Cheetah Ocelot Tiger Lion Asian golden cat	Mink	Mouse	Pig
		Note: **Hamsters and chickens** challenged by parenteral routes did not succumb **Chickens and pigs** challenged by oral route did not succumb **BSE** naturally occurs in no farmed food animal other than domestic cattle *Bos taurus* and in one goat.			

*Unpublished observations courtesy of Dr. C.J. Gibbs, Jr.
Note: "?" indicates that further data is considered necessary to confirm reported results.

Most experimental transmissions have used the optimal intra-cerebral route of infection, often in combination with parenteral (non-oral) challenge. However, since most outbreaks of TSE have followed oral exposure to the infective agent, most epidemiological research focuses on this transmission route. Table 3.2 shows the results and incubation periods following challenge of various species with the BSE agent. Note that BSE has not been transmitted from bovine material to hamsters unless first passaged through mice.

Table 3.2 Results of Experimental Oral or Parenteral Challenge of Various Hosts with the BSE Agent (Minimum Incubation Period – Months)

Species challenged	Oral route	Parenteral route
Mouse	Positive (15)	Positive (10)
Cattle	Positive (35)	Positive (18)
Sheep	Positive (18)	Positive (14)
Goat	Positive (31)	Positive (17)
Pig	Negative	Positive (16)
Marmoset	Not done	Positive (46)
Mink	Positive (15)	Positive (12)
Hamster	Not done	Negative
Chicken	Negative	Negative
Macaque	Positive+ (63)	Positive (30)
Squirrel Monkey	Not done	Positive* (27)
Capuchin Monkey	Not done	Positive*

Source: from Bradley, 1996.
*Data courtesy of Dr. C.J. Gibbs Jr.
+Data from Herzog *et al*, 2005.

3.1.1 BSE in Cattle

The risk associated with BSE depends on several factors. These include the incubation period of BSE, the structure and use of the cattle population, and the average age of cattle at slaughter.

3.1.1.1 Origins of BSE

Bovine Spongiform Encephalopathy was first reported as a new disease in the UK in 1985, with the first clinical cases occurring in April. The first confirmation of the disease following examination of the brain occurred in November 1986. Subsequently, BSE was reported in Switzerland and all of the then Member States of the EU except Sweden. Small numbers of cases have since been reported in some other European countries (now Member States of the EU), Israel, Japan and North America.

BSE is a new disease, completely unknown before 1986. Where did it come from? Before 1986, the only other known animal TSEs were scrapie, which had existed in sheep for over 250 years, and transmissible mink encephalopathy (TME), which was first recognised on a US mink ranch in 1947. Perhaps BSE came from sheep with scrapie. But if it did, it did not come from direct contact between cattle and sheep with scrapie: some 20% of the first BSE-affected farms had not had sheep on them within living memory.

Epidemiological studies showed that recycling of TSE-contaminated tissues as meat and bone meal (MBM) was the most plausible explanation for the onset and spread of BSE. The dispute centres on whether this contamination originated from a species-

crossing infection from scrapie-affected sheep or from a spontaneous case of BSE in cattle.

Moreover, any satisfactory explanation of the origin of BSE must answer two questions: why did BSE begin in the mid-1980s, and why did it begin in Britain? Both the sheep and cattle origin hypotheses have the same answer to the question of timing—that changes in the carcass-rendering system in the 1970s permitted infectivity to survive the process and be recycled.

The scrapie source theory has some advantages: for example, it explains why the UK was the main site of the 1986 BSE crisis. The UK has a comparatively large proportion of sheep to cattle, and scrapie is widespread and endemic there, thus favouring the entry of infected sheep carcasses into the rendering process. Moreover, BSE was unknown until 1986 when the epidemic began. Another argument suggests that unrecognised 'spontaneous' cases of BSE could have been occurring in cattle for decades at the same one-per-million annual frequency as sporadic cases of CJD in humans, and that one such case was a 'founder' of the BSE epidemic. But this theory has a formidable obstacle: sporadic BSE cases cannot have been occurring only in the UK; yet no coincident epidemics occurred in other countries (for example, the US) in which rendering processes were changed at about the same time as in the UK. Moreover, the distribution of early BSE cases in the UK (and subsequently in other countries that unwittingly imported BSE from the UK) is more consistent with multiple initiation points than with a single point source of infection. This would favour scrapie as the source of infection, because scrapie was widespread enough to have entered rendering plants throughout the UK, whereas simultaneous cases of sporadic BSE would have been required to achieve the same effect.

The scrapie source theory also has shortcomings. First of all, on both biological and molecular biological grounds, the strain of TSE that has been consistently isolated from infected cattle differs from all known strains of scrapie (see Figure 3.1) and maintains this distinctiveness when introduced into other species, such as felines, sheep, mice, and humans (see Figure 3.2). This difference argues in favour of the new occurrence of a TSE in cattle, rather than a transmission and transformation of scrapie.

Moreover, all evidence to date indicates that scrapie is not a human pathogen; and if scrapie does not infect humans, why should scrapie passaged through cattle behave any differently? Precedents do exist for TSE in one species that becomes unable to transmit disease to a different species unless it has passed through an intermediate species. However, because this phenomenon is exceptional, it is not as satisfactory as the explanation implicit in the BSE hypothesis, *i.e.*, a new strain of TSE arising in cattle could as easily as not be pathogenic for humans.

A number of alternative hypotheses for the origin of BSE have been proposed and reviewed expertly by the EC SSC (SSC, 2001; Horn, 2001). These include an origin from mammalian species other than cattle (such as a mutant form of scrapie agent); a natural TSE in Bovidae or Felidae or other wild animal, the carcasses of which were rendered into MBM; the existence of a form of sporadic TSE like CJD of humans; and a spontaneous mutation of normal bovine *PrP* into an infectious and protease-resistant TSE prion, *e.g.*, from use of organo-phosporus pesticides. On the basis of current evidence and data the Committee concluded that none of these hypotheses can be substantiated (or be rejected).

The latest in a long line of suggested origins proposes three hypotheses (Colchester and Colchester, 2005). The first is that BSE was acquired from a human TSE; second, that the route of infection was oral, through animal feed containing imported mammalian raw materials contaminated with human remains; and third, that the origin was the Indian subcontinent, from where large amounts of mammalian material were imported during the relevant time period. Human remains are known to be incorporated into meal made locally, and may still be entering exported material. The authors recommend that further investigations are needed into the sources of animal by-products used in animal feed manufacture, and into the transmissibility of human TSE to cattle. This hypothesis has subsequently been refuted by Indian doctors from the National Institute of Mental Health and Neurosciences that deal with CJD cases in India (Shankar and Satishchandra, 2005). The possible origin of BSE from humans has been considered in the past and rejected on the grounds that no evidence existed to support it. Furthermore the BSE epidemic preceded the vCJD epidemic by 10 years. Surveillance for CJD, including vCJD, has been stimulated in all countries (including Asia and the India subcontinent) by the World Health Organization. Classical forms of CJD (and scrapie in sheep) have been reported in India but no cases of vCJD in humans or BSE in cattle have been found. For these and other epidemiological reasons, the hypotheses (other than the oral route of transmission of BSE) are not deemed credible.

At the current time, although the origin of vCJD is generally accepted, the origin of BSE is still something of a mystery and may never be determined with certainty.

Figure 3.1 Biological Strain Typing Field Isolates of Scrapie and BSE in Mice

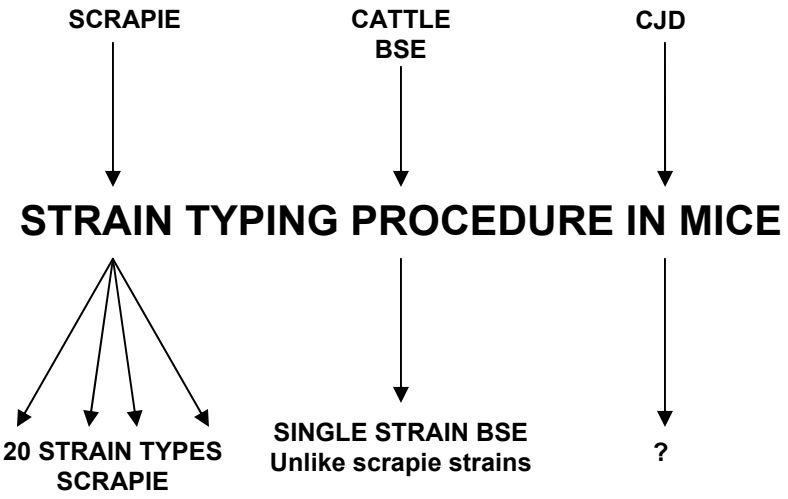
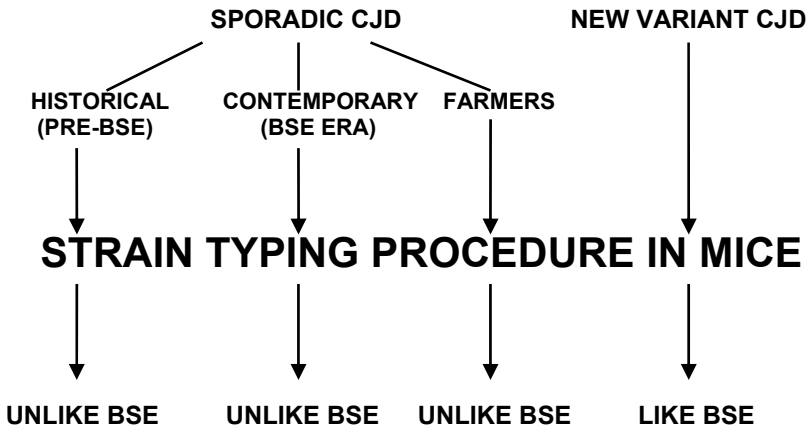


Figure 3.2 Biological Strain Typing of CJD Isolates in Mice



3.1.1.2 BSE incubation period

BSE exposure usually occurs during calfhood and dairy cattle are most at risk in the first 6 months of life (Wilesmith *et al*, 1988; Arnold and Wilesmith, 2004). The incubation period of BSE is long—an average of 60 months under field conditions. Thus, the most common age of occurrence is between 4 and 6 years. However, clinical BSE cases have been found in cattle 20 months of age up to 22 years 7 months. The latter could have been related either to adult exposure with a 'normal' incubation period or exposure as a calf with an exceptionally long incubation period. It may also theoretically have been the result of a cumulative dose effect.

Infectivity during the incubation period has only been detected at distinct intervals in a limited number of tissues: in distal ileum at 6 to 18 months post-challenge (PC); in tonsil at 10 months post-challenge; and in the medulla, spinal cord and dorsal root ganglia from 32 months post-challenge in experimental BSE (Wells *et al*, 1998; SSC, 2002a). Because bioassay studies are expensive and time-consuming, only a very small sample of each of nearly 50 different tissues has been inoculated into a maximum of three to four animals at each age interval. Thus, the possibility of detection failure due to inhomogeneous infectivity distribution or subthreshold test sensitivity infectivity must be factored into the interpretation of negative results.

Collectively, the unknown time of exposure, the long and variable incubation time, and the absence of an *ante mortem* test pose the problem that, for a given clinically healthy animal or an animal found dead, it is impossible to know whether it is TSE-infected and, if so, at what stage of incubation. *Post mortem* examination is required for the confirmation of BSE; but even *post mortem* tests only show the characteristic microscopic and immunostaining features for a period of 3 to 6 months before the onset of clinical signs (*i.e.*, at around 26 to 30 months of age in animals experimentally infected as calves).

3.1.1.3 Age at slaughter, and structure and use of cattle population

The structure of the cattle population in a specific country or region influences the risk of BSE. When the age distribution is skewed toward young animals, the chances of

finding BSE-positive animals are reduced; however, this does not mean there is reduced risk of BSE infection. Calves incubating BSE will not have detectable misfolded protein in the brain. On the other hand, in a herd in which the cattle are relatively old, the chances of finding BSE-positive animals are higher, simply because animals exposed as calves will have progressed towards the end of the incubation period, and misfolded protein will be detectable in the brain even if clinical signs are absent. Dairy and breeding cattle usually reach a higher age than cattle raised only for meat, thereby increasing the chances that exposed breeding cattle will reach the end of the BSE incubation period.

In the UK and most other countries, BSE is far more frequent in dairy herds than in beef suckler herds. This is related to the different systems of management and feeding, especially of calves. In short, offspring of beef suckler cows are suckled, whereas dairy calves are removed from the dam shortly after birth and are fed initially on calf milk replacer, and then on hay and concentrates that may contain MBM. Thus, dairy herds in countries at risk from BSE are more likely to have BSE than are suckler herds. Within these herds, BSE cases are most likely to occur in the breeding animals than in young animals that are slaughtered for meat without breeding, or that die or are culled young.

In addition, the intensity of the production system (feed practices) influences the risk of BSE. The intensity of production systems extends from very high to very low. In high-intensity systems, a high proportion of the concentrate diet consists of animal-derived proteins. In low intensity systems, cattle are generally fed on pasture. In such low-intensity systems, chances of BSE infections are correspondingly low, since cattle have no access to animal proteins that carry a BSE risk.

3.1.2 BSE in Other Animals

3.1.2.1 BSE in zoo ruminants and cats

TSE has been detected in captive greater kudu and nyala, and the agents isolated from these animals are identical with the BSE agent. Several other captive wild ruminants have also developed a spongiform encephalopathy assumed to be equivalent to BSE. It is believed that they all contracted BSE by eating feed containing MBM and contaminated with the BSE agent, in the same way that cattle were infected (Kirkwood and Cunningham, 1994; Kirkwood *et al*, 1993).

Before the BSE epidemic, although domestic cats were known to be experimentally susceptible to TSE, no natural case had ever been recognised. Since the epidemic, approximately 100 domestic cats and several captive wild cats have died of feline spongiform encephalopathy (FSE) (Kirkwood and Cunningham, 1994; Williams, 2001). Most of these animals lived in the UK. However, FSE in one domestic cat in Norway, one in Liechtenstein and two in Switzerland have also been reported. Moreover, there has been some FSE in captive wild cats. The UK exported some apparently healthy captive wild cats (cheetah) to Ireland, France and Australia, and Germany exported one (an Asian golden cat) via the Netherlands to Australia, all of which later developed FSE. These animals are believed to have become infected by eating contaminated feed: MBM in the case of domestic cats, and uncooked central nervous tissue from heads and vertebral columns in the case of large cats.

Despite these deaths, both zoo ruminants and cats are not considered to be a significant risk category for BSE. Worldwide, the total number of infected animals is extremely limited (see Figure 1.1). Moreover, all cases are believed to have been feed-related, most occurring in the UK or clearly linked to the UK or other countries with indigenous BSE. Since these animals are rarely processed as food for other species, the risk of transmission from them to other animals is also low. Furthermore, the small epidemics in these animals are declining, and no new feed exposures are expected.

3.1.2.2 BSE in sheep and goats

Several years ago, BSE was shown to be experimentally transmissible to sheep and goats after both intra-cerebral and oral dosing (Foster *et al*, 2001; Jeffrey *et al*, 2001). Active surveillance within the EU has recently proven that it can also occur under field conditions. Recently, two BSE cases in goats—one confirmed case in France and one suspected case in Scotland—have prompted the EC to increase active surveillance for TSE in goats throughout the EU, at least temporarily. Currently, the prevalence of BSE in goats is believed to be very low and not a cause of serious concern.

3.1.3 Chronic Wasting Disease in Cervidae

The natural hosts of chronic wasting disease (CWD) are mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*). So far, there is no evidence of transmission of CWD to non-cervidae species under natural circumstances. Cattle, sheep and goats that lived for long periods on the same premises as CWD-infected animals did not develop the disease (Salman, 2003). These findings are also supported by a molecular study that showed very low conversion rates of normal protein to misfolded protein in bovine, ovine and human samples exposed to misfolded CWD protein (Miller *et al*, 2000; Raymond *et al*, 2000).

CWD was first reported in the 1960s in free-ranging deer in the US (Williams and Young, 1980). Since then, it has been detected in widely separated parts of the US and Canada (Spraker *et al*, 2002) and very recently in farmed deer in New York State (New York State Department of Environmental Conservation, 2005). There are currently no reports of CWD in Europe; but in 2002 CWD was reported in an elk exported from Canada to the Republic of Korea (Sohn *et al*, 2002), and several more cases have been detected and slaughtered since. In captive populations, the prevalence of CWD can be very high: 90% in mule deer (Williams and Young, 1980), over 50% in white-tailed deer (Nebraska Game and Parks Commission, 2004), and up to 71% in elk (Miller *et al*, 2000; Peters *et al*, 2000; Williams *et al*, 2002). Prevalence in free-living animals has been estimated to be 1 to 17% in mule and white-tailed deer and less than 1% in elk (Miller *et al*, 2000). The incubation period of CWD is estimated at 15 to 36 months (Salman, 2003).

The origins of CWD are not clear. Contaminated feed cannot be the explanation, and maternal transmission is unlikely (Miller *et al*, 1998; Miller *et al*, 2000; Williams and Miller, 2002; Williams and Young, 1992). An important route of transmission may be the alimentary tract by excretion of faeces and saliva. Misfolded protein has been found in brain material, palatine tonsils, visceral and regional lymph nodes, Peyer's patches and other lymphoid tissue of the small and large intestines, and in the spleen of affected animals (Sigurdson *et al*, 2001; Sigurdson *et al*, 1999; Spraker *et al*, 2002). It has not been found in either muscle or antler velvet, both of which are sometimes used

for human consumption. Transmission of CWD to humans has not been shown to occur, but human susceptibility to the disease cannot be excluded. It can be transmitted by i/c inoculation to at least one primate species: the squirrel monkey (Williams and Young, 1992).

3.1.3.1 Surveillance of CWD in the USA and Canada

CWD surveillance in the USA started in 1996, but so far it applies only to farmed cervidae herds. Measures include: 1) mandatory death reporting; 2) testing of all animals that are slaughtered or die on the farm, excluding calves; and 3) an individual animal identification system and an annual census. Some loose controls are currently in place. Deer and elk carcasses are not usually accepted by rendering plants that process animal carcasses into products for ruminant feed (Salman, 2003); but there is no ban on human consumption of meat from CWD-affected animals and no mandated testing of hunted game. The recent discovery of infected white-tailed deer on two farms in New York state will undoubtedly lead to the initiation of a state-wide testing programme applied both to farmed and wild-ranging deer, with appropriate culling and slaughter of affected herds.

In Canada, active surveillance programmes did not begin until 2000; since 2001 CWD has been a reportable disease. Under the new guidelines, a farm that finds an infected animal must quarantine all animals and animal products on the farm, slaughter all Cervidae on the affected farm, and test all slaughtered adult animals. In the provinces of Saskatchewan, Manitoba, and Alberta, all routinely slaughtered adult animals are tested and held until test results are known. Offal does not have to be held until the test results are known: it can be incinerated or taken for deep burial. When a slaughtered animal tests positive for CWD, all Cervidae that have been in contact with it are culled and tested as well. Carcasses are incinerated or taken for deep burial. Antler velvet only from test-negative animals is released for further use. In addition, a voluntary CWD certification programme is in place (Salman, 2003). This is a programme for captive cervid herds. To qualify, a farm must test every cervid that dies on the premises and prove both the absence of clinical signs of CWD among the cervids and the lack of exposure of the herd to possible sources of CWD. As well, animals on these farms have to be individually identified before 11 months of age. After a minimum of 5 years, the herd can reach certification. The CWD eradication programme seems to have been successful, since there are currently no known farms with infected animals in Canada (CCWHC, 2004).

3.1.4 Scrapie in Goats and Sheep

Scrapie was the first recognised TSE, with reports of endemic scrapie in central Europe dating back to the eighteenth century (Brown and Bradley, 1998; Detwiler and Baylis, 2003). Through two centuries of sheep exports, scrapie has spread from Europe to many other parts of the world.

3.1.4.1 Scrapie transmission

Scrapie can be transmitted horizontally from sheep to sheep (Brotherston *et al*, 1968; Dickinson *et al*, 1974) and from sheep to goats (Billinis *et al*, 2002; Brotherston *et al*, 1968). It is widely accepted that infection is mediated by placental contamination (Onodera *et al*, 1993; Pattison *et al*, 1972, 1974; Race *et al*, 1998) and that infection occurs shortly after parturition from an infected mother to her progeny or to other

lambs in proximity (Detwiler and Baylis, 2003). However, experimental BSE in sheep appears not to transmit in this way (Foster *et al* 2004). Similarly, experimentally BSE-infected goats do not transmit BSE via the embryo (Foster *et al*, 1999). For scrapie, the most likely route of infection is oral, with Peyer's patches in the ileum a likely site of initiation (Andreoletti *et al*, 2000; Heggebo *et al*, 2000) (see Table 3.3). A contaminated environment is probably an important means of spreading infection, since both experimental and epidemiological evidence indicates that infectivity can persist in the environment of infected animals for several years (Palsson, 1979; Brown and Gajdusek 1991; Miller *et al*, 2004).

Some sheep genotypes are more susceptible to scrapie than others, and one sheep genotype that has rarely been associated with scrapie is considered to be resistant (DEFRA, 2003). Yet even in susceptible genotypes, exposure to scrapie does not always lead to infection (Foster *et al*, 2001; Hunter *et al*, 1997; O'Rourke *et al*, 1997). It is not clear whether genetic differences diminish goat susceptibility to scrapie; but certainly polymorphisms in the goat *PrP* gene seem to affect the length of incubation (Goldmann *et al*, 1998). Generally, the clinical onset of scrapie occurs in sheep between 2 and 5 years of age (Wineland *et al*, 1998), although a few naturally occurring cases have been seen in one-year-old sheep (Joubert *et al*, 1972; Zlotnik and Katiyar, 1961). Strain variation in the scrapie agent can cause additional variation in incubation times (Bruce, 2003; Bruce *et al*, 2002).

Whether the scrapie agent is present and infectious in faeces, saliva, colostrum or milk, nasal discharge and urine is still uncertain. Significant levels of scrapie infectivity have been detected but not titrated in the faeces of mice for a period of up to 36 hours after an oral challenge with the scrapie agent (Taylor D.M., personal communication). These studies did not address the possibility that infectivity generated in nerves or lymphoreticular tissue in the gastrointestinal tract might be released into the intestine (Taylor D.M. and Dickinson, A.G., unpublished observations).

As in other TSEs, vertical transmission seems not to occur. The scrapie agent has not been detected in semen, testes and seminal vesicles (Hadlow *et al*, 1982; Palmer, 1959). Moreover, the absence of infectivity in foetal tissues from infected mothers indicates that transmission *in utero* does not take place or, if so, only in very rare cases (Hadlow *et al*, 1982). Misfolded protein depositions and infectivity in sheep and goats have been detected in many different tissues, including brain and spinal cord, lymph nodes, intestinal tract, spleen and, very recently, in muscle tissue (Andreoletti *et al*, 2004). With ever-improving detection methods, the misfolded protein (and perhaps infectivity) will likely be found in other tissues.

Table 3.3 Tissue Infectivity in Natural Scrapie

Based upon Limited Tissue Infectivity Studies in Sheep and Goats with Experimental BSE, it is Presumed that a Similar Distribution of Infectivity Would Occur in Natural BSE in Sheep and Goats

NATURAL SCRAPIE IN SHEEP AND GOATS

CLASSIFICATION OF TISSUES BY INFECTIVITY STATUS IN PRE-CLINICAL AND CLINICAL CASES (Number Positive/Number Examined)

INFECTIVITY TITRE*	PRE-CLINICAL				CLINICAL	
	SHEEP				SHEEP	GOATS
	<8M (0/16)	10-14M (8/15)	25M (1/3)	>25M (1/6)	34-57M (9)	38-49M (3)
HIGH 4.1-6.5			Brain Spinal cord		Brain Spinal cord	
MEDIUM 3.2-4.0		Colon-proximal Ileum-distal LN (RP/MP) Spleen	Colon-proximal Ileum-distal LN (RP/MP) Tonsil		Colon-proximal Ileum-distal LN (BM) LN (PF 1/9-ve) LN (RP(MP) (Rectum-distal) + Spleen Tonsil	Colon-proximal Ileum-distal LN (BM) LN (PS/PF) LN (S/mammary) Pituitary (Rectal-distal)+ Spleen
LOW <3.1		LN (PS/PF) Tonsil	Brain (Medulla/ diencephalon) LN (BM) LN (PS/PF) Spleen		Adrenal Bone marrow** Colon-distal CSF Liver** LN (S/mammary x 2) Pancreas** Pituitary Sciatic N# Thymus**	Adrenal Colon-distal CSF Lung** Nasal mucosa Sciatic N Thymus
UNDETECTABLE	Ileum LN (PS/PF) NL (RP/MP) Thymus Tonsil Spleen	Blood clot Brain (medulla) Colon-distal Faeces LN (BM) Serum	Adrenal Brain (cortex mid-brain) Colon-distal LN (S/mammary) Nasal mucosa Salivary glands Spinal cord Thymus	Colostrum	Blood clot Salivary glands Fetus Seminal vesicle Heart Testis Kidney Thyroid Lung Uterus Mammary gland Muscle-skeletal Ovary Placenta 0 Saliva	Blood clot Bone marrow Faeces Kidney Mammary gland Milk Muscle-skeletal Ovary Salivary gland Serum Uterus

* = Log10 Mouse intracerebral LD50/30mg tissue
\+ = not assayed but high content of lymphoreticular tissue
0 = +ve in other studies on scrapie (Pattison et al 1972, 1974)
**= trace or exceptional
= wider range of nerves positive in scrapie (Groschup et al 1996)
M. Simmons, R. Sommerville, personal communication.

BM = Bronchomediastinal
MP = Mesenteric/portal
PF = Pre-femoral
PS = Pre-scapular
RP = Retropharyngeal
CSF = Cerebro-spinal fluid
LN = Lymph node

A clear connection between scrapie-contaminated MBM and transmission of scrapie has not been made. Nevertheless, it seems prudent to prohibit feeding ruminant MBM to any ruminant species, including sheep and goats.

3.1.4.2 Detection and prevalence

Worldwide efforts are being made to eradicate scrapie. Effective eradication programmes start with depopulation, which means culling either entire flocks or smaller groups of high-risk or affected animals. Cleaning and disinfecting the sheep habitat, farm equipment, and processing facilities are the next steps. Finally, restocking is banned for a certain period (usually 2 or 3 years) to prevent reinfection. In Iceland, the farm is permitted to restock only with scrapie-free animals from certified sources after the restock-ban period is over (Palsson, 1979; S. Georgsson, personal communication). Of note, however, is a recent Icelandic study showing the likely

survival of scrapie infectivity in the environment for at least 13 years (S. Georgsson, personal communication).

The prevalence of scrapie in the EU and the US is still unknown (Detwiler and Baylis, 2003); but new slaughterhouse surveillance programmes in these countries will generate new and more reliable data. New Zealand and Australia are considered to be free of scrapie because they have been able to isolate and eradicate imported scrapie cases before further spread had occurred. Iceland might soon be able to eradicate scrapie.

Currently, a great deal of attention is being paid to breeding programmes. Because certain sheep genotypes are more susceptible to scrapie than others, breeders are attempting to breed as much resistance as possible into their breeding flocks. In several European countries and the US, scrapie breeding programmes are in place (Detwiler and Baylis, 2003). One is the National Scrapie Plan in the UK (see DEFRA website at www.defra.gov.uk). A similar US two-part programme certifies flocks with no evidence of scrapie (based solely on the absence of scrapie, not on genetics) while selectively breeding resistant genotypes.

3.1.5 Transmissible Mink Encephalopathy

Transmissible mink encephalopathy (TME) was first recognised in 1947 in farmed US mink. Since then, TME has been reported in Canada, Finland, Germany, Russia, and the US, though outbreaks have always been sporadic (Marsh and Hadlow, 1992). The most recent outbreak was reported in 1985 in the US (Marsh *et al*, 1991).

There is speculation that TME may have been caused by feeding mink with scrapie-infected feed, mostly derived from meat and offal from dead animals (Marsh and Bessen, 1993). Experimental scrapie has never been successfully transmitted to mink by the oral route (Marsh *et al*, 1991). Because the first case of TME was described well before the first BSE case was reported, BSE does not seem to have been the cause of TME. At least one outbreak, in 1985 in Wisconsin, occurred in mink reported to have been fed fallen or sick dairy cattle carcasses and no sheep material (Marsh *et al*, 1991).

TME has never been reported in free-living mink. Moreover, considering that the incubation period of TME is 7 to 12 months, and that the last reported outbreak was 20 years ago, TME is not considered a current threat.

3.1.6 Knowledge Gaps about Animals of Origin

1. There are currently no tests for detecting BSE infection in live cattle. However, several laboratories are working to develop a pre-clinical blood-screening test.
2. The currently available *post mortem* tests are only able to detect BSE-positive animals that have reached nearly the end of the incubation period. BSE-positive animals in early stages cannot be detected, and it would obviously be desirable to develop a test that could be applied to accessible, non-CNS tissues at an early stage of the incubation period.
3. BSE in sheep and goats may have occurred and have gone unrecognised for many years. Up until the feed bans (in the UK in 1988 and 1996; and in the EU in 1994 and 2001), sheep and goats in Europe were almost certainly exposed to feed containing scrapie- and BSE-infected MBM, but not necessarily in doses sufficient to establish and enable amplification of infection. Experimental studies indicate

that BSE in sheep and goats present clinically and pathologically as scrapie. Furthermore, there is continuing difficulty in rapidly distinguishing between TSE caused by the scrapie agents and the BSE agent. So far, no case of naturally occurring scrapie in sheep caused by the BSE agent has been confirmed, but this does not mean that it cannot or does not occur. It is not known whether such putative 'back-crossing' of BSE into sheep would carry with it the same pathogenicity for humans as BSE in bovines.

4. In most countries, there are minimal or non-existent monitoring or surveillance programmes for CWD. As a result, the worldwide prevalence of CWD in either farmed deer or wild deer is unknown. Because CWD occurs in both wild and farmed deer, and because wild deer cannot be thoroughly monitored, the real CWD prevalence in wild deer can easily be under-estimated. The roles of environmental and animal-to-animal transmission are under investigation.

5. The host range and species susceptibility range to CWD, including human susceptibility, has not been fully examined, and thus the possibility of transmission of CWD from deer to humans cannot be ruled out. The possibility exists that wild carnivores could contract CWD from eating fallen wild deer or elk, or that humans could contract CWD from eating their tissues. Knowledge about tissue infectivity levels is incomplete (especially during the incubation period).

6. Knowledge about the susceptibility of *PrP* genotypes in sheep to oral challenge with different TSE agents is as yet incomplete but under investigation.

7. More recent information on new phenotypes of scrapie (for example, NOR98) and the occurrence of misfolded protein in the brain of genetically resistant sheep indicates a need to determine if current breeding programmes are adequate to prevent further spread of scrapie.

8. The occurrence of TSE in animals other than cattle, sheep, goats and *Cervidae* is considered to be very low; but detailed monitoring programmes have not been done to confirm this. In view of the virtual impossibility of testing enough animals to achieve statistical confidence in negative results, it is not expected that such programmes will ever be carried out.

9. The species composition of starting materials for rendering in North America is not accurately known. It is also not known if TSE risk materials predominate in any particular rendering plant or region. Therefore, it may be difficult to estimate the actual or potential TSE risks for North American source material. The National Rendering Association, USDA/FDA, CFIA and Departments of Epidemiology and Risk assessment in North America may be able to research this information if it is perceived as a high priority.

3.2 Tissues of Origin

All TSEs have a similar pathogenesis (disease development pattern). In experimental scrapie in mice, natural scrapie in sheep, experimental BSE in sheep, and vCJD in humans, there is a widespread involvement of the lymphoreticular system (LRS). Other organs or tissues may also be affected. In scrapie, experimental BSE in sheep, and vCJD, blood may become infected during the incubation period; however, blood has not been shown to be infectious in cattle with BSE. Also, the distribution of tissue infectivity is much more restricted in cattle with natural and experimental BSE than in

sheep with scrapie (see Table 3.4, Table 3.5 and Table 3.6). The distal ileum and tonsil become detectably infected about 6 months after experimental oral infection. Later, in the clinical phase, infection is present in the spinal cord, brain, retina and associated ganglia, nictitating membrane (third eyelid) and possibly the bone marrow of the sternum.

Infection of the central nervous system (CNS) in rodents with experimental TSEs, and in sheep with natural scrapie, is first detectable about halfway though the incubation period. In cattle with BSE, however, CNS infectivity does not appear until about 3 to 6 months before the onset of clinical signs. These special features of BSE have importance for testing and surveillance for the disease.

Table 3.4 Tissues of Cattle with Natural or Experimental BSE

(in which Infectivity has been Found by Bioassay in Susceptible Mice or Cattle)

BRAIN
Trigeminal ganglia (not tested in natural BSE)
SPINAL CORD
Dorsal root ganglia (not tested in natural BSE)
RETINA, THIRD EYELID (not tested in experimental BSE)
Distal ileum Palatine tonsil (after bioassay in cattle only)
Clinical phase only: Bone marrow Peripheral nerve* Muscle*

Lower case = experimental BSE only. Bold Upper Case = natural BSE or both (*Fraser and Foster, 1994; EC, 2002; Buschmann and Groschup, 2005; WHO, 2005)

Tissues collected from cattle experimentally infected with BSE during the incubation period (pathogenesis experiment) have been bioassayed in mice (see Table 3.5) and in cattle (some experiments still in progress). Changes to the distal ileum show up first (6 to 18 months after infection), whereas the main signs do not appear until 32 months post-infection. Cattle bioassay, but not mouse bioassay, has detected infectivity in palatine tonsils.

Table 3.5 Clinical Signs, Brain Pathology and Infectivity of Tissues

Including Parts of the Brain [by interval from challenge (4 m old) during the pathogenesis of experimental BSE in cattle following oral exposure to 100g infected brain ($10^{3.3}$ mouse i/c ID_{50}/g)]

	Interval (Month Post-Challenge)										
	2	**6**	**10**	**14**	**18**	**22**	**26**	**32**	**36**	**38**	**40**
Clinical signs											
Brain Pathology and misfolded protein*											
INFECTIVITY:											
Distal Ileum											
Caudal Medulla											
Spinal Cord											
Dorsal Root Ganglia											
Trigeminal Ganglia											
Frontal Cortex											
Bone Marrow											

*Diagnostic spongiform changes were present from 36 months post-challenge.
All other tissues showed no detectable infectivity at all stages.
Unequivocal clinical signs were first detected at 35 to 37 months post-challenge.
Data from Wells *et al* 1994, 1996, 1998, 2005; Terry et al, 2003
NOTE: Selected tissues from selected time intervals post-challenge have subsequently been bioassayed by the i/c route in cattle and results are in broad agreement, though the palatine tonsil collected 10 months post-challenge is also positive. Some studies are incomplete (Wells *et al*, 2005). All other tissues showed no detectable infectivity at all stages.

3.2.1 Infectivity of TSE

Infectivity refers to the relative ease and degree to which disease in an animal or tissue can be passed to another animal. It is not synonymous with contamination. For example, if a TSE-infected material is accidentally or deliberately inoculated into an animal, the tissue into which it is injected will become contaminated. From this point, three main sequences of events can occur. First, the infectious material could become sequestered in place. Second, it could be dispersed elsewhere in the body and be sequestered or disposed of there. Finally, it could replicate. Replication takes on a special meaning in TSE pathogenesis, because it appears that nucleic acid is not involved; instead of the conventional method of replication, the infectious agent multiplies by a mechanism in which the misfolded protein molecules act as templates that somehow recruit normal protein molecules to become similarly misfolded. It is during replication that the disease takes root in the new host.

TSE agents are able to traverse the alimentary tract; thus, if ingested, they could contaminate the environment and become a source of infection for other animals by the oral route. However, this is an unlikely event in BSE; but it cannot be excluded for scrapie or CWD transmission. Under normal circumstances, the lumen of the gut is essentially 'outside' the body, and in a healthy individual it is not permeable to large molecules like proteins. Digestive enzymes, such as protease, normally break down proteins into polypeptides and amino acids, and then the gut absorbs them as simpler molecules.

However, the misfolded prion protein is partially resistant to such protease digestion. The infectious agent appears to enter the body following capture by intestinal dendritic cells, or possibly M cells found in the intestinal epithelium. From there, the cells transport infectivity to the lymphoreticular system (LRS) tissue, such as Peyer's patches within the wall of the intestine, where they can concentrate and possibly replicate in follicular dendritic cells. Spread to other lymphoid tissues, such as lymph nodes, tonsil and spleen, occurs in some species (*e.g.*, sheep), from whence the infection travels to the spinal cord via nerves of the autonomic nervous system or the vagus nerve. Finally, these cells move to target areas in the brain where formidable replication takes place, and pathology develops along with clinical signs, followed by death.

3.2.2 BSE Infectivity in Cattle Tissues

Because the misfolded protein is generally inseparable from infectivity and is widely believed to be the causative agent, its detection in any tissue indicates beyond reasonable doubt that the tissue has been infected with the BSE agent. A feature of all tests for misfolded protein is that none of them has the sensitivity afforded by bioassay. Consequently, a negative test result is not a guarantee that infectivity is not present in a tissue. Most bioassays are done in mice; but, due to the species barrier, these studies are less sensitive than bioassays with cattle. One large comparative bioassay using mice and cattle shows that mice underestimate the infectivity actually present in cattle by about 500 times (Wells, 2001; SSC, 2002a).

Naturally exposed cattle subsequently tested at the clinical stage of BSE have shown infectivity only in a few tissues. The brain has been shown to have approximately 6 $\log_{10}$ cattle i/c LD_{50} per gram of tissue; the cervical and terminal spinal cord, retina and nictitating membranes also show infectivity, but the levels have not been determined.

In experimentally challenged cattle, infectivity has been detected in a similar set of tissues: brain, spinal cord, dorsal root ganglia, trigeminal ganglia, distal ileum and bone marrow by bioassay in susceptible mice. In addition, bioassay in cattle of a restricted range of tissues collected at specific time points has produced similar results, and has also detected infectivity in the palatine tonsil collected at 10 months post-challenge that was not detected in the bioassay using mice (Wells *et al*, 2005).

Designating certain tissues as SRM requires an understanding of the pathogenesis of the disease. Different agencies adopt different tissue lists for SRM and different ages of animals from which they must be removed. Some require removal of tissues that cannot be consistently separated from the higher-risk tissues (see Table 3.6). Central nervous tissues such as the spinal cord, brain, associated ganglia and the eye are almost always included in an SRM list. Some agencies require SRM removal for cattle only, whereas others require SRM from small ruminants to be removed as well, mostly as a risk-reduction measure to protect public health in case BSE is found in sheep at a later date. This measure gives small ruminants additional and immediate protection from scrapie, because these possible SRM would otherwise be processed to produce MBM, which in some countries can be fed to non-ruminant animals and thereby potentially cross-contaminate ruminant diets.

Table 3.6 Summary of Designated SRM in Cattle In Europe (December 2003) and in Switzerland (July 2004)			
	Age above which SRM removal is required		
	European Union	**UK and Portugal**	**Switzerland**
Skull, including brain and eyes	>12 months		>6 months
Entire head, excluding tongue		>6 months	>30 months
Tonsils	All ages	All ages	>6 months
Spinal cord	>12 months	>6 months	>6 months
Vertebral column, including dorsal root ganglia, but excluding vertebrae of the tail, the transverse and spinous processes of the cervical, thoracic and lumbar vertebrae, the wings of sacrum and median sacral crest	>12 months	>30 months	>30 months (includes tail)
Intestines and mesentery	All ages	All ages	>6 months
Spleen		>6 months	
Thymus		>6 months	

3.2.3 Scrapie Infections in Sheep and Goats

Scrapie is the prototype TSE and is thus the best studied. Tissue infectivity is known to be similar in sheep and goats at the clinical and incubation stages of disease, and numerous strains of sheep scrapie have been adapted to mice. The rather broad range of tissues involved in scrapie infection stands in sharp contrast to the restricted number of infected tissues seen in BSE.

In mouse bioassays, scrapie infectivity has been shown to exist in small ruminants during the preclinical stage of infection in brain material, spleen and tonsils; in retropharyngeal, mesenteric and portal, bronchomediastinal, prescapular and prefemoral lymph nodes; in the proximal colon; and in the distal ileum. Infectivity levels in these tissues range from medium to low and have been determined in animals, during the preclinical stage of infection, of 10 to 14 months and 25 months (SSC, 2002a). However, most of the historical studies were done before the discovery of the prion protein, and so no account was taken of the *PrP* genotype of the host, which is now known to influence the timing of infections in different tissues.

Recently, misfolded protein was detected in skeletal myocytes in naturally and experimentally infected sheep (Andreoletti *et al*, 2004) and in orally infected hamsters (Thomzig *et al*, 2004). How this fits with the negative studies using bioassay (Hadlow *et al*, 1979, 1980, 1982) has yet to be resolved.

In sick animals, infectivity has been shown to be present in all tissues that were infectious during incubation, as well as in some additional tissues. These tissues are the pituitary, spinal cord, cerebro-spinal fluid, sciatic nerve, supra-mammary lymph node, distal colon, proximal ileum, distal rectum, adrenal and nasal mucosa. High levels of infectivity have been found in sheep brain and spinal cord, whereas medium to low infectivity levels have been found in other tissues. In rare cases, low infectivity levels have been found in the thymus, pancreas, bone marrow, and liver. Finally, some studies have detected low infectivity levels in the placenta (SSC, 2002a). As a result, and because of concerns about BSE in sheep, some agencies have adopted SRM removal procedures for small ruminants. This entails removal and destruction according to EC rules of the skull (including the brain and eyes), the spinal cord and tonsils of all sheep and goats over 12 months old and the ileum and spleen of sheep and goats of all ages.

3.2.4 BSE Infections in Sheep and Goats

Despite diligent research, BSE has never been identified as having caused infections in sheep, although very recently BSE has been confirmed in a goat in France. A suspect goat case in Scotland is still being studied. These investigations are hampered by the expense and time needed for biological strain typing of isolates; moreover, molecular strain typing methods are still not entirely reliable.

Under experimental conditions, sheep known to be free of scrapie have been successfully infected with BSE, and tissues from these animals have been examined in bioassays, showing a distribution similar to that of natural scrapie. Until more data become available, it should be assumed that tissue infectivity levels of BSE in small ruminants are comparable with those of scrapie.

Because naturally occurring BSE in small ruminants remains under investigation, SRM removal practices for sheep and goats are not the same as those for cattle. Control agencies have made risk reduction their objective, rather than risk elimination. Highest-risk tissues, however, are always removed.

3.2.5 CWD Infectivity in Cervidae (Deer and Elk)

In CWD-infected deer, misfolded protein has been detected during the early stages of pre-clinical infection in retropharyngeal and ileocecal lymph nodes, tonsil and Peyer's patches (Sigurdson *et al*, 1999). Infectivity has been detected in brain and other nervous tissues, including peripheral nerve, palatine tonsils, spleen, lymph nodes, Peyer's patches, and in other lymphoid tissue of the small and large intestine (Salman, 2003; Sigurdson *et al*, 1999). Because the clinical course, neuropathology and distribution of misfolded protein are similar in scrapie and CWD, the pathogenesis of CWD is assumed to be similar as well. However, because CWD has not yet been adapted to laboratory rodents, tissue infectivity levels have not been studied. The development of cervidised transgenic models may eventually solve this problem.

Transmission of CWD to humans or domestic animals (other than farmed Cervidae) under natural circumstances has not been reported, but a theoretical risk cannot be ruled out. Therefore, it seems prudent to exclude from food and feed chains certain tissues from animals that carry a CWD risk. It has been proposed that brain, spinal cord, lymph nodes, spleen, tonsils, and eyes should not be consumed (Williams, 2001). However, in 2003 the Scientific Steering Committee of the EC (SSC, 2003) concluded

that given the wide range of tissues infected in animals with CWD even in the early stages of the disease, no safe SRM list or lower-age cut-off can be defined. Neither is there sufficient knowledge to define exclusions or amendments of any SRM rule on the basis of relative genetic resistance to infection.

3.2.6 TSE Infectivity in Other Animal Species

TSE has been reported in several other animal species. Such infections may be related (*e.g.*, FSE) or unrelated (*e.g.*, TME) to BSE (Bruce *et al*, 1994; Fraser *et al*, 1994). Tissue infectivity studies for these TSEs are rare but not a cause of major concern, because these animal species are not slaughtered for consumption, even though their carcasses may be sent to rendering plants.

3.2.7 Knowledge Gaps about Tissues of Origin

1. The levels of infectivity in non-CNS tissues in most TSEs, during both the pre-clinical and clinical stages of disease, are unknown. Not all tissues have been bioassayed; even when they have, they could have yielded imprecise results due to lower sensitivity associated with the species barrier effect and a sample size that is statistically small. Infectivity determinations for the dorsal root ganglia and placenta of small ruminants merits investigation, as does correlation of the results with genotype analysis. However, because the great majority of body infectivity resides within the CNS, this data would not materially affect risk assessments currently in place.
2. Currently, there is no rapid test to distinguish between BSE and scrapie in sheep and goats. As a result, naturally occurring BSE in small ruminants cannot be ruled out unless complex tests or strain typing studies are done in a specialist laboratory.
3. In goats and Suffolk sheep with natural scrapie, and in cattle with natural and experimental BSE, infectivity has not been detected in skeletal muscle. However, the recent discovery of misfolded protein in sheep skeletal myocytes, from natural cases of scrapie, raises the concern that low levels of infectivity might occur in these tissues. This merits much further investigation.
4. The longevity of TSE agents in the environment is not known with precision, but it appears to be at least several years.
5. CWD is spreading, warranting a much-expanded research programme at all levels—epidemiology, biology, and molecular biology.

3.3 Countries of Origin

A major determinant of BSE risk is its presence or absence within a country. BSE was initially detected in the UK in the 1980s and has been exported to other countries by international trade of live animals and animal products. However, after this initial 'seeding', BSE has spread to even more countries and become an indigenous disease in most of them (see Table 3.7).

There are many reasons for this spread. First of all, BSE is difficult for veterinarians to diagnose without first-hand experience with the disease, because the clinical signs of BSE may be similar to those of other diseases. Results from the UK show that a BSE diagnosis could not be confirmed in about 15% of the slaughtered BSE-suspect cases throughout most of the BSE epidemic. Since then, the percentage of slaughtered BSE suspect cases not confirmed as having BSE continues to increase (Prince *et al*, 2003) and is now at more than 60%. Overall, this suggests that many mistaken clinical diagnoses are made.

Second, the BSE agent is very resistant to traditional disinfection procedures and could therefore easily amplify itself within a system if the implemented measures are inadequate or not rigorously enforced. A long incubation period and a high infectivity add to the problem. Therefore, BSE cattle in the preclinical stage of infection can be imported and exported, which spreads BSE from one country to another. However, due to government policies, epidemic history, and farm practices, some countries have higher TSE risks than others.

3.3.1 Country Statistics on BSE

The following tables summarise the most recent data collected on the worldwide BSE outbreak. Table 3.7 shows the total number of reported cases for each year and each country starting in 1989, and Table 3.8 gives the number of cases per country/region, relative to the total herd size in that country.

Table 3.7 Number of Reported Cases of BSE in Farmed Cattle

By Country, as Reported by September 19, 2005

Country/Year	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Austria	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
Belgium	0	0	0	0	0	0	0	0	1	6	3	9	46	38	15	11	1
Canada	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	1	1
Czech Republic	0	0	0	0	0	0	0	0	0	0	0	0	2	2	4	7	5
Denmark	0	0	0	1	0	0	0	0	0	0	0	1	6	3	2	1	
Finland	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	…[2)]	
France	0	0	5	0	1	4	3	12	6	18	31	161	274	239	137	54	
Germany	0	0	0	1	0	3	0	0	2	0	0	7	125	106	54	65	
Greece	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	
Ireland	15	14	17	18	16	19	16	73	80	83	91	149	246	333	183	126	47
Israel	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Italy	0	0	0	0	0	2	0	0	0	0	0	0	48	38	29	7	3
Japan	0	0	0	0	0	0	0	0	0	0	0	0	3	2	4	5	5
Liechtenstein	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	
Luxembourg	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
Netherlands	0	0	0	0	0	0	0	0	2	2	2	2	20	24	19	6	
Poland	0	0	0	0	0	0	0	0	0	0	0	0	0	4	5	11	14
Portugal	0	1	1	1	3	12	15	31	30	127	159	149	110	86	133	92	28
Slovakia	0	0	0	0	0	0	0	0	0	0	0	0	5	6	2	7	
Slovenia	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	2	1
Spain	0	0	0	0	0	0	0	0	0	0	0	2	82	127	167	137	66
Switzerland	0	2	8	15	29	64	68	45	38	14	50	33	42	24	21	3	3
United Kingdom[1)]	7228	14407	25359	37280	35090	24438	14562	8149	4393	3235	2301	1443	1202	1144	611	343	121
United States																	1

* Source: OIE, 2005, www.oie.int
[1)] As reported by June 15, 2005
[2)] Not reported.

Table 3.8 Annual Incidence Rate of BSE in OIE Member Countries

Number of Indigenous Cases per Million Bovines Aged over 24 Months as reported by August 30, 2005

Country/Year	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004
Austria	0	0	0	0	0	0	0	0	0	0	0	0	0.96	0	0	0
Belgium	0	0	0	0	0	0	0	0	0.61	3.69	1.84	5.53	28.22	25.75	10.54	7.882
Canada	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.33	0.149
Czech Republic	0	0	0	0	0	0	0	0	0	0	0	0	2.85	2.50	5.78	10.234
Denmark	0	0	0	0	0	0	0	0	0	0	0	1.14	6.77	3.35	2.39	1.296
Finland	0	0	0	0	0	0	0	0	0	0	0	0	2.39	0	0	...[2)]
France	0	0	0.45	0	0.09	0.27	0.27	1.09	0.54	1.64	2.82	14.73	19.70	20.96	12.01	4.736
Germany	0	0	0	0	0	0	0	0	0	0	0	1.07	19.97	17.02	8.71	10.915
Greece	0	0	0	0	0	0	0	0	0	0	0	0	3.3	0	0	0
Ireland	4.41	4.12	5.00	5.14	4.57	5.43	4.57	20.28	21.39	20.79	22.83	38.17	61.80	88.39	57.81	43.327
Israel	0	0	0	0	0	0	0	0	0	0	0	0	0	6.25	0	0
Italy	0	0	0	0	0	0	0	0	0	0	0	0	14.1	10.60	9.86	2.348
Japan	0	0	0	0	0	0	0	0	0	0	0	0	1.44	0.97	1.96	2.491
Luxembourg	0	0	0	0	0	0	0	0	10	0	0	0	0	14.54	0	...
Netherlands	0	0	0	0	0	0	0	0	1	1.01	1.03	1.07	10.25	13.19	10.86	3.399
Poland	0	0	0	0	0	0	0	0	0	0	0	0	0	1.28	1.49	3.578
Portugal	0	0	0	0	0	15.06	18.82	38.90	37.64	159.35	199.50	186.95	137.88	107.80	137.19	94.901
Slovakia	0	0	0	0	0	0	0	0	0	0	0	0	18.34	18.73	6.74	24.635
Slovenia	0	0	0	0	0	0	0	0	0	0	0	0	4.34	4.44	4.39	9.170
Spain	0	0	0	0	0	0	0	0	0	0	0	0.59	24.23	37.95	46.31	38.945
Switzerland	0	1	9.2	15.5	30.3	67.6	73.6	48.5	45.4	16	58.7	40.6	49.1	27.93	24.86	3.750
United Kingdom[1)]	1264.13	2506.97	4467.15	6636.07	6264.08	4277.78	2582.02	1416.78	794.43	585.65	416.36	270.56	232.76	228.24	122.25	68.799

*Source: OIE, 2005, www.oie.int
[1)] As reported by March 9, 2005
[2)] Not reported

3.3.2 Risk Assessment of BSE

The risk of BSE within a country should be determined by a formal risk assessment (Morley *et al*, 2003). The assessment is a decision-support tool, not a guarantee of safety. It is a scientifically supportable means by which to assess the chance that a given event will occur. The quality of the input information determines the quality of the risk assessment's outcome. Risk defined by a *qualitative* assessment is categorised as *highly unlikely, unlikely, low, moderate* or *high*, whereas assessments with more exact input information can be categorised quantitatively, with a number, *e.g.*, the number of individuals in a given population that might be exposed to the risk in question.

Although a risk assessment gives an indication of the risk, it does not conclude whether or not something should be considered safe. Decision-makers have the role of translating the risk assessment's results into conclusions, subject to societal, cultural and political influences (Brunk, 2002). Ultimately, these decisions reflect the public's tolerance for risk in that area. For example, although the general risk of traffic accidents is high, most people consider the risks tolerable and continue to drive. But risks related to food products generally must be extremely low before the public will consider a food product safe. In a risk assessment on the human risks related to the combustion of SRM, a likelihood of contracting vCJD that is lower than the likelihood of contracting CJD has been considered to be negligible (Cummins *et al*, 2002). However, most consumers feel that any risk of vCJD is too high.

BSE risk assessments generally include two main elements: assessment of the *external challenge* (the risk of BSE being introduced into a country), and assessment of the *domestic amplification* (the risk embedded in structural features of the cattle/beef industry, precautionary government regulations, quality of surveillance, etc). One obviously crucial risk factor is whether live cattle or cattle products have been imported from countries that either have BSE or were later found to have BSE. During the 1980s, importing countries might not even have been aware that imported MBM originated from the UK, because it was not mandatory for brokers to advise their customers about the country of origin. Even in situations where imports were stopped before the first BSE case had been detected in the exporting country, incubating cattle or BSE-contaminated products could have been imported and fed to ruminant animals, including cattle.

After BSE has been imported into a country, control of domestic amplification risks associated with the BSE agent determines whether or not the problem will spread. Amplification occurs for three reasons: (1) incubating animals cannot be or have not been identified; (2) rendering and inactivation procedures are not adequate in reducing TSE agent infectivity in MBM; and (3) feed for cattle is contaminated with infected MBM. The degree of control over these three amplification risks must be evaluated to determine the overall risk status of a country.

3.3.3 Risk Management of BSE

To manage the BSE risk within a country—either to eliminate it or to prevent BSE from entering and being amplified—certain control measures should be implemented (Heim and Kihm, 2003). First, BSE must be treated as a statutorily notifiable disease, and an effective surveillance system must be in place. Second, passive surveillance systems, which rely on farmers and veterinarians to report clinical suspect cases and

subsequent *post-mortem* testing, should be complemented with active surveillance systems, in which all cattle in risk categories are targeted for *post-mortem* BSE examination, with or without a direct clinical suspicion of TSE. As an extra safeguard, high risk tissues or specified risk material (SRM) must be prevented from entering any food or feed chain and must be destroyed. During slaughter, SRM must be removed and processed separately for destruction. SRM are defined differently in different countries, although all countries with a ban include at least the brain and spinal cord.

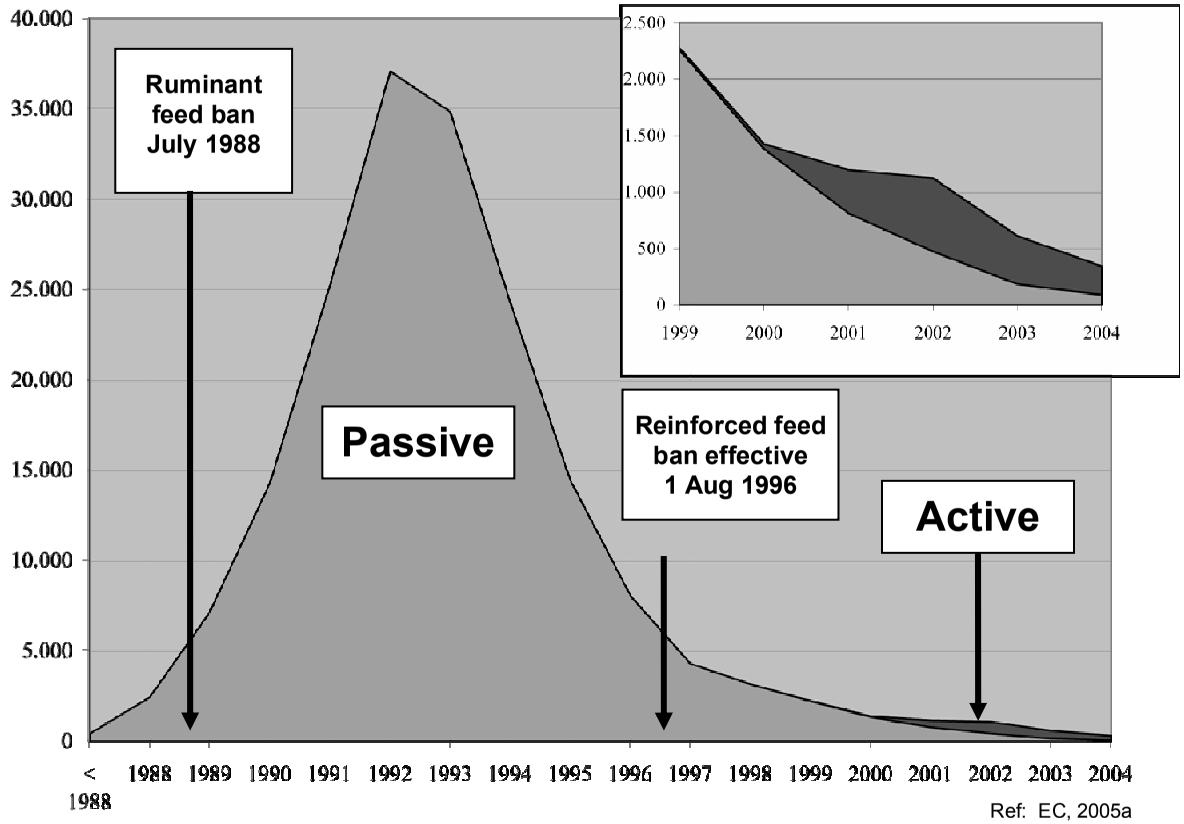
Standardising rendering procedures for cattle by-products is another important step in preventing BSE spread. Some countries have legislated nation-wide requirements for animal by-product rendering; in other countries, rendering processes and practices vary from plant to plant, resulting in wide variations in the TSE safety of their protein products. Although infectivity cannot be fully reduced in the greaves/MBM fraction of the cattle by-products, it can be substantially reduced if 'best-practices' rendering processes are legislated and enforced.

In addition, to prevent new BSE infections, cattle must be prevented from ingesting BSE-infected feed. Infected MBM is considered to be the most common vehicle of infection. Many countries now have a ruminant-protein-to-ruminant feed ban in place, which means MBM derived from ruminants cannot be fed to ruminants. This control measure has significantly decreased new infections in countries in which it is practiced. But experience in Europe has shown that an exclusively ruminant-to-ruminant feed ban is not sufficient to eliminate all new infections (Heim and Kihm, 2003). When ruminant MBM is still permitted in feed for non-ruminants, cross-contamination is possible in feed mills preparing both ruminant and non-ruminant feed, even when upgraded separation procedures are used. In addition, cross-contamination during transport, on-farm storage, and feeding cannot be consistently eliminated. Moreover, it is difficult for farmers and rendering plant workers to distinguish between ruminant and non-ruminant MBM; thus, errors can occur. For these reasons, many countries have extended their feed ban controls to all mammalian MBM or processed animal protein in feed products for farmed, food-producing animals.

Finally, BSE risk management requires a strong legal framework to regulate and enforce control measures. Implementation of programmes cannot be left to self-regulation by industry. The relevant authorities must oblige all parts of the food industry to comply with legislation (Heim and Kihm, 2003). The UK BSE Inquiry (2000) showed that a 1988 control programme banning SRM from ruminant feed failed to protect animal health because it lacked effective inspection and control of the different categories of material for rendering. It was not until 1995, when a very thorough inspection system (National Meat Hygiene Service) was introduced, that the control measures were finally appropriately implemented. Once the reinforced 'real' control ban was in place, new exposures as a result of indigenous cross contamination of feed were eliminated, and confidence that BSE could be eliminated increased. The effect of the implementation of feed bans and surveillance systems in the UK and the rest of the EU is illustrated in Figure 3.3 and Figure 3.4.

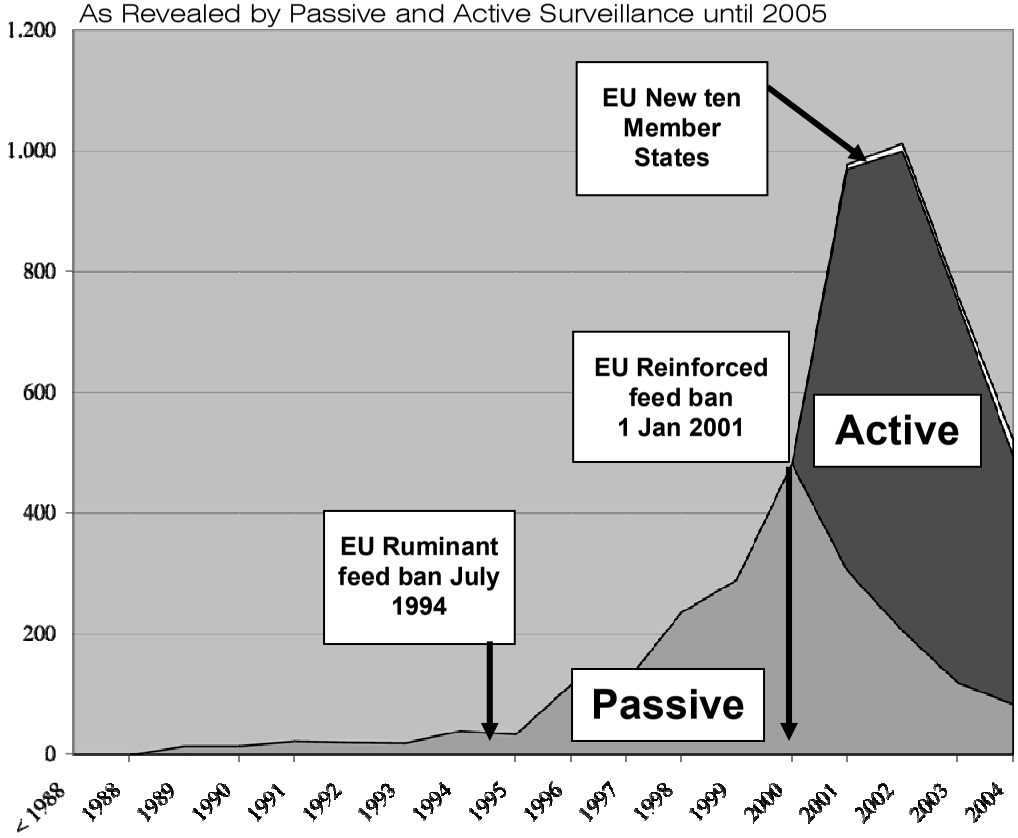
Figure 3.3 Evolution and Decline of BSE in Cattle in the UK

As Revealed by Passive and Active Surveillance until 2005



Ref: EC, 2005a

Figure 3.4 Evolution and Decline of BSE in the EU (excluding the UK)
As Revealed by Passive and Active Surveillance until 2005



Ref: EC 2005a

3.3.4 Internationally Recognised BSE Risk Classification Systems

Two organisations have been significantly involved in developing BSE risk classification systems to minimise the risk of introducing and amplifying BSE into, and amplifying it in, countries without the disease through trading practises involving animals (especially cattle) and their products. The first is the World Organisation for Animal Health (OIE), to which virtually all trading nations belong. The second is the European Commission (EC), which was originally advised by the Scientific Steering Committee, whose role is now taken over by the European Food Safety Authority.

Apart from the three versus four classes of risk determined by the two agencies, there is considerable agreement between them, and the classification systems can facilitate international trade because trading partners can know each other's BSE status. The EC has declared that it intends to adopt the OIE three category system by July 2007 if it has not done so before (EC, 2005b).

3.3.4.1 World Organisation for Animal Health (OIE) BSE Evaluation

Through the OIE's Terrestrial Animal Health Code, and especially Chapter 2.3.13 on BSE, the OIE determines the BSE risk status of the cattle population of a country or zone on the basis of certain criteria. The criteria include the outcome of a risk assessment (reviewed annually), based on Section 1.3 of the Terrestrial Code, which identifies all potential factors for BSE occurrence and their historic perspective. The Code also specifies the recommended requirements for safe trading in various bovine commodities.

The risk assessment identifies all possible historical and current risk factors for the BSE agent, including *external risks* (importing BSE and the subsequent release of the BSE agents into the country) and *internal risks* (the likelihood of exposure of the BSE agents to cattle). External risk factors include the presence or absence of animal TSE agents in the country and their prevalence; and importation of live animals, MBM or greaves, animal feed or feed ingredients, and products of ruminant origin for human consumption. To assess the likelihood of internal risk factors, the following parameters need to be assessed: the potential for spreading the BSE agent by ruminant-to-ruminant feeding practices or cross-contamination of ruminant feed; the origin and use of animal carcasses (including fallen stock), by-products and slaughterhouse waste; rendering parameters and methods of animal feed manufacture; and the level of surveillance for BSE conducted and the results of that surveillance.

After the risk assessment, the OIE determines the presence or absence of an ongoing awareness programme for veterinarians, farmers, and workers involved in the transportation, marketing, and slaughter of cattle. The OIE then examines systems of compulsory notification and investigation of all cattle showing clinical signs consistent with BSE. Finally, the OIE considers data from brain and tissue examinations collected by the surveillance system and tested in an approved BSE laboratory.

The outcome of this evaluation leads to a three-category qualitative classification system:

1. Countries or zones with a negligible BSE risk
2. Countries or zones with a controlled BSE risk
3. Countries or zones with an undetermined BSE risk.

3.3.4.2 Geographical BSE Risk (GBR) Assessment by the European Commission (EC)

The EC operates on behalf of the Member States of the EU by formally classifying countries wishing to trade with them in live cattle or cattle products, using a four-category Geographical BSE Risk (GBR) assessment. The GBR defines the qualitative likelihood of the presence of one or more BSE-infected cattle in the native cattle population of a country, either pre-clinically or clinically, at a given point in time. When the presence of BSE has been confirmed in a country, the GBR gives an indication of the level of infection. The calculation of the GBR is based on two risk factors: the chance that BSE has ever been imported into the country; and the chance that BSE has the opportunity to amplify itself within the country.

The GBR assumes that BSE can only occur in a country into which either infected cattle or MBM have been imported. The only exception to this assumption is the United Kingdom; however, it is now known that both live cattle and MBM imported from other European countries have been responsible for a small number of UK cases of BSE. The chance of importing BSE is called the *external challenge* of a country, and the external challenge is assumed to be independent of the size of the challenged system.

After BSE enters a country, a GBR considers the country's ability to prevent or reduce the speed of spreading the BSE agent within its boundaries. This is called the *stability of a system*. Aspects considered are surveillance, SRM removal, and rendering and feeding practices within the country. The only route of transmission considered in a GBR assessment is oral transmission through contaminated feed, because feed has proven to be the most likely cause of infection.

For all aspects of the external challenge and internal stability, the country has to provide information for every year since 1980. In the evaluation, the information provided is assumed to be correct (although it can be compared to other sources). In case of conflicting data from equally reliable sources, the worst-case scenarios are used. To complement insufficient information, estimated worst-case scenarios are also employed. GBR assessments are possible for countries that have not yet reported their first BSE case, by assessing the external challenge potential, the internal stability of the system, and hypothetical assumptions based on these two assessments.

The resulting classification of a country is based on the interaction of the external challenge and the internal stability. Countries with a low external challenge and a high internal stability will have a very low risk of having BSE. Countries with a high external challenge and a low internal stability will be very likely to have BSE. The GBR assessment defines four risk categories:

1. *Highly unlikely* that one or more clinically or pre-clinically BSE infected cattle are present in a country;
2. *Unlikely* but not to be excluded that one or more clinically or pre-clinically BSE infected cattle are present in a country;
3. *Likely* but not confirmed that one or more clinically or pre-clinically BSE infected cattle are present in a country OR presence of BSE has been confirmed at a lower level;
4. *Presence* of BSE has been confirmed at a higher level (SSC, 2000a, 2002b).

Many countries within and outside the EU have been assessed by this method (see Table 3.9), and some countries have even been reassessed when changes in the external challenge or the internal stability outdated the previous assessment (SSC, 2004).

The validity of the GBR assessment has been proven over time, since several countries that had been classified as GBR III soon afterward reported their first occurrence of BSE. Note that classifications change regularly. A change in the BSE status of an exporting country might influence the GBR classification of other countries importing from it, and in-country changes can lead to a reclassification. The most up-to-date publications on GBR classifications can be found on the websites of the SSC and the European Food Safety Authority (EFSA) [efsa.eu.int/index_en.html].

Table 3.9 GBR Assessments in 2005 by Country

Country	Classification	Year of Adoption
Argentina	I	2005
Australia	I	2004
Iceland	I	2002
New Caledonia	I	2003
New Zealand	I	2005
Panama	I	2005
Paraguay	I	2005
Singapore	I	2003
Uruguay	I	2005
Vanuatu	I	2002
Botswana	II	2005
Brazil	II	2005
Colombia	II	2001
Costa Rica	II	2005
El Salvador	II	2005
India	II	2001
Kenya	II	2001
Mauritius	II	2001
Namibia	II	2005
Nicaragua	II	2005
Nigeria	II	2001
Norway	II	2004
Pakistan	II	2001
Sweden	II	2004
Swaziland	II	2005
Albania	III	2001
Andorra	III	2002
Austria	III	2000
Belarus	III	2003
Bulgaria	III	2002
Belgium	III	2000
Canada	III	2004
Chile	III	2005
Croatia	III	2002
Cyprus	III	2003
Czech Republic	III	2001
Denmark	III	2000
Estonia	III	2003
Finland	III	2000
Former Yugoslav Republic of Macedonia	III	2003

Table 3.9 GBR Assessments in 2005 by Country

Country	Classification	Year of Adoption
France	III	2000
Germany	III	2000
Greece	III	2000
Hungary	III	2001
Ireland	III	2000
Israel	III	2002
Italy	III	2000
Latvia	III	2002
Lithuania	III	2003
Luxembourg	III	2000
Malta	III	2002
Mexico	III	2004
Netherlands	III	2000
Poland	III	2001
Republic of South Africa	III	2004
Romania	III	2001
San Marino	III	2002
Slovak Republic	III	2001
Slovenia	III	2002
Spain	III	2000
Switzerland	III	2000
Turkey	III	2002
United States of America	III	2004
Portugal	IV recommended for III	2005
United Kingdom	IV recommended for III	2005

- Source: SSC, 2004; EFSA, 2005b (as of 17 August 2005)
- **Note:** Because the occurrence of BSE in countries is dynamic and because effective risk management measures may lag behind new BSE occurrences, the GBR is dynamic too. Consequently some countries may improve or worsen their GBR category over time.

3.3.5 Knowledge Gaps about Countries of Origin

1. Without adequately enforced monitoring systems that follow international standards, it is difficult to determine with certainty the level of BSE infection in a particular country.

2. A current problem in risk assessments is the lack of a single internationally accepted and applied procedure. Each country gathers information differently, and the quality of the data is thus difficult to assess objectively. The quality of a risk assessment depends heavily on the quality of the input data. For this reason, a universal standard is needed.

3. Many countries have not performed BSE risk assessments because they assume they do not have a risk for BSE. This assumption puts the cart before the horse: all countries should conduct a risk assessment for BSE, as recommended by the OIE.

4. Many countries report that they have implemented a BSE surveillance system. However, to date there is no way to evaluate the quality of these surveillance systems other than in the EU, Norway and Switzerland. A legal framework for the implementation of BSE measures does not a guarantee a good surveillance system, which includes enforcement and compliance. To date, there is also no compulsory international evaluation for national BSE surveillance systems.

3.3.6 Sources of TSE Risk: Conclusions

TSE risks from source materials can be reduced by selecting input materials from low-risk countries as determined by OIE or GBR assessments of countries of origin (SSC, 2000a, 2002b; EFSA 2004: OIE, 2004). Additional security is afforded by appropriate selection of animals and of tissues from those animals.

The use of porcine and avian starting materials, separately or together, is theoretically a way to reduce risk, since neither of these species is naturally affected by TSE, and neither is experimentally susceptible to oral challenge with the BSE agent (Bradley, 2004; Dawson *et al*, 1994; Wells, 2003). Furthermore, orally challenged pigs showed no detectable infectivity in the tissues that were selected for bioassay in susceptible mice (Wells, 2003). However, there is one important point of caution: In countries with BSE or other animal TSE, or at risk of BSE or other animal TSE, and where there is no effective ban on feeding ruminant protein to non-ruminant species, there is a potential risk that infected MBM fed to pigs or chickens could still be in the alimentary tract at slaughter. Thus, additional measures might be required in this circumstance.

In many rendering plants, however, the input material is consistently of mixed species origin. In the EU, this applies particularly to Category 1 processing plants (plants in the EU approved to process Category 1 high risk material – see p. 82 4.1.3.1 Risk categories), where SRM from cattle, sheep and goats is processed, as well as fallen farm stock and food animals slaughtered as part of a programme of infectious disease control. Wastes from circus animals, wildlife, road kills, fallen stock, farmed mink and even pet and laboratory animals have been rendered in the past. Most of these materials would now be considered as high-risk waste. Generally speaking, the separation of animal species for rendering would be impractical.

Thus, although zero-risk input material is theoretically possible by adherence to a strict selection of animals, tissues and source countries, biodiesel production aims to use precisely these potentially infected and low-value tissues. In consequence, rigorous selection procedures are inconsistent with the objective of using low-value animal fats for biodiesel production.

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4 PROCESS: CONVERTING ANIMAL FEEDSTOCKS INTO BIODIESEL

4.1 Rendering

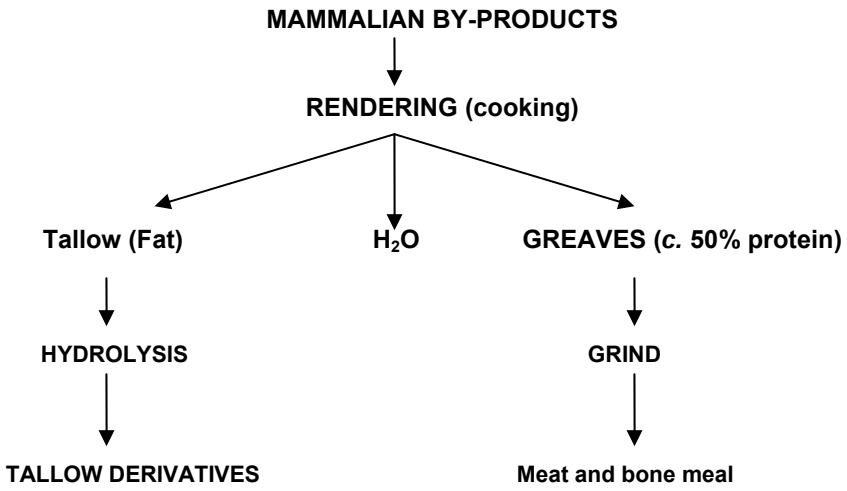
There are four approaches to dealing with the possible presence of TSE infectivity in animal tissues. The first approach is to ensure that safe sources are used, a practice adopted by manufacturers of vaccines for human use (Bradley, 2001, 2006). A second approach is to separate safe from unsafe tissues at source, such as is adopted by the human food and animal feed industries in the EU and Switzerland. This method differentiates waste streams by degree of risk and removes and destroys specified risk material (SRM) (EC, 2002, 2003; SSC, 1997, 2000b). A third approach is to process the infected starting material in such a way that the infected component is separated or partitioned into one stream, leaving one or more other streams without or with greatly reduced levels of TSE infectivity. A fourth approach is to include one or more steps in the process to reduce or eliminate infectivity. In practice, the third and fourth approaches are often combined, such as in the rendering industry.

There are several different types and manufacturers of rendering equipment, and the same equipment can be used in different ways. Despite the fact that TSE agents are much more resistant to inactivation than conventional microbiological agents (Kimberlin *et al*, 1983; Taylor, 2000), infectivity can be reduced under certain conditions, including by many processes used in rendering plants (SSC, 1998; Taylor, 2000). But each process must be examined separately to determine its potential to reduce or eliminate TSE infectivity.

4.1.1 Types of Rendering

Rendering is generically a cooking and separating process. Waste animal material is cooked, the water content is evaporated, and fat (tallow) is extracted, mainly by pressing or centrifugation, leaving protein-rich greaves that are ground to produce meat and bone meal (MBM) (see Figure 4.1). There are several popular methods, including batch and continuous processes, vacuum and pressure systems, and processes that use added fat.

Figure 4.1 Rendering of Mammalian Animal By-products and Product Streams



The principal rendering process used in the EU is pressure-cooking, which is the only method authorised for more hazardous materials, such as SRM. It uses the following parameters: particle size of the starting material ≤ 50mm, 133 ºC, 3 bar, 20 min. Throughout this document the term 'pressure-cooking' refers only to these parameters used in a batch or continuous system. In pressure-cooking, the cooking vessel is hermetically sealed before heating. Consequently, as heat is applied, water inside is converted to steam under hyperbaric conditions. Thus, the contents are cooked using the high latent energy in the steam (Taylor and Woodgate, 1997).

Pressure-cooking completely inactivates all conventional animal pathogens and inactivates ≥ 3 logs of TSE infectivity. Of all the available rendering processes, it is considered the most efficient means to achieve maximal reduction of TSE infectivity (scrapie and BSE) in MBM. In order to have the maximal effect on TSE infectivity, pressure-cooking must be used as the primary rendering process.

But pressure-cooking is not used in rendering plants in North America, where most processes today are continuous and automated, using air- and water-pollution prevention systems. These procedures have largely replaced older 'wet' batch rendering, in which the starting material was sealed in a pressure vessel into which superheated steam at 110 to 120 ºC is injected. In these earlier systems, cooking took 4 to 6 hours and resulted in a three-layered product with fat in the top layer, water in the middle, and greaves in the bottom. Some of the rendering methods that contributed to the BSE epidemic in the UK originated in North America, where some may still be in use. However, they are now banned throughout the EU.

Before the BSE epidemic, a high proportion of MBM in the UK was produced using hydrocarbon solvents to extract greater amounts of tallow from the greaves, thus increasing tallow yield and profitability (while reducing the MBM fat content from 15% to < 3%). The hydrocarbon solvents were subsequently evaporated under steam for recovery and re-use. In early analysis of the BSE epidemic, this additional wet-heat

step was believed to have helped reduce any residual TSE infectivity present in the greaves that had survived initial rendering. Further evidence supports this hypothesis. The hydrocarbon solvent extraction system declined from the late 1970s onward, and by 1981 was largely extinct except in Scotland. The incidence of BSE in native-born Scottish cattle was significantly lower than in the rest of the UK, leading some epidemiologists to believe that the reduced use of hydrocarbon solvent extraction from the early 1980s in the rest of the UK may have resulted in increased amounts of TSE agent in MBM, thereby initiating and subsequently amplifying the BSE epidemic (Wilesmith *et al*, 1988). Although subsequent laboratory studies have suggested that the infectivity reduction was slight (Taylor *et al*, 1998a), it remains possible that even this marginal inactivation, coupled with the temperature vicissitudes of continuous (as opposed to batch) rendering, might have been enough to prevent the threshold level of input infectivity from surviving the rendering process.

Fat melting is another processing method, one that extracts fat for human use. Excluding mesenteric or midrum fat, which in the EU is considered SRM, it uses only discrete adipose tissue from carcasses passed as fit for human consumption, including major fat depots and trimmings from abattoirs, cutting rooms and butcher's premises. Essentially, this fat is devoid of other tissues and is simply melted and collected in a hygienic manner. Untreated 'clean' beef fat can be sold as suet (see Table 4.1). However, fat melting is not considered rendering. Moreover, for biodiesel production, this process produces end products of too fine a quality and too high a price to be useful. For this reason, despite the low TSE risk of fats derived from this process, clean fats will not be considered for biodiesel production.

Table 4.1 Physical and Chemical Properties of Beef Tallow

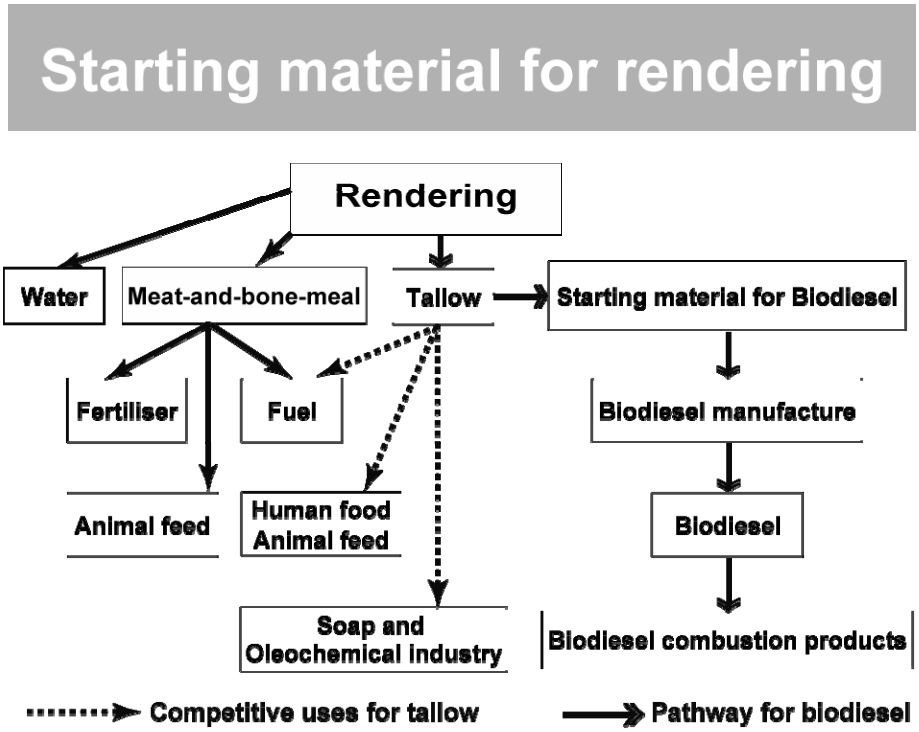
Derived by Different Methods (Quoted by the SSC (2001b) and Derived from Data Provided by the European Fats and Proteins Research Association)

TALLOW TYPE	PREMIER JUS	BEEF TALLOW	CALF FAT	TALLOW
Usual production process	Wet melting	Dry rendering	Wet/dry melting	Wet/dry rendering
Quality characteristics				
FFA (%)	Max 0.5	Max 2	Max 0.75	Max 15
POV (meq/kg)	Max 2	Max 3	Max 2	Max 10
IV (g I_2/kg)	37-42	40-50	47-54	--
Slip melting pt (^oC)	Min 43	Min 40	Min 35	--
Colour	Yellow	Yellow/brown	White/yellow-brown	Brown
Gardner colour	Max 3	Max 8	Max 2 / 7	Max 9
Insoluble impurities (%)	Max 0.02	0.15	0.02	0.15
Odor/flavour	Fresh, slightly butter-like	Fresh, fried greaves-like	Fresh typical	Typical
Fatty acid composition (%)				
Lauric acid C-12:0	4	3	2	
Myristic acid C-14:0	26	26	8	
Palmitic acid C-16:0	22	24	25	
Stearic acid C-18:0	36	36	16	
Oleic acid C-18:1	3	2	34	
Linoleic acid C-18:2			6	

4.1.2 TSE Risks in Starting Material for Rendering

TSE agents are found exclusively in particular tissues from a limited number of species, so contamination of the starting material with plant or mineral matter will not affect TSE risk (see Figure 4.2).

Figure 4.2 Major Pathways for Rendered Products



Specified risk material (SRM) is animal material or tissue identified as having a real or potential risk of being infected with a TSE agent (EC, 2002, 2003; SSC, 1997, 2000b). It can include whole animal carcasses, specific tissues or parts of carcasses, carcass trimmings, or liquids contaminated by such material. The definition of SRM varies among different countries and agencies; in this report, SRM is assumed to be those tissues that are or might be infected with a TSE agent if there is a TSE risk in the country or region in which the animal was born, reared or died.

Moreover, anything that comes in contact with SRM is usually regarded as SRM and should be treated as such. This means that a whole bovine, ovine or caprine carcass would be regarded as SRM if SRM tissues were not removed. If disposable materials such as gloves come in contact with SRM, unless alternative arrangements are made they should enter skips used for receipt of SRM. Equipment such as stun guns, knives, buildings and vehicles should be dedicated for SRM use and then decontaminated after use.

In countries operating an SRM ban and separating SRM from non-SRM for rendering, SRM might be expected to contain variable amounts of TSE infectivity. The amount of infectivity will depend on the TSE status of the country, the dynamic state of the epidemics in the country, the TSE status of the source animals, and the tissues selected as SRM. However, it cannot be assumed that there would be no TSE infectivity in the non-SRM material, because the bans in place may be imperfectly enforced. Moreover, ban requirements, especially those involving the designation of given tissues as SRM and the minimum age of removal, may not have been selected on a worst-scenario basis. Nevertheless, despite possible further cross-contamination risks from certain

penetrative stunning methods at slaughter, there is certainly a much lower TSE risk in non-SRM than in SRM (SSC, 2001c).

In addition, the rendering process may concentrate any infectivity present in starting materials into one or another component product. TSE agents seem to be entirely proteinaceous (Prusiner, 1982, 1998), and infectivity is always associated with the misfolded protein (McKinley *et al*, 1983); so any infectivity present in starting materials is more likely to partition with the protein fraction (greaves/MBM) than with the tallow (Wilesmith *et al*, 1988, 1991; Taylor *et al*, 1995, 1997). In addition, natural post mortem autolytic processes that destroy most tissues and pathogens may concentrate infectivity because TSE agents are resistant to these processes.

Although tissues and tallow are unlikely to be infected, for the purpose of this report all starting materials for rendering will be assumed to be contaminated with TSE agents as a worst-case scenario, whether or not they are designated SRM or non-SRM. The only exception will be cases where the country of origin is officially classified as BSE highly-unlikely (SSC, 2000a, 2002b). **Note**: the role of assessing the Geographical BSE Risk (GBR) previously undertaken by the EC Scientific Steering Committee (SSC) has now been taken over by the European Food Safety Authority (EFSA). To determine the latest Opinion on the GBR of different countries, the EFSA website should be consulted. The latest opinion for several countries have been updated to 2005.

4.1.3 Risk Management in Animal By-products

4.1.3.1 Risk categories

To manage health risk issues in animal by-product production, the EC classifies animal by-products and slaughter waste into three categories. Category 3 is the lowest-risk group, and includes meat and meat products that are fit for human consumption, as well as melted edible fats and food-grade gelatin obtained from bones (and skin or hides) from edible carcasses. It excludes vertebral columns and skulls from animals of specified ages. The two other categories are reserved for products not intended for human consumption (Categories 1 and 2). The three categories have been defined in Regulation of the Parliament and of the Council EC No. 1774/2002 and subsequent amendments, which should be consulted for details.

Category 1 materials are animal by-products that are regarded as high-risk. For TSE risks, these include all body parts, including hides and skins, of the following: animals that are suspected or confirmed to be infected with a TSE (including fallen ruminant stock); and animals that have been killed in the context of TSE eradication measures, including pet, zoo, circus, experimental, and wild animals suspected of being infected with a zoonosis. Also included is SRM (or the entire body of an animal if the SRM is not removed separately), and all animal material recovered from drains and waste-water in premises where SRM are removed. Category 1 waste also includes material with non-TSE risks, such as animals or parts of animals with prohibited residues, and catering waste from international transport. Other than catering waste (which can be buried in a licensed landfill), it has been required that all Category 1 materials must be incinerated; rendered and subsequently incinerated; co-incinerated; or buried under strict conditions. However, some alternative technologies and uses have recently been authorised in Europe (EC, 2005). These include high-pressure hydrolysis biogas, high-pressure high-temperature hydrolysis, Brookes gasification, and biodiesel and alkaline

hydrolysis. A recent Opinion from the EFSA (EFSA, 2004) approves the use of pressure-cooked Category 1 tallow for manufacture of biodiesel under strict conditions. This is endorsed for biodiesel and the other technologies in Commission Regulation 92/2005 (EC 2005). Scientific evidence from a spiking experiment supporting the safe use of tallow produced by primary pressure-cooking of Category 1 waste for biodiesel production has been provided to the EFSA (B. Seidel, personal communication). This is described in detail in "Steps in the biodiesel process" (page 103) and in the following publication: Seidel *et al,* 2006.

Category 2 materials are animal by-products that still pose a risk (but not a TSE risk). This category includes manure and digestive tract contents; fallen stock of non-ruminant species and non-ruminant animals killed to eradicate an epizootic disease not included in Category 1; products of animal origin that contain certain residues or drugs; mixtures of Category 2 and 3 materials; and any material that is not either Category 1 or 3. There are several options for disposal of Category 2 materials, including those used for Category 1 materials. They can also be processed in an approved Category 2 processing plant and, if rendered by approved methods (not necessarily pressure-cooking), the tallow can be further processed in a Category 2 oleochemical plant, after which they can be used in organic fertilisers or technical products other than cosmetics and medicines, or transformed to fuel in a biogas plant. Thus, Category 2 tallow prepared by pressure-cooking is also approved for biodiesel manufacture.

Category 3 materials are animal by-products that pose a low risk. These by-products are derived from animals considered fit but not chosen for human consumption. This category includes by-products from the slaughter process, degreased bones, former foodstuffs of animal origin devoid of an infectious risk to humans and animals, fresh fish by-products, and catering waste other than of international transport origin. Materials in this category can be used for pet-food production, animal feed for non-food-producing animals, compost, or technical products after appropriate processing in an approved Category 3 processing plant. The material can also be disposed like Category 1 or 2 materials, and a range of less severe options is also permitted. Tallow from Category 3 material produced in a Category 3 rendering plant is permitted for use to make biodiesel.

4.1.3.2 Starting materials for rendering outside the EU

Outside of the EU, Norway, Switzerland, and some other countries, where categorisation of animal by-products governs the processing method (see above), different groups of products can be used as input materials for the rendering industries. These groups are as follows:

- *Waste from abattoir and butchery premises*. Abattoir and butchery waste is the major source of material for the rendering industry and consists almost entirely of animal material with no other uses. However, abattoir and butchery waste of small ruminants differ from that of cattle, because meat from small species tends to be sold on the bone. Other than brisket, ribs and T-bone steaks, cattle meat is usually sold off the bone.
- *Waste from human food and fats used to cook human food*. It is possible that starting materials other than animal waste could be used in rendering, including plate waste and cooking grease from restaurants. As long as these commodities are

not contaminated with TSE-infected material, they are safe for rendering. However, without strict controls, errors could happen. There have been fatal human incidents when mineral and other toxic oils have been mistakenly recycled into the food supply.

- *TSE-risk animals and products*. TSE-risk animals and products include the following: clinically suspected BSE-affected cattle; cattle that fail a BSE 'Rapid' test; fallen stock; feed and birth cohort animals of confirmed BSE cases; offspring of confirmed BSE cases; cattle infected with BSE but compulsorily slaughtered as part of a disease control programme for other diseases; SRM; and MBM and feed containing MBM officially seized for destruction.
- *By-products from the bone gelatin industries*. Porcine and bovine bones used as a source for gelatin manufacture are first crushed to form bone chips and pressure-hosed with water at 85 °C to remove fat. It is theoretically possible that this fat/water run-off (which may be contaminated with other animal tissues) could be included as a starting material for rendering.
- *Other animal material*. Unless prohibited by legislation, whole carcasses of other species may form part of the starting material for rendering, including captive wild animals, pets, circus animals, wild animals, road kills, and experimental animals (some species of which can be naturally or experimentally infected with TSE agents).

4.1.4 Tallow from Rendering

Tallow is animal fat separated from protein and other animal parts through rendering. Tallow is the most important product of rendering for biodiesel production, because only fats can be converted into biodiesel.

4.1.4.1 Quality of tallow

Tallow varies in quality depending mostly on the nature of the starting materials for rendering and their state of preservation. Its colour, melting point, and some other characteristics may be influenced by the breed of animal and type of feed provided.

Commercial tallow must be graded, with edible tallow and lard (exclusively from pigs) at the top (Grades 1 and 2) and choice white and yellow greases at the bottom (Grades 12 and 13). The different grades have different uses. Grades and specifications are determined by five major factors: colour, from 1 (lightest) to 45 (darkest) using odd numbers only; free fatty acid (FFA) content; solidification temperature (tallows > 40 ºC and greases < 39 ºC); and the content of moisture, unsaponifiables and impurities (insolubles).

A recent international bulletin listed available tallows and greases as follows (with the FFA content in parentheses):

- Pure beef tallow (1 or 2%)
- Extra fancy tallow, top white tallow (1%)
- Technical tallow, edible tallow (1%)
- Bleachable fancy tallow (2 or 4%)

- Low grade tallow (10%)
- Medium gut tallow (10 to 15%)
- Yellow grease (6 to 15%)
- K grade tallow (21%).

So-called green tallow is a generic term relating to tallow mostly generated from alimentary tract material containing feed with chlorophyll. Because in the EU SRM includes intestinal material, SRM tallow is likely to also be 'green' tallow.

Tallow can also be measured and specified to suit particular commercial applications. These include pH, filtration rate, iodine value and levels of specific impurities, of which polyethylene (from meat packaging) is one of the most important because it is soluble in tallow but can become precipitated at some late stage of an industrial process.

In the EU, most tallow industries purify food-grade tallow to a maximum level of 0.02% insoluble impurities. Additional steps, such as filtering and centrifugation, may reduce residual TSE risk in the tallow to negligible proportions. Nevertheless, because tallow varies in nature and quality, the precise characteristics should be studied and specified for each intended use. For biodiesel production, small impurities may be important unless they can be removed because of the effects they could have on engine performance.

4.1.4.2 TSE infectivity in tallow

TSE infectivity is closely associated with misfolded protein. Cleaned, filtered tallow is presumed to be devoid of protein and thus presumably devoid of TSE infectivity. Furthermore, studies have shown that lipid molecules are not part of any TSE agent (Stahl, 1987; Prusiner, 1998; May *et al*, 2004), and thus do not contribute to any inherent infectivity present in starting materials. Thus, clean tallow can be presumed to be TSE-free; and the question of tallow infectivity should focus on either cross-contamination or impurities.

But fat cells can be closely associated with some proteins. Before separation and rendering, fat is attached to other tissues in the body, including protein. Moreover, fat is stored within cells of adipose tissue, the cell membrane of which is comprised of protein that would have to be broken during processing to release the fat. Fat is also found in other cells, such as hepatocytes of the liver, which also contain protein. Furthermore, the CNS and myelinated peripheral nerves have a significant fat content as well as potentially infected proteins. Thus, to reduce the risk of TSE contamination of tallow, it is important to remove all insoluble material, especially protein, before or during refining.

Modern rendering processes remove these impurities from tallow very efficiently: filtration and other secondary cleaning steps reduce infectivity to negligible levels. For international trading, the OIE requires that tallow is free of protein and the level of insolubles is set at a maximum of 0.15% by weight.

Fat could conceivably protect TSE infectivity from the effects of heat, as it does for conventional pathogens such as the poliomyelitis virus (Kaplan and Melnick, 1954) and bacterial spores (Sendhaj, 1997; Sendhaj and Levin, 1977). In the same way,

tallow might be able to protect the prion protein which, despite its hydrophobic nature (Hope *et al*, 1988; Safar, 1996), is more likely to partition with the protein fraction of rendered material than with the tallow.

Tallow storage offers another opportunity for TSE risk reduction. Tallow is stored in sealed tanks of various capacities on a 'tank farm'. It is solid at room temperature; therefore, short-term storage in tanks requires that it be continuously heated to a temperature above the melting point. This can be achieved by circulating steam through a sealed pipe system in the lower part of the tanks. As a result, insoluble material, which might include protein and thus TSE infectivity, sediments to the bottom of the tank and is called a 'tank bottom'. Material forming the 'tank bottom' is likely to have a higher TSE risk than the tallow stored above it. Tank bottoms should be avoided for use as feedstock for biodiesel production.

4.1.5 Studies on TSE in Tallow

Rendering studies to date suggest that any residual BSE infectivity in tallow would be too low to infect cattle by the oral route. For this reason, it is even less likely to infect other species, including humans, which would be partially protected by the species barrier effect.

4.1.5.1 Epidemiological studies

General studies about BSE have indicated that tallow is not likely a carrier of infectivity. In 1987, a few months after BSE was first discovered, the UK conducted an exhaustive investigation of its origins. This study indicated that cattle had been exposed to a scrapie-like agent in MBM in the winter of 1981 to 1982, with the epidemic of subsequent clinical disease commencing in 1985 (Wilesmith *et al*, 1988). Additional epidemiological studies by Wilesmith *et al* (1991, 1992) supported this theory and further suggested that the cessation of hydrocarbon solvent extraction may have resulted in enough increased exposure to infectivity to initiate the epidemic. These studies excluded tallow as a cause, since the geographical distribution of BSE cases was not consistent with the distribution and use of tallow (Wilesmith *et al*, 1988). Further research showed that scrapie-like agents were intimately associated with animal cell membranes (Stahl *et al*, 1987); therefore, they were more likely to partition with the cellular residues of the MBM fraction than with the lipids of tallow.

One set of studies in 2003 (Paisley and Hostrup-Pedersen, 2004) considered tallow a possible cause of new BSE cases, but this theory had several shortcomings. Paisley and Hostrup-Pedersen proposed that calf milk replacers incorporating tallow were historically associated with BSE transmission via feed. Although no evidence was provided to support this statement, it found some favour in Germany (Kamphues *et al*, 2001), Denmark, (Paisley and Hostrup-Pedersen, 2004) and to some extent in Japan (BSETC, 2003).

Paisley and Hostrup-Pedersen also proposed that tallow-based milk replacers could account for the occurrence of BSE in UK cattle born after the 'real' (reinforced) feed ban (Paisley and Hostrup-Pedersen, 2004; SSC, 2001). But milk replacers are not the only possible explanation. The 'real' feed ban was introduced in the UK in March 1996 and accepted to be effective starting 1 August 1996; but a similar EU-wide ban was not introduced until 1 January 2001. Thus, calves in the UK born after the 1996 ban could have been infected following consumption of contaminated feed imported from

countries without an effective feed ban (Wilesmith 2002; Prince 2004). As a result, the infected contaminating material in the 'post-ban' cases was more likely MBM than tallow.

Overall, several highly questionable assumptions were made in the Paisley and Hostrup-Pedersen modeling exercise. They assumed: 1) that spinal columns were included in tissues used for SRM at the time of the BSE cases; 2) that BSE infectivity is associated with insoluble protein impurities in tallow; and 3) that accumulation of subthreshold doses of infectivity can accumulate to transmit disease. In reality, spinal columns were not included in SRM tissues until after 1996; infectivity has never been found in unfiltered tallow containing insoluble impurities; and the relationship between cumulated subthreshold doses and increased transmissibility is based on a hamster model in which transmissibility was strongly influenced by intervals between doses and showed a 'trend' rather than true statistical significance (see also Gravenor *et al*, 2003 and Kimberlin, 1996).

Nevertheless, Paisley and Hostrup-Pedersen (2004) recommended that all milk replacer ingredients be pressure-cooked, including tallow derived from discrete adipose tissue containing no more than 0.02% of insoluble impurities. These measures seem extreme. First of all, fat in supermarket meat is not treated in any way, though it is usually cooked before consumption. Secondly, the SSC has not recommended pressure-cooking meat or fat derived from discrete adipose tissue (which has the same negligible BSE risk as meat) before or after sale to the consumer. Thus, it seems unreasonable to expect measures of risk reduction for calf milk replacers where none are in place for humans. Currently, none of the control agencies in the world recommend including discrete adipose tissue fat in SRM.

4.1.5.2 Experimental studies

Studies specifically examining fat and tallow for infectivity have shown negative results. In 1994, infectivity in body fat from a confirmed field case of BSE was undetectable by mouse bioassay (Fraser and Foster, 1994). Discrete adipose tissue of the midrum fat (mesenteric fat) was used in the study, with the mesenteric lymph node tissue removed. But as a precaution, and following an Opinion from the EC Scientific Steering Committee, mesenteric fat was nevertheless designated as SRM because of the risk that small amounts of infectivity might be present in the splanchnic and other autonomous nerves that connect the intestine to the CNS, through which infectivity in the gut might be transported. These nerves run within the mesenteric fat. But this requirement may be unnecessary: the 1994 bioassay showed no detectable infectivity even though no attempt was made to remove nerve tissue from the sample.

The UK and the Netherlands have since undertaken comprehensive studies of the effect on TSE agents of rendering processes representative of those used in Europe (Taylor, Woodgate and Atkinson 1995; Taylor *et al*, 1997; Schreuder *et al*, 1998). The two studies by Taylor and colleagues revealed that tallow derived from starting materials spiked with either BSE or scrapie-infected brain contains no detectable infectivity before or after filtration, even when the rendering process fails to inactivate TSE infectivity in MBM. All the studies (see below) came to the same conclusions about residual infectivity in MBM and clearance factors. It is to be noted, however, that the studies had a few weaknesses. For example, the starting titre in the BSE brain material used in the UK studies was relatively low; and mice were used in the bioassays, introducing a species barrier effect and consequent loss of test sensitivity.

4.1.5.3 The EURA Survey (1988)

In 1988, a EURA survey was conducted of the rendering processes used in the UK (Wilesmith *et al*, 1991) and, later, of those used in other EU countries (except Spain, Portugal and Greece) (Krenk, 1991; Taylor *et al*, 1995). These studies noted that rendering equipment and processes had changed over the previous 20 years and attempted to devise experiments to determine the effectiveness of these processes in the face of the new challenge of the BSE agent in starting materials. Their effectiveness for reducing scrapie infectivity was also examined.

For example, the dry rendering batch process had largely been replaced by advanced continuous systems for four main reasons: the new processes were cheaper to operate (requiring less fuel); the quality of the end products was improved; odour control was more effective; and the process could be automated and computer-controlled (Krenk, 1991). Another improved continuous rendering procedure, pressure-cooking, previously mostly a batch process, was now used in plants in several continental countries, though not in the UK at that time.

The EURA surveys concluded that, despite the relatively limited range of rendering equipment available, there was a rather wide variation in how the equipment was used. For this reason, it was not possible to design an experiment to test the effectiveness of each and every process. Instead, the survey recommended selecting a small number of generic processes for study under experimental conditions using a small-scale model rendering plant.

4.1.6 The EC Rendering Experiments

Subsequent to the EURA Survey, in the early 1990s the EC began a series of studies on rendering in collaboration with the UK Ministry of Agriculture, Fisheries and Food (MAFF); the European Rendering Association (EURA); the rendering industry (Prosper de Mulder); and the Neuropathogenesis Unit (NPU) of the then Agricultural and Food Research Council (AFRC). The goal of these experiments was to determine the clearance factors achievable in MBM and tallow. The starting material used in the processing was spiked with infected brain material from cattle with natural BSE, and subsequently from sheep with natural scrapie. The processes tested were representative of actual rendering processes and employed protocols that mimicked the average and minimal conditions of each rendering process.

In the first experiment, a BSE spike was used (Taylor, Woodgate and Atkinson, 1995). In the second experiment, a scrapie spike was used (Taylor *et al*, 1997). The parameters and results of these experiments are outlined in simplified form in Table 4.2 and Table 4.3. The experiments were intended to imitate rendering practices used in the EU at the time and to determine the clearance factors resulting from processing of spiked starting material.

4.1.6.1 Methodology of the rendering experiments

The general principles were the same in both the BSE and scrapie experiments. The BSE spike consisted of 861 cattle brains from BSE-suspect cattle, in which BSE was confirmed in 85%. The brainstem had been removed for statutory diagnostic purposes.

The scrapie spike consisted of 2 687 brains from sheep with clinical signs of scrapie. In each case, homogeneity of the spike was achieved by randomizing the brains and

mincing them to produce six equal aliquots. These were then remixed and again divided into six lots for the different studies. Samples from a 10% homogenate in antibiotic solution from each aliquot were taken for bioassay in weanling RIII/FaDk-ro mice (RIII mice) by the combined i/c (0.02 ml) and i/p (0.5 ml) routes. Ten-fold dilutions were also made of some inocula for infectivity titrations by mouse inoculation. The tissue composition of the starting materials (brain spike/intestine/bone in a ratio of 1:3:6) was typical of that used in the rendering industry. The particle sizes were usually in the range of 10 to 50 mm. Cooking of the starting material mimicked a full-scale rendering plant in all material respects, despite the 1:20 pilot scale of the equipment. For each protocol, both MBM and free-running tallow were collected for storage and bioassay.

In the BSE experiment, the low-temperature 72 ºC protocol (Protocol M), which was supposed to produce an infected starting material, revealed no infectivity by bioassay, so the planned additional studies could not be completed. However, in the scrapie study, the same protocol produced the required TSE-infected greaves, thus enabling the planned studies to be completed.

In addition to standard pressure-cooking (Protocol Q), two further hyperbaric processes were included (Protocols R and S), even though these processes were never used in industry. They were included because laboratory studies had suggested they were likely to achieve a high degree of inactivation. In the scrapie study, these two hyperbaric processes were also used to process infected MBM prepared by Protocol M (Protocols T and U). If these processes were able to inactivate the TSE agent, then the study would be able to recommend that a pressure-cooking stage be added to the end of existing rendering processes (a technically simple task). This additional step would then inactivate any residual infectivity not inactivated by the standard process.

Tallow bioassay comprised only a small part of the overall experiment; but the results are important for questions about the use of tallow for biodiesel production. The tallow collected in the experiments was only bioassayed from two protocols (Protocols I and S), since these were representative of high- and low-temperature rendering at the two extremes of the rendering spectrum. Samples of tallow (10% homogenate in saline) were then inoculated into mice both before and after vacuum filtration through diatomaceous earth (Kennite 1000; Diacel), in imitation of industrial practice. A total of 144 mice were used in each bioassay. After inoculation, all mice were observed for clinical signs of TSE for up to 904 days. The shortest incubation time recorded following challenge of RIII mice was 317 days, so brains from all mice surviving over 300 days were examined by light microscopy for evidence of TSE.

4.1.6.2 Results: the BSE experiment

The experimental study is reported by Taylor *et al* (1995). The protocols used for each of the rendering processes—batch atmospheric and batch pressure (natural fat); continuous atmospheric (high fat); continuous vacuum (high fat); and continuous wet rendering (natural fat)—are listed in Table 4.2. Other than for the batch atmospheric process, more than one set of protocols was used.

Table 4.2 summarises the results. Protocols in **bold** show residual infectivity in MBM. Tallow from Protocols I and S showed no detectable infectivity. Tallow was not bioassayed using the other protocols. Protocol M was designed to produce greaves for Protocols N, O and P and MBM for Protocols T and U.

Table 4.2 Experimental Rendering Protocols and Results Using Raw Materials Spiked with BSE Agent

(from Taylor, Woodgate and Atkinson, 1995)

PROTOCOL	PROCESS	PARTICLE SIZE (mm)	FAT CONTENT	TIME (min)	END TEMP ºC	BIOASSAY MBM	BIOASSAY TALLOW^
B	Batch atmospheric	150	Natural (N)	150	121	NDI	Not tested
CE	**Continuous atmospheric**	**30/30**	**N**	**50/50**	**112/122**	**Positive**	Not tested
DF	Continuous atmospheric	30/30	N	125/125	123/139	NDI	Not tested
GH	Continuous atmospheric	30/30	High (H) 1:1**	30/120	136/137	NDI	Not tested
I	**Continuous vacuum**	**10**	**H 4:1****	**20**	**120**	**Positive**	**NDI**
J	**Continuous vacuum**	**10**	**H 4:1****	**57**	**121**	**Positive**	Not tested
KL	Continuous wet, low temp	20/20	N	120/240	101/119	NDI	Not tested
M	Continuous wet, low temp	20	N	240	72	NDI	Not tested
QRS	Batch pressure (raw)	50/30/30	N	30*/28*/28*	133/135/145	NDI	NDI (S)

MBM = meat and bone meal
NDI = no detectable infectivity
^ = filtered and unfiltered tallow
* = includes 10 minutes for heat penetration
** = Ratio of fat to raw material

Note: Some protocols (listed in column 1) were multiple and slightly different variables of particle size, time and end temperature are reported. These should be read respectively, *e.g.* protocol Q used a particle size of 50mm, the time was 30 min and the end temperature was 133 ºC; the parameters for R and S were particle size 30 mm, time 28 min for both and end temperatures were 135 ºC and 145 ºC respectively.

861 BSE-suspect cow brains (10%) were mixed with intestines and beef bones (90%) in ratios of 1:3:6 or, for protocols K, L, and M, 1:5:4. The titre of the spike of brain material was $10^{2.7}$ mouse ic/ip ID_{50}/g. The titre of the spiked starting material calculated to be $10^{1.7}$ mouse ic/ip ID_{50}/g,
Protocols in **bold** showed **infectivity** in MBM. Tallow from Protocols I and S showed no detectable infectivity. Tallow was not bioassayed in the other protocols.
Protocol M was designed to produce greaves for protocols N, O and P but contained no infectivity – results not reported.

4.1.6.2.1 BSE infectivity in the spiked input material

The infectivity titre of the infected brain pool macerate was $10^{2.7}$ mouse ic/ip ID_{50}/g. The infectivity titre of the spiked starting material was calculated to be $10^{1.7}$ mouse ic/ip ID_{50}/g, since the brain pool comprised 10% by weight of the total mass of the starting material. If no inactivation occurred, the titre in the solid end product would be 101.7 mouse ic/ip ID_{50}/0.3g. (because the weight of the solid end product was 30% of that of the starting material). The bioassays were calculated to detect just under a 100-fold reduction in titre.

4.1.6.2.2 BSE infectivity in MBM

The study showed that MBM produced by protocols C, E, I and J were still infected, and that MBM from protocol J had a titre of $10^{1.6}$ mouse ic/ip ID_{50}/ml of 30% MBM, closely similar to that of the starting material. Titrations of material from protocols C, E and I were not attempted. No infectivity was detected in MBM using any of the other protocols.

4.1.6.2.3 BSE infectivity in tallow

Tallow was only bioassayed from two processes, I and S. No infectivity was found in either the filtered or unfiltered samples from Process I (in which infectivity was present in MBM at nearly the same titre as in starting material) or Process S (pressure-cooking at 145º, 4 bar, for 28 minutes holding time, including 10 minutes to permit heat penetration to the 30 mm particles).

4.1.6.3 Results: the Scrapie experiment

The experiment is reported by Taylor *et al*, (1997). Table 4.3 shows the rendering processes and the associated protocols used—batch atmospheric and batch pressure, with starting material (natural fat) and meal (natural fat); continuous atmospheric (high fat); continuous vacuum (high fat); and continuous wet rendering (natural fat). Table 4.3 summarises the results. Protocols in **bold** showed no detectable infectivity in MBM.

Table 4.3 Experimental Rendering Protocols and Results Using Raw Materials Spiked with Scrapie Agent

(From Taylor *et al*, 1997)

PROTOCOL	PROCESS	PARTICLE SIZE (mm)	FAT CONTENT	TIME (min)	END TEMP (º C)	BIOASSAY MBM	BIOASSAY TALLOW^
B	Batch atmospheric	150	Natural (N)	150	114	Positive	Not tested
CE	Continuous atmospheric	30/30	N	50/50	102/100	Positive	Not tested
DF	Continuous atmospheric	30/30	N	125/125	121/138	Positive	Not tested
GH	Continuous atmospheric	30/30	High (H) 1:1*	30/120	134/138	Positive	Not tested
I	Continuous vacuum	10	H 4:1*	27	117	Positive	**NDI**
J	Continuous vacuum	10	H 4:1*	61	126	Positive	Not tested
KL	Continuous wet, low temp	20/20	N	120/240	103/120	Positive	Not tested
M	Continuous wet, low temp	20	N	240	72	Positive	Not tested
N	Solvent extraction (greaves)	20	Greaves from M	10	80	Positive	Not tested
O	Steam treatment (greaves)	20	Greaves from N	20	100	Positive	Not tested
P	Steam treatment (greaves)	20	Greaves from O	40	100	Positive	Not tested
QRS	**Batch pressure (BP)**	**50/30/30**	**N**	**30/18/18**	**134/136/145**	**NDI**	**NDI (S)**
TU	**BP (MBM from M)**	**2.2/2.2**	**MBM from M <10%**	**20/20**	**136/145**	**NDI**	Not tested

MBM = meat and bone meal NDI = no detectable infectivity * = Ratio of fat to raw material

^ = filtered and unfiltered tallow

Note: Some protocols (listed in column 1) were multiple and slightly different variables of particle size, time and end temperature are reported. These should be read respectively *e.g.,* protocol Q used a particle size of 50mm, the time was 30 min and the end temperature was 134 ºC; the parameters for R and S were particle size 30 mm, time 18 min for both and end temperatures were 136 ºC and 145 ºC respectively.

2 687 scrapie suspect sheep brains (10%) were mixed with porcine intestines and bones (90%) in ratios of 1:3:6 or, for protocols K, L, and M, 1:5:4. Titre of the spike of brain material was $\geq 10^{4.1}$ mouse ic/ip ID_{50}/g. The infectivity titre of the spiked starting material was calculated to be $10^{3.1}$mouse ic/ip ID_{50}/g,

Protocols in **bold** showed **no infectivity** in MBM. Tallow from Protocols I and S showed no detectable infectivity. Tallow was not bioassayed in the other protocols. Protocol M was designed to produce greaves for protocols N, O and P and MBM for protocols T and U.

4.1.6.3.1 Scrapie infectivity in the spiked input material

The infectivity titre in the scrapie brain pool macerate was $\geq 10^{4.1}$ mouse ic/ip ID_{50}/g, which was 1.4 logs higher than that in the BSE brain pool. The infectivity titre of the spiked starting material was calculated to be $\geq 10^{3.1}$ mouse ic/ip ID_{50}/g, since the brain pool formed 10% of the total mass of the starting material. If no inactivation occurred, the titre in the solid end product would be $\geq 10^{3.1}$ mouse ic/ip ID_{50}/0.3g (because the weight of the solid end product was 30% of that of the starting material).

4.1.6.3.2 Scrapie infectivity in MBM

Scrapie infectivity was found in the MBM after all protocols from A to P inclusive but not in MBM from starting material subjected to pressure-cooking (Protocols Q to U inclusive). In contrast to Protocol J in the BSE study (in which there was virtually no reduction in the infectivity in the MBM), in the scrapie study there was a clearance of about 1.5 logs of infectivity, suggesting that the thermostability of the BSE agent might exceed that of the scrapie infectivity in the brain pool. However, the study uses a large number of sheep brains, presumably containing several strain types of agents; moreover, the strain type of agents present is unknown, so a cautious interpretation should be made.

4.1.6.3.3 Scrapie infectivity in tallow

No infectivity was found in unfiltered or filtered tallow samples from Protocol I, in which infectivity was detected in the MBM, and all inoculated mice went on to develop scrapie; nor from Protocol S (pressure-cooking), where there was no detectable infectivity in the MBM. These negative results for tallow, coupled with the same results in the BSE study, suggest that infectivity in starting materials that is not inactivated does not partition with the tallow fraction but rather with the MBM fraction. The absence of detectable infectivity before filtration, evident in both studies, suggests either that the residual solids contain no infectivity or that the titre present was below the sensitivity of detection in the mouse bioassay. The findings are consistent with the view of Wilesmith *et al*, (1988) that tallow is not a causal factor in the occurrence of BSE.

Even though the scrapie and BSE studies taken together suggest that pressure-cooking is the most effective of the rendering processes for inactivating TSE agents in starting material, it cannot be concluded that pressure-cooking is completely effective under all conditions, especially if high levels of infectivity were in the starting material (Schreuder *et al*, 1998). Furthermore, results obtained by laboratory autoclaving TSE-infected cattle brain tissue with a high infectivity titre ($10^{5.2}$ mouse, ic/ip, ID_{50}/g) at 135ºC for 18 minutes showed that approximately 2 logs of infectivity remained after treatment (Taylor *et al*, 1994).

4.1.6.3.4 Protocols M and N: the effect of hydrocarbon solvent extraction

Protocol M was not used in commercial rendering. It was included in both the BSE and scrapie experiments in order to produce greaves which could then be treated with hydrocarbon solvent and steam to study the effects of these protocols on infectivity. But these complementary studies could not be completed in the BSE experiment because the greaves produced by Protocol M did not contain any detectable infectivity.

In the scrapie experiment, the infectivity in the greaves produced by Protocol M was $10^{0.3}$ mouse ic/ip ID_{50}/g. Treatment of the greaves from Protocol M with the hydrocarbon solvent heptane at 80 ºC for 10 minutes (Protocol N), and then with steam at 100 ºC either for 20 minutes (Protocol O) or 40 minutes (Protocol P) did not reduce the infectivity further in any experiment. These results suggest that hydrocarbon solvent extraction of tallow from MBM is not effective in preventing infectious oral doses of any TSE agent surviving into feed containing MBM or tallow, as previously believed (Wilesmith *et al*, 1988). Infectivity was still present in greaves after solvent extraction (Protocol N) and in the MBM derived from it after treatment with steam at 100 ºC for 20 minutes (Protocol O) or 40 minutes (Protocol P). These results suggest that the withdrawal of these practices was not instrumental in initiating the epidemic (Taylor *et al*, 1997). Further studies on solvent extraction of tallow from greaves using a spike with the murine strain of BSE 301V are in progress (D. Matthews, personal communication).

Furthermore, Taylor *et al* (1998) reported laboratory studies on the effect of hot hexane, heptane, petroleum spirit, perchlorethylene (hydrocarbon lipid solvents used by part of the rendering industry), steam, and saline (as a control) on the inactivation of murine BSE strain 301V and the 22A strain of scrapie agent in mouse spleens. The average degree of inactivation was less than 1 log of infectivity. The authors concluded that the solvent extraction process had little capacity to inactivate these strains of TSE agent.

4.1.7 Other Rendering Experiments

Independent studies in the Netherlands by Schreuder *et al* (1998) have shown that rendering conditions which include pressure-cooking (133 ºC, 3 bar, 20 minutes) are not always effective at completely inactivating BSE infectivity. However, BSE infectivity was reduced by pressure-cooking using the above parameters by approximately 150 fold for diluted material and by about 1 000 fold for undiluted material. The main difference between these studies and those of Taylor *et al* (1995, 1997) was the use of higher-titre brain stem tissue. This study concluded that the BSE agent consistently appeared to be more resistant to heat inactivation processes than the scrapie agent, particularly at the lower temperatures and shorter times that were also investigated. Tallow was not bioassayed.

4.1.8 Other Inactivation Studies

4.1.8.1 Autoclaving

A study (Taylor *et al*, 1994) was undertaken to investigate the effect of recommended autoclaving parameters (134 ºC to 138 ºC for one cycle of 18 minutes, or six sequential cycles of 3 minutes) used to decontaminate surgical instruments possibly exposed to CJD agent in UK hospitals (DHSS, 1984). This study used mouse-adapted strains of scrapie and BSE agents, which can be grown to a high titre. Following exposure to 134ºC, 136 ºC or 138 ºC for times ranging between 9 and 60 minutes, samples were inoculated by the i/c route into healthy mice. Because the strains were already adapted to mice, the absence of a species barrier maximised the sensitivity of the system to detect small amounts of residual infectivity. The data from these experiments (Taylor, 1994, 2000) showed that the BSE 301V strain can survive exposure to 138 °C for an hour.

Studies of different TSE strains conducted at high temperatures (115 to 134 °C) under ultra-high pressure conditions (100 000 to 170 000 psi) also showed that BSE was the most resistant of all tested strains, including 263K strain hamster-adapted scrapie (Brown *et al*, unpublished data).

Other data have shown that infectivity that survives one round of autoclaving is much more difficult to inactivate during a second round (Taylor *et al*, 1998b). The above data collectively demonstrate that high titres of BSE infectivity can survive vigorous pressure-cooking and lend further support to the notion that the BSE agent is more thermostable than murine scrapie agent strains. The data also support the conclusion that the murine BSE strain 301V is the most thermostable TSE agent known (Taylor, 2000). Thus, it can be claimed with some justification that there are no known autoclaving protocols that can reliably guarantee sterilisation of the most heat-resistant strains of TSE, perhaps because of their strong tendency to 'fix' to plastic and metal surfaces, such as those of surgical instruments (Zobeley, *et al*, 1999; Weissmann *et al*, 2002). However, no instrument cross-contamination cases of CJD have been identified anywhere in the world during the past 30 years, including the EC, where systematic CJD surveillance has been ongoing since the mid-1990s. The appearance of vCJD, however, has led to more research in this area.

4.1.8.2 Hydrolysis

Hydrolysis of tallow to produce tallow derivatives using highly rigorous processes of extraction and purification (WHO, 1997) is likely to be effective for the inactivation of TSE agents. The UK Medicines Control Agency (MCA Position Paper on Tallow and Tallow Derivatives for Use in Pharmaceutical Products, 1996) specifies using water at temperatures ≥ 250 ºC and pressure of 50 bar for 3 hours though other similar severe processes are also used and approved.

4.1.8.3 Phosphoric acid precipitation

An interesting but untested possibility for reducing infectivity in tallow is precipitation of protein with phosphoric acid (SSC, 2001b). This treatment may permit further removal by filtration or centrifugation of the precipitate. Phosphoric acid treatment of tallow has been reported to leave a residual nitrogen content of 0.01% (SSC, 2001b). Thus, a phosphoric acid treatment may be useful preceding a second diatomaceous earth filtration step. However, no information is available on the effectiveness of this approach.

4.1.9 Criticisms and Responses

1. *The experiments used mice to assay the infectivity. This could underestimate the real infectivity levels in tallow due to the inefficiency of the assay generated by the species barrier effect.*

Response: Based on the results of a comparative bioassay of the same brain material from confirmed cases of BSE in mice and cattle, the underestimate is in the range of 500 times (Wells, 2001). However, the transmissions used the efficient combined i/c-i/p routes, rather than the inefficient oral route, which would be most likely route of exposure in practice. In mice, the efficiency difference between the i/c route and the oral route is 10^5 times (Kimberlin, 1994, 1996; Kimberlin and Walker, 1983, 1988, 1989a,b). Preliminary data from cattle on the relative efficiency of i/c versus oral

routes of infection yield a similar difference. Thus, the criticism of using mice rather than cattle for the bioassay is counterbalanced by the fact that any infectivity in the tallow fraction (or biodiesel produced from it) would only reasonably result in exposure by the relatively much less efficient oral route. Note that exposure in this way from biodiesel is unlikely.

Furthermore, cattle-based studies have confirmed the results of this mouse-based study. In one such study, a selection of tissues from an experimental challenge of cattle by the oral route (pathogenesis study), already bioassayed in mice, was inoculated into cattle by the i/c route with the same result to date as the result obtained using mice. There are two exceptions. Tonsil tissue (which is SRM), 10 months after challenge, showed detectable infectivity in the cattle bioassay but not in mice; however, there was a low attack rate and a long incubation period in the cattle study, suggesting a low titre of infectivity (Wells *et al*, 2005). Sternal bone marrow from cattle at one point in the clinical stage of disease has failed (so far) to be detected by the cattle assay; but infectivity in the sternal bone marrow was detected by the mouse assay (Wells *et al*, 1999). Overall, these results indicate that despite the difference in efficiency induced by the species barrier effect, mice are excellent and accurate predictors of BSE infectivity in cattle tissues. Therefore, the conclusions of the report on tallow safety are defendable and valid.

2. *The percentage of insoluble solids in unfiltered tallow can be as high as 0.5%. Moreover, these solids could include protein content from the starting materials, the infectivity of which has not been determined.*

Response: The EC does not permit insoluble content levels in usable tallow of more than 0.15%, the level which must be achieved for international trading. In addition, these impurities must be protein-free (OIE, 2004). Similar criteria are specified for the production of biodiesel from tallow prepared from Category 1 starting material (EC, 2005). In practice in the EU, most tallow for human food or feed use has ≤ 0.02% insolubles; but these extremely low levels are likely to be achieved most readily as a result of using high-quality starting materials.

The protein content of the insoluble fraction of tallow has been estimated as high as 85% (SSC, 2001b). However, since the protein content of MBM is only about 50%, and MBM is the most likely source of the insoluble fraction, this 85% figure seems unrealistically high. Industry experts suggest that the protein content of the insoluble fraction ranges between 5% and 16%, rather than 85% (SSC, 2001b), based on analysis of a limited number of samples. These lower estimates were produced by measuring nitrogen levels and, as such, they could be over-estimating the percentage of protein because of the presence of non-protein nitrogen. In a worst-case scenario, using the 85% estimate figure, filtered tallow containing 0.15% of insoluble impurities would contain 0.128% protein. Yet industry-based figures for impurity content are much lower than 0.128%: Piva (quoted as Piva 1997 unpublished, in SSC, 2001b) analysed three samples of rendered tallow for animal feed and measured the total nitrogen content as 0.01% to 0.02%, a figure which corresponds to an impurity level of 0.15% insolubles. The SSC (2001b) quoted industry-derived figures in a range of from 0.001% to 0.004%, a figure which corresponds to an impurity level of 0.13 to 0.27% (see Table 4.1).

In addition, filtration helps reduce any risks present in tallow precipitate or sediment. But if filtration practices were improved to yield a maximum insoluble solid content of

0.02%, then a further clearance of just under ten times could be achieved (see Table 4.4). Because improved filtering technology is already used for food- and feed-grade tallow in Europe, it could be applied to lower grades of tallow as well. Food- and feed-grade tallow is usually prepared from high-quality starting materials. After pressing the greaves, then centrifuging or decanting it to extract the crude fat, and finally filtering the fat under vacuum through diatomaceous earth, the resulting tallow has an insoluble fraction of about 0.02%. The equivalent process with SRM-type, low-quality-fat starting materials would yield a total insoluble content of closer to 0.15%. Thus, to reduce the total insoluble content in this material to 0.02%, the industry would need to add one or more robust filtration stages to the process.

Table 4.4 Infectivity Clearances

Achieved by Various Physical Processes and Derived from Various SSC Opinions

Production Process	**Infectivity Clearance Factor**
Saturated steam (Pressure-cooking) @ 133 ºC, 3 bar, 20 min	≥ 10^3
Tallow post-sterilisation	> 1 and probably ≥ 10^3
Tallow + filtration to ≤ 0.15% insolubles	$10^{2.8}$
Tallow + filtration to ≤ 0.02 % insolubles	$10^{3.7}$
Tallow derivatives	Total safety assumed under specified conditions
*Hydrolysis using 1M HCl for 1 hour @ ≥65 ºC	Almost complete inactivation
*Hydrolysis using 6M HCl for 6 hours @100 ºC	Almost complete inactivation
**Hydrolysis @ ≥140 ºC, ≥3.6 bar, ≥30 min	≥ 10^3
***Hydrolysis @ ≥ pH 11, ≥80 ºC, ≥3 hours	Further reduces risk

* Hair and skin ** Proteins *** Hydrolysed proteins
Data courtesy of P. Vossen

More studies on the chemical analysis of the insolubles in tallow are required to determine precisely the amount and nature of any residual protein (*e.g.*, if they are aggregated and sediment on standing in molten tallow). To date, there is no evidence that TSE infectivity exists even in unfiltered tallow from a range of rendering processes. If protein were present in the insoluble fraction, further filtration to remove more insolubles would likely reduce protein content and thus improve the expectation that tallow even from SRM would be a safe starting material to make biodiesel.

3. *Appel et al (2001) suggested that, under lipid-rich conditions, scrapie agent in the form of concentrated extracts of 'prion rods' from hamster scrapie (not cattle BSE)*

might survive autoclaving at temperatures of up to 170 °C for 20 minutes. The more fat present, the less destruction of misfolded protein. It was further suggested that the hydrophobic misfolded protein might migrate into the tallow fraction, rather than remain in the greaves.

Response: In actuality, the proteins would be more likely to aggregate and fall to the bottom of the tallow tank as sediment. Appel *et al* (2001) used purified prion rods (analogous to scrapie associated fibrils) and based their conclusions upon biochemical observations rather than bioassays. Results from this type of study cannot be directly applied to the BSE-related safety of tallow and tallow-derived products, because the aggregated form of the misfolded protein used in these studies represents an artificially high degree of challenge compared with the infected brain-tissue that would be used in more relevant studies.
Furthermore, the studies of Taylor *et al* (1995), which used brain tissue from naturally infected cattle as a spike for rendering SRM, failed to detect any infectivity in tallow. In their study, 12 mice were injected with a total of 6.24 ml of 10% unfiltered tallow. Since no mice became infected, there must have been less than one intra-cerebral infectious dose (1 ic ID) in this amount of 10% tallow (or 0.624 ml of neat tallow). The equivalent oral ID dose would be lower by a factor of about 5 logs (Kimberlin, 1994, 1996; Kimberlin and Walker, 1978, 1983, 1988, 1989a,b). For this reason, it is unlikely that infectivity sufficient to infect cattle by the oral route could migrate to the tallow fraction.

4.1.10 Knowledge Gaps Related to Rendering

1. It will never be known with certainty whether the cessation of hydrocarbon solvent extraction in the UK in 1980 permitted scrapie-like agents to reach levels of infectivity in MBM that may have caused the BSE outbreak. Epidemiological and experimental evidence are in disaccord. Ongoing experiments on the effect of hydrocarbon solvents on the mouse-adapted 301v strain of BSE may help to resolve the controversy.

2. The precise chemical composition of the insoluble impurities in tallow from rendering processes is disputed, and incompletely studied and reported. Because pressure-cooking is not used in North America, TSE infectivity in North American starting materials is unlikely to be completely inactivated in the MBM; and any proteinaceous material present in the tallow as part of the insoluble fraction will potentially not be inactivated either. A legitimate TSE risk assessment of tallow produced by North American rendering methods will require information about the actual chemical composition of the insoluble impurities in tallow made by different processes. A number of institutes have the capability to initiate studies in this area and could perhaps determine how potential risks might be reduced. If analysis showed that no residual protein was detectable and that any polypeptides present had an M_r of $\leq 10\,000$, then infectivity could be confidently assumed to be absent or at such a low level as not to constitute a significant risk (SSC, 2001b).

3. The optimum conditions (time and temperature) to achieve a maximum sedimentation of insolubles from tallow are not known. Research on this issue could be conducted by the rendering industry or appropriate research institute/laboratory.

4. More investigation is needed into methods for separating insoluble impurities from tallow in an efficient and cost-effective way, with additional attention to methods of filtration, centrifugation or both. Research could be conducted by a university department, in collaboration with the rendering industry.

5. Phosphoric acid treatment of tallow could benefit from further studies to determine if any nitrogen is present in the form of protein, polypeptides and amino acids and, if so, in what proportion and molecular weight. A university chemistry department might be a suitable place to conduct such studies. The stage during processing that could achieve the most efficient removal of protein should be determined.

6. The effect of rendering on CWD and TME agents, or on atypical forms of BSE and scrapie, is not known (Casalone *et al*, 2004; Benestad *et al*, 2003). On the basis of broad biological similarities among all TSE agents, it is assumed that they do not behave differently from the tested strains; but formal proof is lacking.

7. The specifics of rendering tallow and reducing the insoluble fraction in tallow merit further study. These topics are still largely under-examined. If misfolded protein is indeed present in the insolubles fraction, then further studies may be needed to determine the most effective removal method. These specifics still need investigation:

- The rate of sedimentation of insolubles in liquid tallow during storage under various conditions (*e.g.*, temperature);
- The benefit of using protein precipitants before sedimentation or filtration and at what stage they should be applied;
- The most effective single or combination methods of removal;
- The achievable clearance after a second diatomaceous earth filtration;
- The chemical composition of the insoluble fraction by nitrogen, protein, polypeptide and amino acid content;
- The possibility that misfolded protein could reside within the fat component of tallow (such as in a lipoprotein), as distinct from the insoluble fraction;
- Quick, accurate and inexpensive methods to determine the protein content and possible misfolded protein content of tallow for use in a quality control system using the Hazard Analysis and Critical Control Point (HACCP) principles; and
- The possibility, value, cost and effectiveness of using high pressure and temperature to inactivate any TSE infectivity in tallow.

4.1.11 Conclusions: Rendering

Although the ensemble of studies summarised in this chapter show that pressure-cooking is an effective way to inactivate TSE agents in contaminated tissues, it cannot be concluded that it is fully effective under all conditions. In the absence of complete inactivation, it is obviously important to know where any remaining infectivity is concentrated. The negative results for tallow in both the scrapie and BSE studies suggest that residual infectivity in starting materials does not partition with the tallow fraction, but rather with the MBM fraction. The absence of detectable infectivity could

mean either that the residual solids in the tallow contain no infectivity, or that the titre present was below the sensitivity of detection in the mouse bioassay.

Research on TSE inactivation associated with a variety of rendering process conditions leads to the following conclusions:

1. *The BSE agent is the most thermally-resistant TSE agent known*. Thus the outcome from rendering BSE-infected material, using methods other than pressure-cooking, can be assumed to be a worst-case scenario.
2. *Pressure-cooking reduces TSE infectivity*. Infectivity was not detectable in either the MBM or the tallow from experiments involving pressure-cooking.
3. *Rendered tallow is TSE-free*. Rendering separates the cooked constituents into three fractions: water, greaves and tallow. The efficiency of the separation of greaves from tallow is high. In the experiments that demonstrated virtually no reduction in titre of the MBM compared to starting material, infectivity was not detectable in the tallow, whether filtered or not.
4. *All rendering processes produce TSE-free tallow.* The two EC rendering experiments tested methods at either end of the rendering process spectrum used historically in the EU. The process of separation/collection of the tallow was the same in all experiments; therefore, it can be assumed that tallow produced by the processes between these two extremes would not have had any detectable infectivity. The rendering processes used in the study are likely to be representative of the range of rendering processes used throughout the world.

Thus, according to the results of the experiments of Taylor *et al* (1995, 1997), tallow can be regarded as safe feedstock for biodiesel production even if the starting material is designated SRM.

Since 2001, several agencies in Europe have revisited the issue of tallow safety, its starting materials, its processing conditions, and its uses. The emphasis has been placed upon tallow intended for consumption in human food or animal feed, which can now only be prepared from Category 3 material. A recent application to use Category 1 tallow for biodiesel manufacture was initially rejected by the European Food Safety Authority (EFSA). However, since then EFSA has received additional data from the industry (Seidel *et al*, 2006) and has conditionally authorised biodiesel production from Category 1 tallow prepared by pressure-cooking (EFSA, 2004). This change is endorsed by recent EC legislation (EC, 2005).

4.2 Producing Biodiesel from Animal Fats

If the tallow used as starting material for making biodiesel is not infectious, then consideration of infectivity reductions in the biodiesel manufacturing process becomes irrelevant. However, in spite of all of the evidence favouring the absence of infectivity in tallow, it is still not possible to state that such infectivity could never be present. Therefore, a discussion of the capacity for the manufacturing process to inactivate TSE agents in biodiesel and any of its by-products or waste products is appropriate.

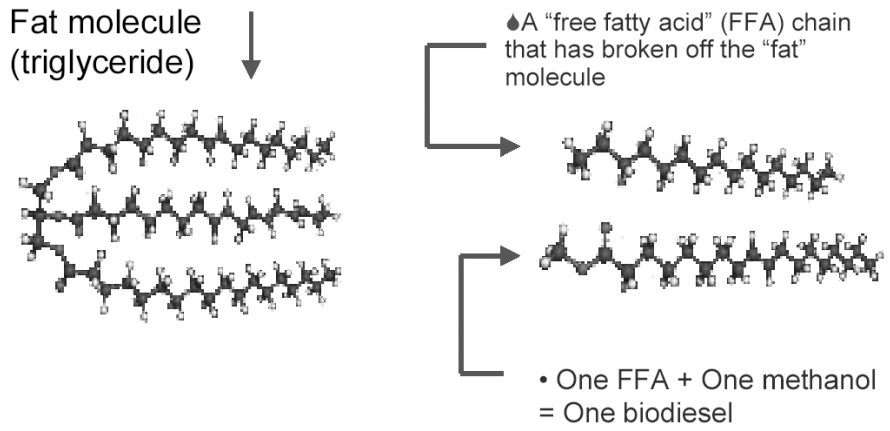
4.2.1 Feedstocks in Biodiesel Production

Over 90% of biodiesel produced in Europe and in North America uses vegetable oil (rape or soy) as the feedstock. For this reason, most biodiesel procedures have been

developed specifically for plant oils. Europe and North America also produce some biodiesel using tallow and/or yellow grease as the feedstock (about 10% of total biodiesel). The advantage of these animal fat feedstocks is that they cost less than vegetable oils; the disadvantage is that they have a *higher free fatty acid* (FFA) content, which complicates the esterification process.

All fats and oils, whether animal or plant, are composed of *triglyceride molecules*. Each triglyceride is composed of three long-chain fatty acids of 8 to 22 carbons attached to a glycerol backbone. A free fatty acid (FFA) is a fatty acid chain which has broken away from the triglyceride through hydrolysis (see Figure 4.3). Hydrolysis can be created by steam from cooking, salts, some chemicals, or heat. The rendering process involves heat and steam, and so increases the FFA content of the tallow. When feedstocks with high FFA levels are esterified in a base-catalysed system, the FFA tend to form soaps; thus, special processing methods have been developed for high FFA feedstocks.

Figure 4.3 Fat Molecule and Free Fatty Acid



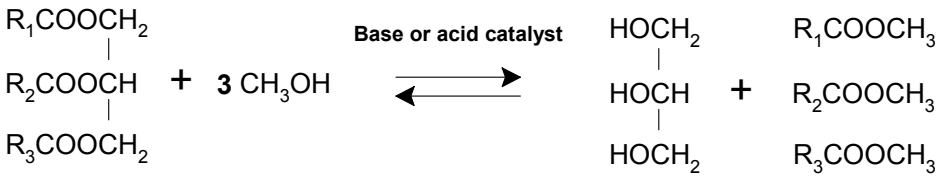
4.2.2 Technologies for Biodiesel Production

The production of biodiesel is an old and well-known process. The first patents for the process were issued in the 1940s to researchers from DuPont (Van Gerpen *et al*, 2004). The basic chemical reaction is depicted in Figure 4.4. One hundred kilograms of a fat or oil is reacted with an alcohol (10 kg of alcohol when methanol is used) in the presence of a catalyst to produce 10 kg of glycerine (a soap by-product) and 100 kg of methyl esters (biodiesel). The methanol is charged in excess to increase the rate of conversion, and is recovered for reuse. The catalyst is usually sodium hydroxide (NaOH) or potassium hydroxide (KOH), which has already been mixed with the methanol. R_1, R_2, and R_3 indicate the alkyl molecules associated with an individual fat.

Figure 4.4 The Transesterification Reaction

The reaction occurs between triglyceride and alcohol molecules

(Rn symbolizes different alkyl chains)



Several conditions hinder transesterification, including the poor solubility of alcohol in oil, the presence of free fatty acids, and the presence of water. To overcome these conditions, process developers use variations of the basic chemical process. Some of these variations are already commercially available and in use. Other variant methods that may offer improved economics are under development.

The chemicals used in transesterification are later removed from the final product. Most biodiesel production uses methanol to produce *methyl esters* (a biodiesel); but other alcohols can be used, such as ethanol, which produces *ethyl esters*. Catalysts are typically strong acids or bases, such as sodium hydroxide or sulphuric acid. In order to reduce production costs, process developers are continually finding new ways to speed up the reactions, use lower temperatures, and reduce the amounts of acid or base catalysts required.

Currently, there are three well-established methodologies that can be used to produce biodiesel from different sources of lipid feedstocks:

- The base-catalysed process;
- The acid-catalysed process;
- The acid-base integrated process: acid-catalysed pre-esterification and base-catalysed transesterification.

Although all three methods can convert triglycerides and FFA into esters, only the base-catalysed and the acid-base integrated processes are commercially practiced.

4.2.2.1 The basic biodiesel production process

4.2.2.1.1 Chemical ingredients

The alcohol most commonly used in the biodiesel production process is methanol. Producing biodiesel with ethanol is not a common practice because 44% more ethanol by weight is required than methanol to complete the reaction, and because ethanol is generally more expensive and difficult to process than methanol.

The catalyst is usually KOH or NaOH, with different process developers having different preferences. NaOH is used by most biodiesel producers; but some process developers that have processes capable of processing feedstocks with high FFA content, such as tallow, prefer KOH. Approximately 50% more KOH than NaOH, by weight, is required for the reaction. Moreover, KOH is usually more expensive than

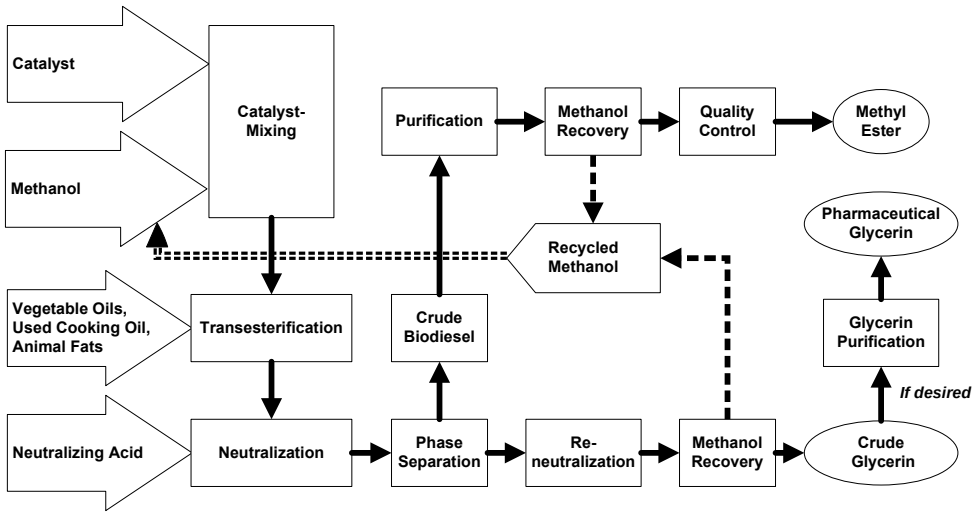
NaOH. But it has one advantage: it is more soluble in methanol. Sodium methoxide is also sometimes used as a catalyst.

Both the oil and the methanol must be extremely dry for biodiesel production, because even 0.5% water can halt the reactions. For the base-catalysed processes, the FFA level must also be very low. The final separation of the glycerine is more difficult with ethyl esters than with methyl esters.

4.2.2.1.2 Steps in the biodiesel process

The general biodiesel production process, as described by the National Biodiesel Board (2002), is shown in Figure 4.5.

Figure 4.5 Biodiesel Production Process



1. Mixing of methanol and catalyst. NaOH or KOH is dissolved in the methanol (or ethanol) by simple mixing. Care must be exercised to ensure that the NaOH (typically pellets or flakes) does not absorb too much water in storage, which can cause large clumps that are difficult to dissolve. Water can also pose problems in the later stages of processing.

2. Reaction. The methanol/catalyst mix is charged into a reactor, either continuously or in batches, and the oil or fat is added. Depending on the process supplier, the reaction mix is kept at an elevated temperature of up to 65 °C for between 1 and 8 hours under vigorous agitation. Excess methanol is normally used to ensure total conversion of the fat/oil to esters.

The catalyst will first react with any free fatty acids in the oil to form soap. There must be enough additional catalyst to catalyse the reaction after reacting with the FFA. If the FFA level is too high (above 0.5% to 1%), or if any water is present, the soap formed will begin to form emulsions with the methanol and oil, preventing the reaction from occurring. In some cases, the emulsion can be so strong that it becomes unbreakable and forms a cottage-cheese-looking product. In this case, the product must be physically removed from the system and most likely scrapped. Most tallow will have

FFA levels between 3% and 15%. Therefore, it is necessary to treat the incoming feedstock to remove free fatty acids and to keep all feed streams free of water.

3. Methanol removal. In some systems, excess methanol is removed at this stage via a simple flash process or distillation. In other systems, the methanol is removed after the glycerine and esters have been separated. In either case, the methanol is recovered and reused using conventional equipment. Care must be taken to ensure that no water accumulates in the recovered methanol stream.

4. Separation. Once the reaction is complete and the methanol has been removed, two major products are left: glycerine and methyl esters. Due to the density difference between glycerine (1.0 kg/l) and methyl esters (0.88 kg/l), the two are allowed to gravity separate, and glycerine is simply drawn off the bottom. In some cases, a centrifuge is used to separate the two layers. Any rag layer (the unseparated interface between the two products) is either recycled or sent to sewage treatment.

5. Glycerine neutralisation. The resulting glycerine contains unused catalyst and soaps, which are neutralised with an acid (usually hydrochloric or phosphoric) to form salts and sent to storage as crude glycerine. In some cases (for example, if potassium hydroxide is used as the catalyst rather than sodium hydroxide, and phosphoric acid is used as the quench acid), the salt is recovered for fertiliser. In most cases, however, a caustic soda catalyst and hydrochloric acid are used, creating sodium chloride, which is simply left in the glycerine. The glycerine is typically 80 to 88% pure and ready to be sold as crude glycerine.

6. Methyl ester wash. Once separated from the glycerine, the methyl esters are washed gently with warm water, dried, and sent to storage. In some cases, the esters are distilled under vacuum to achieve even higher purity. The washing step can be greatly affected by the free fatty acid level of the feedstock, since all the free fatty acids form soaps in the reaction. If the soap content in the washing step is too high, a water wash will entrain the esters, and yields will be diminished, sometimes severely. Some processes eliminate this washing step by using very clean feedstock. In these situations, the resulting biodiesel product is typically 98% ester and ready to be sold as fuel.

4.2.2.2 The Base-catalysed process

Most of the biodiesel produced today uses the base-catalysed process, which works well with vegetable oils, the most commonly used feedstock. Base-catalysed production processes typically operate with large excesses of alcohols and with a base catalyst concentration of 0.5 to 2.0% wt (of the oil feedstock) for sodium or potassium hydroxide and 0.5% or less for sodium methoxide. The concentrations of the catalyst in the reactor vessel are lower because of the dilution of the methanol: with a 6 to 1 molar ratio of methanol to oil, the concentration would be approximately 0.3 to 1.4% wt. This is equivalent to 0.09 to 0.36 M concentration of the NaOH catalyst.

Base-catalysed processes can be operated in a batch or continuous mode. Batch systems are usually used in small plants and have two advantages: they do not require continuous supervision, and they have somewhat greater flexibility for fine-tuning the process. Continuous processes are used in larger plants and allow the use of more sophisticated separation systems, such as centrifuges, to improve quality, increase throughput, and lower capital costs.

One advantage of alkaline catalysts is that they result in a relatively fast reaction. The process typically takes 4 to 8 hours to complete at ambient temperature. Increasing the temperature reduces the time needed. But since methanol boils at 65 ℃, pressure vessels are required at elevated temperatures. In general, the base-catalysed reaction can be carried out at moderate temperatures and pressures (60 to 65 °C and 140 to 400 kPa, respectively) with low catalyst concentrations (about 0.5 to 2.0% by weight of the lipid feedstock). High ester yields are obtained with alcohol-to-triglyceride molar ratios of at least 6:1. Typical conditions applied to the base-catalysed process are shown in Table 4.5.

Table 4.5 Typical Reaction Conditions for a Base-Catalysed System

Feedstocks	(1) Lipid feedstocks with low free fatty acid contents (≤ 1 wt%) (2) Methanol
Alcohol to oil molar ratio	At least 6:1
Temperature	60-65 ºC
Time	1 to 4 hours
Pressure	140-400 kPa
Catalyst	NaOH (most common)
Catalyst concentration (weight % of lipid feedstock)	0.5-2 wt%, 0.09 to 0.36 M NaOH
Conversions	≥ 95% can be expected at 1h reaction time

Because the base catalysts used are sensitive to water and free fatty acids, this production method poses problems for animal-based feedstocks. Whatever free fatty acids are present will react with the alkali to form soaps; and whatever water is present will hydrolyse fats to form free fatty acids and eventually soap. Soaps can gel at ambient temperatures causing the biodiesel to form a semi-solid mass. For this reason, the base-catalysed process must be modified for use with animal-based feedstocks.

4.2.2.2.1 Potential effects of the base-catalysed method on TSE infectivity

1. Bases: The base catalyst is solubilised in the methanol, which in turn is not soluble in the oil. The low solubility of the base and alcohol in the oil not only slows the reaction times but also reduces the potential for base reaction with and deactivation of any TSE infectivity in the oil. However, most process developers employ mechanical

systems to increase the mixing and facilitate the catalytic reactions between the oils and methanol, so there may be some possibility of contact between the base and any TSE agents in the oil.

Because 1% NaOH by weight is added to the lipid feedstock in this process, one might conclude that this results in a molarity of 0.25M in the reaction mixture; however, there is also the dilution of the alcohol to consider, so that the average solution molarity is 0.18M. The reaction mixture is not homogeneous but biphasic in nature. Thus, there would be an oil/fat phase and an alcohol phase into which the NaOH (or any other base or even an acid) would partition. There would probably be a point at which the NaOH present in the alcohol phase would to some extent permeate the oil/fat phase, but quantitative information on this subject is lacking. The molarity of the NaOH that would eventually pass into the oil/fat phase is also unknown.

What happens to pH in a biphasic mixture including an oil/fat phase is a key question that Goodwin *et al* have recently considered. It is unlikely that any existing data for the inactivation of TSE agents by NaOH could be applied to this undefined biphasic situation.

2. Alcohol: Alcohol (usually methanol) has no detectable inactivating effect on aqueous suspensions of tissues contaminated with TSE agents. Moreover, it is possible that, like formaldehyde, it may actually protect against inactivation by subsequent exposure to heat by virtue of stabilising the prion protein (Taylor, 1996).

A major problem in evaluating any such protective effect concerns the biphasic nature of the reaction mixture in biodiesel production. Although the constituents of the alcohol phase must eventually permeate the oil/fat phase to some extent, the degree and timing of this permeation is unknown. In the meantime, one has to assume that sufficient alcohol permeates into the oil/fat phase to cause infectivity 'fixing' problems for any TSE agents present.

3. Temperature and pressure: The temperature and pressure levels used in the biodiesel production process would not, in themselves, result to any significant extent in reducing TSE infectivity levels. The process is carried out at 60 to 65 ºC. No inactivation of TSE agents would be achieved by exposure to such temperatures, especially for the very thermostable BSE agent. Even though the temperatures are also accompanied by hyperbaric air-pressures of 135 to 400 kPA, published data show that even steam pressures of up to 30 kPa, with temperatures of 133 ºC or higher, are required to achieve significant reductions in the titre of TSE agents. Even under these conditions, the degree of inactivation can be relatively small, especially when thermal resistance to inactivation has been induced by 'fixation'.

4. Antioxidants: Antioxidants such as t-butyl hydroquinone or tocopherol may be added to biodiesel to prevent oxidative degradation during storage. There is no evidence about the effect that these (or similar) compounds would have on TSE infectivity.

In general, biodiesel plants using the base-catalysis process generally refine the crude oils that they receive for processing before subjecting them to the biodiesel production process. The refining techniques include washing, centrifugation, caustic treatment, filtration and bleaching. All of these procedures could have some potential for reducing or removing TSE infectivity in the raw materials, but there is insufficient technical data for an assessment.

4.2.2.3 The Acid-catalysed process

The acid-catalysed process is similar to the base-catalysed process, except that an acid (usually sulphuric acid) is used in place of the base catalyst. This process is not used for commercial production because it is much slower than the base-catalysed process and requires higher temperatures and pressures. The temperatures need to be 200 to 250 °C, and the pressure up to 1 000 kPa. Moreover, all the materials of construction need to be acid resistant. The overall process of converting both FFA and triglycerides to methyl esters is quite slow—up to 48 hours at 60 °C. However, since the acid-catalysed process is insensitive to the presence of free fatty acids in the feedstock, there is no need to remove or treat the FFAs before the biodiesel manufacturing process can begin.

4.2.2.3.1 Potential effects of the acid-catalysed method on TSE infectivity

Much of the information about inactivation of TSE agents in the base-catalysed process also applies to the acid-catalysed process. But there are questions about when and how much acid permeates into the oil/fat phase. In addition, the alcohol-to-oil molar ratio used in the acid-catalysed process is 50:1 (compared with 6:1 in the base-catalysed process). Thus, the concern regarding the alcohol-related 'fixation' and stabilisation of infectivity relating to the base-catalysed process is even greater for the acid-catalysed process.

Another significant difference between the acid and base processes is that, because the acid-catalysed process is insensitive to the presence of free fatty acids in the feedstock, there is no need for complex refinement (as is required in the base-catalysed process) before the biodiesel manufacturing process can begin. The potential downside of this absence of a refinement step is the absence of procedures such as washing, centrifugation, caustic treatment and bleaching that might potentially remove or inactivate at least some TSE infectivity.

Acids: The inactivating effect of hydrochloric acid (HCl), pH 2, at ambient temperature is minimal. Although sulphuric acid has not been studied, the effect is probably similar, because it is the pH rather than the chemical that is critical. In contrast to low pH at ambient temperature, hot (1M) hydrochloric acid appears to have a significant infectivity-reduction effect at temperatures above 60 °C. Since the process is run at more than 200°C, HCl may achieve a significant amount of infectivity reduction. However, the use of HCl is largely precluded because it reacts with the methanol to produce chloromethane, which makes the recycling of methanol more difficult. In addition, the use (or formation) of chlorinated organic compounds in the process should be avoided because of environmental concerns relating to dioxin formation.

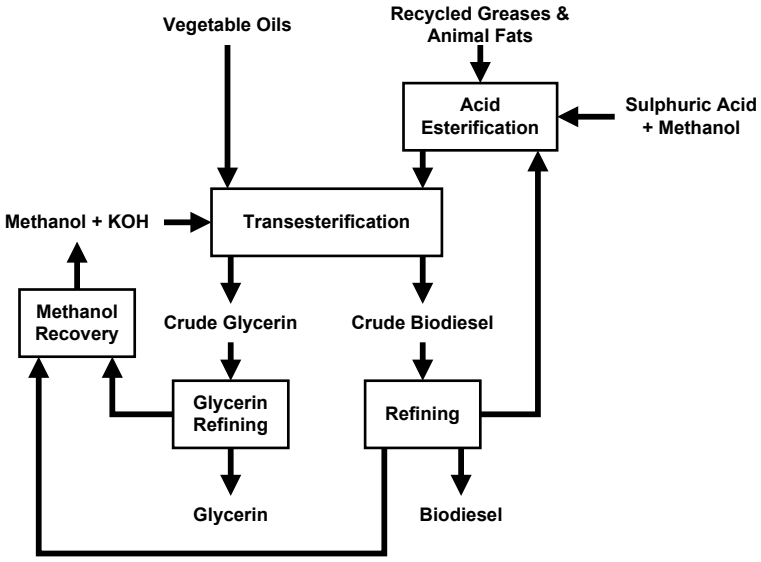
Near the end of the process, the sulphuric acid is neutralised with calcium hydroxide, and calcium sulphate is formed. This compound has no known inactivating effect on TSE infectivity.

4.2.2.4 The combined acid-base-catalysed process

Base-catalysed processing is the preferred method, due to speed and cost factors. But high FFA feedstocks, such as tallow, can only be effectively esterified in the base-catalysed process if they have been pre-processed to remove or reduce the free fatty

acid content. Although several pre-processing technologies have been developed (see Emerging and Experimental Technologies pg. 113), the acid-catalysed pretreatment is the most commonly used production method for making biodiesel from animal fats. This two-step process is used for most of the animal-based biodiesel produced worldwide. An acid catalyst pretreatment is followed by base catalyst transesterification to process feedstocks with up to about 20% FFA. The acid catalysts used are most commonly sulphuric and phosphoric acids (Figure 4.6).

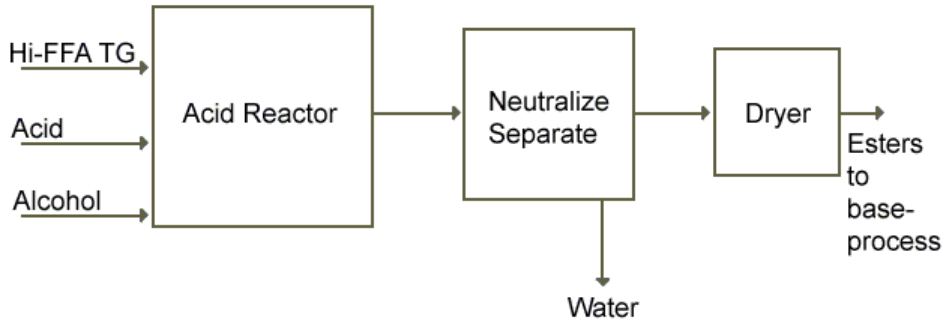
Figure 4.6 Acid-Base Esterification Process



4.2.2.4.1 Advantages of acid-catalysed pretreatment

Acid catalysts have one important commercial advantage over base catalysts: they can convert free fatty acids directly to biodiesel. Although the process of converting all the fats (FFA and triglycerides) to methyl esters is quite slow (as described above), the acid esterification of the FFA alone is quite rapid (< 2 hours); thus, acid catalysation is most useful as a pretreatment (see Figure 4.7). Because the base catalyst process works well with triglycerides, it overcomes the disadvantages of the acid treatment. However, slightly higher levels of the base catalyst are required to neutralise the acids used in the first step.

Figure 4.7 Acid Catalyst FFA Pretreatment



For feedstocks that require a pre-esterification pretreatment, reaction conditions depend on the free fatty acid concentration of the feedstock. In general, the more free fatty acids the feedstock contains, the higher the concentration of sulphuric acid required in its pre-esterification. One or two pre-esterification steps are usually applied to lower the free fatty acid concentrations to acceptable levels for the subsequent base-catalysed transesterification. Two steps (the second is a repeat of the first) are recommended to ensure that the resulting free fatty acid concentration is below 1 wt% (Canakci and Van Gerpen, 2003).

In addition, water produced by the process has to be removed between steps. Otherwise, it slows the reaction and can seriously affect the subsequent base-catalysed transesterification. Glycerol extraction can be used to remove the water (Zhang *et al*, 2003); or the reaction mixture can be settled in a tank for 24 hours until the methanol and water form an easily separable top layer (Canakci and Van Gerpen, 2003).

The typical reaction conditions for the acid pretreatment steps are shown in Table 4.6.

Table 4.6 Typical Reaction Conditions for Pre-esterification		
Feedstocks	Lipid feedstocks with high FFA contents (> 4%) Methanol	
First esterification step		
	Alcohol to oil molar ratio	Catalyst concentration
Yellow grease	20:1	5 wt% of feedstock
Brown grease	20:1	10 wt% of feedstock
Reaction time	1h	
Temperature	60-70 °C	
Pressure	140-400 kPa	
Catalyst	H_2SO_4	
Catalyst Concentration	0.25 to 0.5 M	
Second esterification step		
	Alcohol to oil molar ratio	Catalyst concentration
Yellow grease	40:1	1 wt% of feedstock
Brown grease	40:1	2 wt% of feedstock
Reaction time	1h	
Temperature	60-70 °C	
Pressure	140-400 kPa	
Catalyst	H_2SO_4	
Catalyst Concentration	0.03 to 0.07 M	

These reaction conditions are relatively severe, with acid concentrations that are proportional to the quantity of FFA present and ranging up to 10% of the oil feedstock. The second stage should have lower acid concentrations, since the first stage converts more than 75% of the FFA to esters. The methanol concentrations are much higher than typically used in the base-catalysed process. This will result in some dilution of the acid in the total mixture. Because of the poor solubility of the methanol and acid with the oil, the mass transfer process between the acid catalyst and any protein material that would be present in the oil will be slow.

4.2.2.4.2 Potential effects of the acid-base-catalysed method on TSE infectivity

The acid-based integrated process combines acid-catalysed pre-esterification and base-catalysed trans-esterification. Therefore, the conditions that apply to these two processes individually also all apply in this process.

4.2.2.5 Separation and purification stages

4.2.2.5.1 Separation

Once the trans-esterification reactions are completed, either the mixture is allowed to settle and form two distinct phases, or it is processed with a centrifuge to create the two layers. The bottom heavy layer is mainly glycerine and methanol; but ester residues as well as most of the catalyst, water and residual soap are also in this layer. The upper phase contains the ester products with unreacted oil and residual methanol, glycerine and catalyst. If residual proteinaceous matter containing TSE infectivity is present in the initial lipid feedstock and survives pre-esterification and transesterification processing, it is likely to remain in the top hydrophobic, or biodiesel, layer.

The top biodiesel layer is usually washed with water to remove any remaining methanol, glycerine, salts and catalyst. The washing step is often combined with the required neutralisation acid. After the washing steps, the biodiesel is dried to remove any traces of methanol and water, usually accomplished by heating the product or using some combination of heat and vacuum.

The bottom glycerine-rich layer must also be treated after separation. The remaining catalyst in the crude glycerine is typically neutralised by adding phosphoric acid, which converts the residual soap back into free fatty acids. Enough phosphoric acid is added such that complete neutralisation of the remaining base is achieved. After neutralisation, three distinct phases form: a top layer of free fatty acids; a bottom layer containing glycerine, water and alcohol; and a sedimentary layer containing salt precipitates. The non-glycerine layers are treated as waste and the bottom glycerine phase from the first separation can be combined with the wash water and then distilled to recover the excess methanol (for recycling through the process) and a crude glycerine.

4.2.2.5.2 Biodiesel purification

The biodiesel that results after the washing stage usually meets the quality requirements of the ASTM D6751 standard for biodiesel; but sometimes further purification of the biodiesel is needed. The lower the quality of feedstock, the more likely that some degree of purification will be required to meet the final product specifications.

There are several options for biodiesel purification:

- Passing the biodiesel over an absorbent that removes glycerol and mono- and di-glycerides, and then filtering it;
- Passing the biodiesel through activated carbon to improve the colour of the product;

- Using vacuum distillation to deodourise the biodiesel, remove minor contaminants, and ensure that the final product has a sulphur content of less than 15 ppm.

Most production facilities will pass the biodiesel through a final filter of about 5µm prior to the product leaving their control.

Because of the adherent nature of the TSE agent, the absorbent or activated charcoal steps might significantly reduce any infectivity in the purified product, a possibility that merits investigation.

The vacuum distillation process is used to remove the light ends (methanol and water) from the biodiesel. The biodiesel itself is not distilled. Any protein that might be present in the biodiesel would remain with the biodiesel after this distillation step.

4.2.2.5.3 Glycerol purification

The recovered glycerol from the transesterification reaction contains residual alcohol, catalyst residue, carry-over fat/oil and some esters. The glycerol from rendered feedstocks may also contain phosphatides, sulphur compounds, proteins, aldehydes and ketones, and insolubles (dirt, minerals, bone, or fibres). The proteins are significant for considerations of TSE infectivity. The glycerol must be purified before it can be used in most applications. Several approaches are used in the industry, including chemical refining, physical refining and purification.

Chemical refining is used to neutralise the catalyst which tends to concentrate in the glycerol phase, and this neutralisation step precipitates salts. Also, soaps in the glycerol must be removed by coagulation and precipitation with aluminium sulphate or ferric chloride. The removal may be supplemented by centrifuge separation. Control of the pH is very important because low pH leads to dehydration of the glycerol, and high pH leads to polymerisation of the glycerol. As a final step, the glycerol may be bleached using activated carbon or clay.

Physical refining involves removal of fatty, insoluble or precipitated solids by filtration and/or centrifugation, and may require pH adjustment. The water is removed by evaporation. All physical processing is typically conducted at 65 to 95 ℃, where glycerol is less viscous, but still stable.

Purification of glycerol is completed using vacuum distillation with steam injection, followed by activated carbon bleaching. The disadvantage is of this process is that it is capital- and energy-intensive.

Ion exchange purification of glycerol is an attractive alternative to vacuum distillation for smaller capacity plants. The ion exchange system uses cation, anion, and mixed-bed exchangers to remove catalysts and other impurities. The glycerol is first diluted with soft water to a 15 to 35% glycerol-in-water solution. The ion exchange is followed by vacuum distillation or flash drying of the glycerol for water removal, often to an 85% partially refined glycerol. Ion-exchange chromatography is known to absorb up to 3 to 4 logs of infectivity in contaminated tissue fluids (Flan, 2005).

4.2.3 Emerging and Experimental Technologies

Besides the catalysation and purification processes already discussed, there are several new and emerging technologies for biodiesel production. Most are not currently being used commercially, except in an experimental manner.

4.2.3.1 Emerging preprocessing technologies

4.2.3.1.1 Caustic stripping

Caustic stripping is most often used for feedstocks that are relatively low in FFA (<4%) rather than for high FFA feedstock, such as tallow. The caustic reacts with the FFA to form soap, which is removed prior to transesterification. The process has a lower yield because of the conversion of the FFA to soap, and higher cost because of the caustic consumption. Caustic consumption is about 0.3 kg for every kg of FFA in the feedstock. Tallow would need to have a FFA content of less than 10% for this approach to be useful; thus, it is not likely to be used by plants designed to process low-quality feedstocks. Feedstock with 10% FFA content would require a 0.7 M (3 wt%) caustic dosage for FFA content removal.

4.2.3.1.2 Enzymatic

Some new enzymatic catalysts have been developed for preparing feedstocks high in FFA for esterification. Commercial lipases have been shown to be capable of treating feedstocks with FFA contents as high as 50% (DOE, 1995).

Researchers at USDA's Eastern Regional Research Center conducted bench-scale experiments on biodiesel production using a variety of commercial lipase preparations. The use of enzymes is very costly, and thus the technology application is not widely used, although it does have some interesting advantages. More data is required on the applicability of the process, and more work is required to reduce the costs of this approach before it can be used commercially.

4.2.3.1.3 Glycerolysis

Glycerolysis is a commercial process not currently used for biodiesel production. The process converts FFA to monoglycerides and then transesterifies the feedstock to biodiesel. The process is slow and generally requires high temperatures (250 to 260 °C). It is mostly used to produce monoglycerides and not for biodiesel production.

4.2.3.2 Emerging processing technologies

4.2.3.2.1 BIOX co-solvent process

The BIOX process, a new biodiesel process developed by Professor David Boocock of the University of Toronto, has been successfully demonstrated in a laboratory and pilot setting. The first commercial plant is under construction in Canada. Co-solvent processes are also being developed by Resodyn in the United States (Tyson, 2003).

The BIOX process can be applied to feedstocks containing low FFA, in which case a single-step process can be applied to feedstocks. It can also be applied to high-FFA feedstocks, where a two-step, acid-base catalysed process can be used. In the two-step process, FFA are converted using a 30-minute exposure to methanol at 60 °C and a methanol-to-triglyceride molar ratio of 20 to 30. Catalyst concentrations of 2% by

weight of feedstock (0.05 M sulphuric acid in total solution) are claimed using sulphuric acid, but the real concentration will likely depend on the feedstocks being used.

The use of a co-solvent to create a single-phase solution has the benefit of exposing any protein present to the acid catalyst; but at the same time, the high methanol-to-triglyceride ratio reduces the molarity of the acid in the single phase, which may reduce the acid's deactivation potential. The base-catalysed step uses 1 to 2% NaOH (0.07 to 0.14 M NaOH) at 15 to 65 °C and is complete in seconds.

4.2.3.2.2 Super critical processing

Super critical processing uses very high temperatures and pressures so that the reaction times are very short. It produces a fatty acid ester from oils and fats, but through a process different from those described earlier in this section. There is some concern about the final quality of the biodiesel, because the temperatures are high enough for pyrolysis reactions to form non-ester products. But pyrolysis appears not to occur in the process. Solvents explored so far include methanol, ethanol, propane and carbon dioxide.

Experimental work indicates that the composition of the final biodiesel product is similar to that obtained in commercial processes (Kusdiana, 2001). The process shown in Table 4.7 was complete in 4 minutes, and the yield was 98.5% biodiesel (traditional processes need processing times of several hours with yields of about 97%). There are as yet no commercial supercritical plants in operation for the production of biodiesel.

Table 4.7 Features of Supercritical Processing: Bio Super 3000

	Conventional Processes	**Supercritical Processing**
Reaction time	1-8 hours	2-4 minutes
Reaction conditions	0.1 MPa, 30-65 °C	>8.09 MPa, >240 °C
Catalyst	Acid or Alkali	None
Free fatty acids	Saponified products	Methyl Esters
Yield	Normal	Higher
Removal for purification	Methanol, catalysts and saponified products	Methanol

4.2.3.3 Emerging non-ester biodiesels

New processes can produce biomass-derived, diesel-like fuels that are sometimes loosely classified as biodiesels. The two pathways described below, Fischer-Tropsch distillates and catalytic hydrotreatment, both transform the biomass into a long-chain hydrocarbon rather than into an ester. Neither of the processes is currently being commercially practiced, although a catalytic hydrotreatment plant has been announced by Fortum, the Finnish oil company.

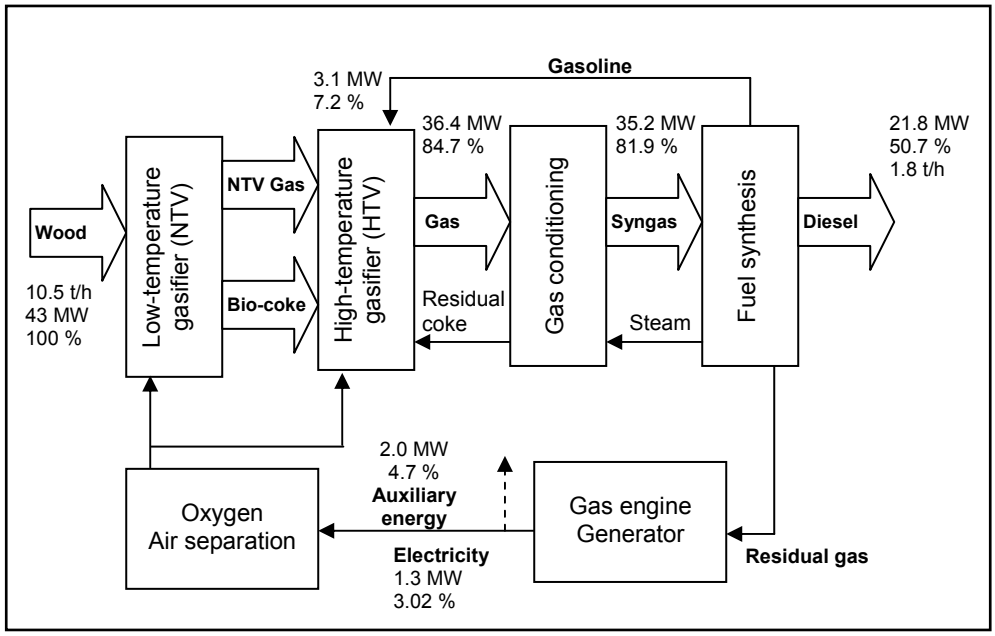
4.2.3.3.1 Fischer-Tropsch distillates

A diesel-like fuel can be produced by gasifying biomass material and then synthesising it into a fuel using a Fischer-Tropsch synthesis step (or the similar Shell Middle Distillate Synthesis technology). The fuel produced by these processes is a *high cetane*

hydrocarbon that can be used as a diesel fuel component. Like the supercetane produced by catalytic hydrotreatment (see below), it has clean burning properties but does not contain any oxygen. The one advantage of this process is that the feedstock is not limited to oils and fats; it can also produce fuel from agricultural residues, such as straw or wood chips. Companies in South Africa and Malaysia are currently using this method, with coal and natural gas as the feedstocks.

The technology has not yet been combined commercially with biomass gasification, although some product has been produced in a pilot plant unit operated by the German company Choren (http://www.choren.de/). The process is shown in Figure 4.8. The temperatures for the various chemical reactions that occur are in the range of 500 to 1400 ℃. The gas composition is determined, in large part, by the temperature of operation.

Figure 4.8 Choren Biomass to FT Distillate Process



Shell, Volkswagen and DaimlerChrysler have shown interest in this fuel production method in some parts of the world. Volkswagen has named the fuel Sunfuel (www.sunfuel.de). An alliance including ECN, Ecofys, Rabobank, Shell and Volkswagen has come up with a similar concept called BIG-FiT (Biomass Integrated Gasification – Fischer-Tropsch). It has completed a preliminary evaluation that included a life cycle assessment, process design studies and costing studies (SDE), but has not yet published a report.

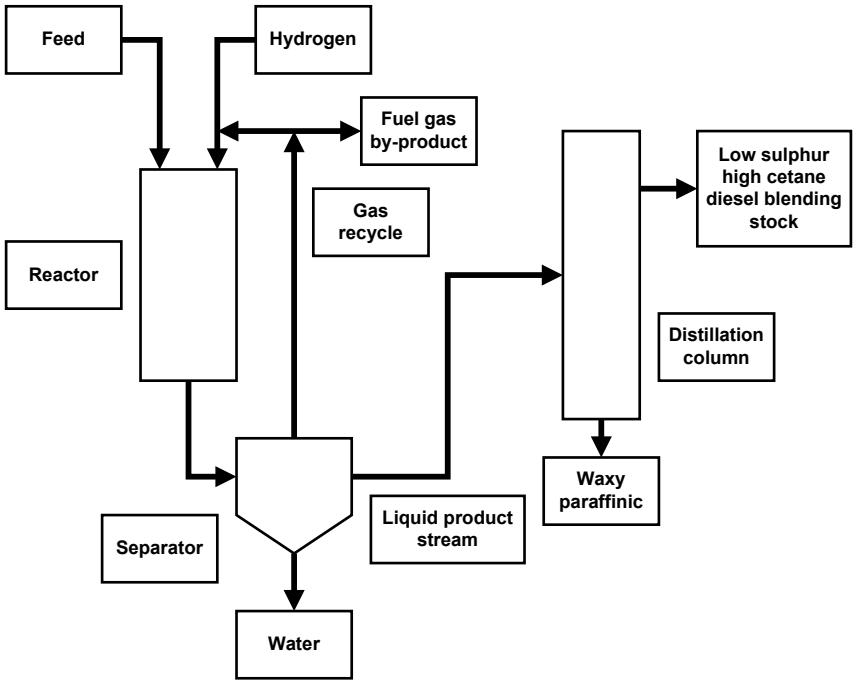
4.2.3.3.2 Catalytic hydrotreatment

Natural Resources Canada (NRCan, 2003) has developed a procedure that can process fats into fuels by hydrotreating them to produce paraffins. The product is called *super cetane*. The paraffins, which only contain carbon and hydrogen, can be used as a

diesel-fuel-blending compound; they have a very high cetane, but also function poorly in cold weather.

A number of feedstocks have been successfully processed using the technology, including canola oil, soy oil, yellow grease, animal tallow and tall oil (a by-product of the Kraft pulping process). Yields of 75 to 80% based on feedstock input have been achieved (see Figure 4.9).

Figure 4.9 Super Cetane Process



Several reactions occur in the process, including hydrocracking (breaking apart of large molecules), hydrotreating (removal of oxygen), and hydrogenation (saturation of double bonds). The catalyst used in the process is a conventional, commercial, refinery hydrotreating catalyst. Hydrogen is the only other input.

The hydrotreatment process is typically operated in a continuous mode at moderate to high pressure (2 to 8 MPa) and high temperature (250 to 420 ℃) for at least 20 minutes, producing isoparaffinic diesel fuel. The process has been successfully scaled up in a one-barrel/day hydrotreating pilot reactor using depitched tall oil as the feedstock. A similar processing concept has been developed in Finland and is being commercialised there (R. Semotiuk, personal communication). The Finish process may also involve an isomerisation step to improve the cold weather properties of the finished product.

The catalytic hydrotreatment process may be able to generate a TSE-safe product even if the starting materials contained residual infectivity. The method may be completely effective as is, and would at the very least provide improved assurance in the event that infectivity is found to survive the rendering process. This process is akin to the commercial method for making tallow derivatives accepted as TSE-risk-free.

4.2.4 Proteins and Infectivity in the Biodiesel Production Process

Overall, transesterification offers an uncertain opportunity for significant inactivation of TSE agents during biodiesel manufacture. The possible exceptions reside in methods used to refine feedstock in the acid-catalysed process. However, much more information about these procedures is required before a confident scientific opinion can be provided.

The base-catalysed process and its subsequent separation protocols include some steps with the potential to reduce or eliminate TSE infectivity. Notably, NaOH used at up to 0.36 M concentration during transesterification might have a significant inactivation effect. A number of studies summarised by Taylor (2004) have shown that exposures to 1 M and 2 M sodium hydroxide for periods of an hour or more result in a significant loss of infectivity in saline suspensions of contaminated tissue suspensions. NaOH concentrations as low as 0.25 M have shown partial reduction of infectivity (Kasermann & Kempf, 2003).

But the two-phase system of biodiesel production may inhibit the exposure of protein-associated infectivity to the alkali. Experimental data on rendering practices (Taylor *et al*, 1995, 1997) indicate that TSE infectivity does not preferentially migrate into tallow but tends to remain within the proteinaceous by-product of rendering. Other data raise the possibility that the infectivity in these impurities might actually be protected from inactivation during the production process. For example, the protective effect of lipids in preventing conventional microorganisms from inactivation is well known; and TSE infectivity suspended in tallow becomes much more resistant to inactivation by autoclaving (Appel *et al*, 2001). Clearly, reducing the level of suspended solids in tallow to $\leq$ 0.15% and free of protein content before commercial use is vital for reducing any TSE-related risks. Table 4.8 summarises process steps that can positively impact TSE deactivation, with qualitative descriptions of the possible effects.

Table 4.8 Some Process Steps Applied to Tallow
Using the Acid-Base Integrated Process

Process	Characteristic	Limitation	TSE Deactivation
Tallow Pretreatment			
Particle filtration using a 20-micron cellulose filter	Removes particulate matter	Does not remove colloidal dispersed proteins in the fat	Little
Steam distillation	Oxidizes proteins	---	Partial to total
Bleaching	Removes spoiled proteins	Depends on the effectivity of protein-bleaching clay contact	Partial
Biodiesel Synthesis			
Acid-catalysed esterification	Degrades proteins due to high sulfuric acid concentration	There is an initial tallow/methanol-catalyst phase separation	Partial
Base-catalysed transesterification	Deactivate TSE agents due to alkali concentration, and applied temperature and pressure	There is a glycerin/biodiesel phase separation in the final stage of the reaction	Partial

4.2.4.1 The Saria Bio-industries study

The only directly applicable data on TSE inactivation during the biodiesel production process come from experiments initiated by Saria Bio-industries using tallow spiked with the 263K strain of hamster-adapted scrapie. The full report on the Saria Bio-industries experiments has been published (Seidel *et al*, 2006).

The rendered fats were filtered to establish a maximum insoluble fraction of ≤ 0.15%, the agreed-upon level for international trading; and each of the major steps was evaluated for TSE infectivity reduction. The process involved one esterification step, two transesterification steps, three washing steps and distillation. Alm (2004) reported a protein clearance factor of at least a 4 log reduction for each chemical step, 1 to 3 logs for each washing step, and 1 log for the distillation step (EFSA, 2004; Seidel *et al*, 2006).

The study has two main caveats. First, reductions were measured by Western blot assay of misfolded protein rather than bioassay. Although clearance of the protein provides an excellent clue to clearance of infectivity, the protein and the infectivity may in practice be dissociated, especially under non-aqueous conditions. Second, it is invalid to add together the reductions in individual steps to yield an overall process reduction, because the fraction of infectivity surviving a given process may be unpredictably resistant to a subsequent process.

4.2.5 Knowledge Gaps for Biodiesel Production

There are large gaps in the understanding of how TSE infectivity would survive the biodiesel production process.

1. Acids, bases, elevated temperatures and pressures have been shown in other applications to reduce infectivity levels. But exposure to alcohols may prevent the reduction of infectivity levels. Experimental work is required to understand what role the combinations of acids or bases and temperature, pressure and time play in regards to TSE infectivity. Similarly, the role that alcohols and other co-solvents play in regards to TSE infectivity needs to be investigated.
2. Once a better understanding of the fundamentals of biodiesel production and TSE infectivity are understood, then experimental work is also required to examine the deactivation capabilities of different commercially viable biodiesel production processes, including pre-treatment steps, to establish clearance levels. This would require carrying out an initial immunoassay programme followed up by a bioassay to validate findings and verify clearances.
3. It may also be advantageous to determine if the commercial biodiesel production processes could be optimised for biodiesel production efficiency and TSE infectivity reduction. This could include the investigation of separation methods to remove any proteins in the final product.
4. Some, but not all, fuel quality standards for biodiesel limit the quantity of insoluble materials that can be tolerated in the final product. It is not clear if a test would capture any proteins that might be present. Test methods that could identify protein levels in the final biodiesel product and a specification level in the product, should be investigated as a means to enhance biodiesel product quality.

5. The biodiesel production process also produces glycerine and some other co-products and waste streams. The potential for infectivity in these streams is unknown but may warrant further investigation. If the combined effects of acids, bases, temperatures, pressures and time on TSE infectivity in spiked tallow is investigated, an improved judgement could be made.

4.2.6 Conclusions: Biodiesel Production

Only the base-catalysed and the acid-base integrated processes are commercially practised for the production of biodiesel fuel. For production using animal-based feedstocks, the integrated process is nearly always used, since tallow from animal by-products results in a higher free fatty acid (FFA) content in the biodiesel production starting material. High FFA poses difficulties for processing because of the tendency to form soaps.

Current processes used to produce biodiesel from tallow may or may not reduce TSE infectivity, if such infectivity were to be present. Based on currently available information, the following conclusions can be drawn:

1. In the base-catalysed process, the low solubility of the base and alcohol in the oil reduces the potential for base deactivation of any TSE infectivity in the fat.
2. The TSE inactivation effects of acids used in the acid-catalysed process have not been proven.
3. The alcohol used in acid-catalysed pre-esterification and base-catalysed transesterification may have a counter-productive stabilising effect on infectivity.
4. The temperature and pressure levels used in the base-catalysed process would not in themselves result in reducing TSE infectivity levels to any significant extent.
5. Preprocessing steps associated with the combined acid-base method could have a beneficial effect in reducing infectivity.
6. Some emerging and experimental technologies for biodiesel production have the potential to reduce TSE infectivity that may be in feedstocks; but determination of specific effects on TSE will require research and tests.
7. Production processes that involve hydrotreating the feedstock use temperatures and pressures that should be effective in reducing TSE infectivity. The product that is produced by these processes is not an ester but rather a paraffin that is blended with diesel fuel.

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5 USE OF BIODIESEL: COMBUSTION

This chapter examines the potential for the combustion of tallow-based biodiesel fuel in compression-ignition (CI, or 'Diesel') engines to destroy TSE infectivity.

Only one related assessment has been published, in which a combustion engine was modeled on a thermal disposal (incineration) process for which the ability to reduce TSE infectivity was known. A theoretical reduction in TSE infectivity from CI engine combustion was calculated (Cummins *et al*, 2002). There are serious limitations in assuming that exposing active TSE agents to dynamic engine combustion process temperatures is the same as subjecting TSE agents to steady-state incineration process temperatures (see Other Factors Affecting Engine T,P,t Operating Conditions p. 128).

5.1 Incineration Studies

Two studies (both using scrapie-infected brain tissue) have been conducted at temperatures similar to those employed for incineration. The first study, in which heat was applied at ambient pressure in an insulated laboratory oven, showed a significant loss of infectivity in brain tissue macerates after 5 to 15 minute exposures to progressively higher temperatures: only a minute amount of infectivity survived a temperature of 600 ℃, and none survived a temperature of 1 000 ℃ (Brown *et al*, 2000).

Recently, the study was repeated and extended to examine both the residual ash and the vented emissions in an apparatus designed to mimic actual incinerator conditions. Samples of hamster brain tissue containing more than 109 mean lethal doses (LD_{50} – mean lethal dose per g of tissue at which 50% of exposed animals become infected) were exposed for 15 minutes to temperatures of 600 °C and 1 000 °C in both oxidative and reducing environments. Residual ash, outflow tubing residues, and vented emissions were collected, and the entire volume of each sample was bioassayed in healthy weanling hamsters. Only the ash from the 600 °C sample transmitted disease— to 2 of 21 hamsters. No transmissions occurred from either the reactor tube residue samples or vented emissions samples exposed to either 600 °C or 1000 °C. The authors concluded that, at temperatures approaching 1 000 ℃ under the air conditions and combustion times used in the experiments, contaminated tissues could be completely inactivated with no release of infectivity into the environment from emissions. Exposure at 600 °C could leave an almost undetectably low level of infectivity in the ash but none in the vented air emissions (Brown *et al*, 2004).

A second part of this experiment removed the air conditions and tested TSE tissues burned with nitrogen gas. Since no transmissions resulted, the authors concluded that the presence of oxygen was not required to inactivate any TSE agents present. They further concluded that reduction to carbon (ash) during incineration did not protect infectivity.

The most common incinerators in the US are controlled-air, two-stage, modular incineration systems. The first stage dries, volatizes, pyrolyses (reduces) and partially combusts waste in an environment containing less than the stoichiometric amount of air required for complete combustion. The air temperature above the waste is typically 760 to 980 °C. In the second stage, air is added to the gases produced in the first chamber to complete combustion, with the resulting gas temperature reaching 980 to 1095 °C. Thus, the gases from the first stage have a heating value high enough to

'fuel' the secondary burn phase of this incineration process, essentially acting as an after-burn stage.

In the UK, non-medical waste streams possibly containing TSE-contaminated livestock, carcasses, or meat and bone meal (MBM) are incinerated in dual-chamber incineration facilities with primary and secondary chamber temperatures of 850 ^{o}C and 1 000 ^{o}C, respectively, for a residence time in-chamber of one hour (Brown *et al*, 2004).

5.2 Basic Compression-Ignition (CI, or Diesel) Engine Combustion Process

Combustion in a compression-ignition engine involves several dynamic processes governed by chemical reaction kinetics, thermodynamics, and fluid mechanics. The time interval during which the reactant air/fuel mixture is converted to combustion products is generally on the order of milliseconds, and is mainly influenced by the engine speed (measured in revolutions per minute, or rpm) and chemical reaction rates of the species involved in the combustion reactions.

The chronology of combustion events in the CI engine cylinders is as follows:

1. Fuel is injected directly into the cylinder after the intake "charge" of air is compressed to a pressure of about 4 MPa and a temperature of about 800 K (527°C).
2. The fuel auto-ignites a fraction of a millisecond later from the temperature and pressure resulting in the compressed intake air charge. Auto-ignition causes the cylinder pressure to rise to approximately 7 to 10 MPa. The in-cylinder temperatures during this phase of combustion range from 2 800 to 3 600 K (2 527 to 3 327 °C).
3. The combustion phase finishes as the piston reaches the end of its stroke at the bottom of the cylinder. The in-cylinder temperatures during this phase of combustion range from 1 600 to 2 100 K (1 327 to 1 827 °C).
4. At this point, the expansion phase begins, in which the piston withdraws along the cylinder length, with a resulting temperature and pressure drop of the gases within the cylinder. The average temperature of the gases leaving the cylinder is 100 to 400 °C.

In contrast to spark-ignited (SI) gasoline engine combustion, which consists of burning a fuel/air mixture in known (stoichiometric) premixed proportions, CI engine combustion comprises auto-ignition of an air-fuel mixture that is not stoichiometric throughout the engine cylinder. In practical terms, this results in very large temperature variations in space and over time during CI engine combustion (Hountalas *et al*, 2001). In a new or ideally performing engine, typically 98% of the fuel will be oxidised to CO_2 and H_2O, with some parcels of the cylinder gases experiencing temperatures exceeding 2 400 K (2 127 °C). The remaining 2% of the fuel may be converted to pyrolysis products, such as soot and polycyclic aromatic hydrocarbons. Thus, a non-uniform temperature profile within the cylinder over time, and a short duration of combustion events, characterise the transient, dynamic nature of this process.

5.3 Newer CI Engine Technology

Over the past 30 years, CI engine technology has advanced to accommodate more stringent fuel economy, emissions, and performance criteria demands, which are both market- and legislation-driven. Engine T/P/t (temperature, pressure, time) conditions for different engine technologies are listed in Table 5.1, which includes data obtained from both experimental and modeling results. New engine technologies covered in Table 5.1 include electronic fuel system (EFS) control strategies, such as multiple fuel injections (MI) (includes split and pilot fuel injections), combustion rate shaping (CRS), and start of injection (SOI) timing.

Some recent improvements in diesel engine designs, developed as part of manufacturer strategies to reduce engine emissions, involve recirculating the exhaust gases in-cylinder and using after-treatment devices.

5.3.1 Exhaust Gas Recirculation (EGR)

Exhaust gas recirculation generally affects the range of in-cylinder flame temperatures. For example, a study varying EGR rates between 0 and 50% showed that

EGR rate = 0%	**T_{flame} ~ 1 980 – 2 410 ^{o}C**
EGR rate = 30%	**T_{flame} ~ 1 630 – 2 130 ^{o}C**
EGR rate = 50%	**T_{flame} ~ 1 530 – 1 980 ^{o}C**

In general, the range of flame temperatures decreases with an increase in the percentage of exhaust gas that is recirculated (McTaggart-Cowan and Hill, 2005). This would suggest that in-cylinder temperatures in general would also fall as a function of increased EGR rates. Thus, the potential for TSE agent inactivation in an EGR engine might also be a function of EGR rate.

5.3.2 Exhaust Gas After-Treatment (EGA) by Catalysis

One example of a modern exhaust after-treatment technology is the hydrocarbon-selective catalytic reduction (HC-SCR) device. This device decreases emissions of NOx, CO, and hydrocarbons at both low and high temperatures. These devices typically operate at 150 to 550 °C (Westerberg *et al*, 2003; Koenig *et al*, 2001). Based on studies of dry heat inactivation of TSE agents, these temperatures would reduce but not sterilise TSE infectivity (Brown *et al*, 2000).

5.3.3 Diesel Exhaust Oxidation Catalysts

Oxidation catalysts target reduction of soot emissions from CI engines. Typical operating temperatures for these oxidation catalysts are between 240 °C and 700 °C (Johnson, T.V., 2004). However, the ideal oxidation temperature for targeted soot particles depends on the certification class of the engine; for example, a modern EURO IV engine produces much different soot than that generated under fuel-rich engine operating conditions from a EURO III engine. Both soot types begin oxidising at temperatures around 330 °C; but the EURO IV engine reaches a maximum oxidation rate at 560 °C, whereas the older EURO III engine reaches a maximum oxidation rate at 660 °C. Again, it is difficult to predict TSE inactivation at these varying temperatures, since the temperatures are both above and below 600 °C.

5.3.4 Diesel Particulate Filters (DPFs)

Diesel particulate filters remove particulate matter (PM) from the exhaust stream, with an added regeneration process at the filter inlet surface that clears it of accumulated PM. However, the regeneration temperatures at this location depend on the engine operating condition (engine load, speed combination) and the concentration of soot in the engine exhaust. One study indicated that for engine idling condition (*i.e.*, no load on the engine, engine speed of 600 rpm) and 4 gram/L soot loading, DPF inlet temperatures had to be between 600 and 670 °C in order for the DPF to achieve complete regeneration. In this particular case, there was also a stipulated maximum DPF temperature of 900 °C, for safety reasons (Johnson, T.V., 2004).

5.4 Other Factors Affecting Engine T,P,t Operating Conditions

5.4.1 Extreme Operating Conditions for CI Engines: Cold Start

Table 5.1 includes data obtained from experiments involving engine start-up from cold conditions. Exhaust temperatures in this case (not the same as in-cylinder temperatures) can be as low as 20 to 156 °C. Cold-start testing of a class A compression-ignition truck engine at 10 °F (-12 °C) resulted in unburned hydrocarbon emissions on the order of 1 000 ppm. This represents approximately 18% of the initial fuel mass burned during this cold startup period, which in this case was assumed to last for 2 minutes (Whitfield, S. 2005).

5.4.2 Fuel Type/Properties

Table 5.2 shows experimental data on the effect of fuel type/fuel properties on engine operating temperatures and combustion auto-ignition delay (a function of the diesel fuel property known as 'cetane number'). However, the in-cylinder temperatures do not differ greatly from those of standard diesel fuels, ranging from 827 to 2 130 °C.

5.4.3 Unburned Fuel in 'Open System Process' vs. Closed Incineration Process

Not all combustion gases in a CI engine pass through the diffusion flame, where temperature is typically around 2 100 °C. Some fuel can escape combustion if it is mixed with too much air or is quenched (cooled) by the cylinder walls. This unburned fuel may not experience temperatures much higher than the exhaust temperature, which suggests a low probability that any TSE infectivity within that unburned fuel would be inactivated.

In addition, typical CI engines operating under fuel-lean conditions (*i.e.*, ideal operation involving excess air for most complete fuel-burning possible) have a combustion efficiency of 98% (Heywood, 1988). This means that typically 2% of fuel injected into the engine cylinder exits the exhaust tailpipe either as gaseous 'unburned hydrocarbon' emissions or as a portion of the heterogeneous particulate matter emissions.

This observation, however, describes CI engines that are operating under ideal fuel conversion conditions (98% efficiency). The deviation of engine combustion performance and emissions from the ideal case is another factor that may effectively increase a CI engine's tendency to emit unburned fuel. Examination of data from the

Greater Vancouver Regional District's AirCare programme showed that, for a mixed fleet of vehicles (both SI and light-duty CI engines), the number of vehicles in disrepair by model year (from an emissions standpoint) ranged from 0.5 to 36%, depending primarily on vehicle age. This result was based on 999 007 vehicles tested during 1999 and 2000 for emissions of unburned hydrocarbons, carbon monoxide and oxides of nitrogen (Stewart *et al*, 2001). This statistic suggests that true fuel conversion efficiencies are likely lower than 98% as engines increase in age and vary in terms of disrepair. This reduced efficiency may increase the unburned hydrocarbon emissions in the exhaust.

5.5 Combustion vs. Incineration

Incineration is a steady-state process. In contrast, CI engine combustion is a series of short-lived chemical reactions occurring far from steady-state conditions. The conditions in the incineration studies described above involve time durations on the order of minutes, which are far greater than those associated with in-engine fuel combustion events, which are typically on the order of milliseconds, or 10^{-6} seconds. Experimental data emphasising the contrasting characteristics of each of the two processes are listed in Table 5.1 and Table 5.2.

Table 5.1 Engine Temperature, Pressure and Time Conditions

Experimental and Modeling Results Found in the Literature for Various CI Engine Technologies

EngineTechnology Variable or Operating Condition	Temperature (°C)	Pressure (MPa)	Combustion Duration (crank angle degrees)	Engine speed (rpm)	Time duration	Reference
SOI timing	-	1.2 - 6.8	90	1 800	8.3 ms	(Long, 2000)
SOI timing	527 – 2 227	1.6 – 6.5	80	1 500 1 200	8.9 ms 11.1 ms	(Ishii, 2001)
SOI timing	340 – 850	2 – 42 2 - 60	280 280	1 500 1 500	31.1 ms 31.1 ms	(Hountalas, 2001)
Pilot injection Split injection	727 – 2 327 727 – 2 127	2.2 – 7.5	60	900	11.1 ms	(Yamane, 2000)
Cold start	20 – 156 β	4	360	800	75.0 ms	(Han, 2001)
Fuel type/ fuel properties	827 – 2 127	5.4 -14.4	50	4 000 4 000	2.1 ms	(Habchi, 2004)

β – Temperature indicated here is **exhaust temperature**, which is not analogous to in-cylinder temperatures indicated in the table.

Table 5.2 Incineration Temperature, Pressure and Time Conditions

Experimental Results for Steady-State Incineration Conditions

Process	Temperature (°C)	Pressure (MPa)	Time duration	Comment	Reference
Incineration	600	0.1	15 min.	TSE transmitted to 2 of 21 inoculated hamsters; no infectivity remaining in vented emissions or residues	Brown *et al* (2004)
Incineration	1000	0.1	15 min. (9×10^5 ms)	No infectivity in ash, vented emissions, or residues	Brown *et al* (2004)

At first glance, the upper temperature range in Table 5.1 (156 to 2 327 ºC), which represents data from a survey of newer engine technologies, may seem adequate for inactivation of TSE infectivity. However, the lower portion of this temperature range falls into a range that is at best only partially inactivating (Brown *et al*, 2000; Brown *et al*, 2004). Moreover, the temperatures listed in Table 5.1 are spatially variable within the engine cylinder (Hountalas, 2001; Ishii, 2001; Long, 2000; Yamane, 2000).

In addition, the time duration values shown in Table 5.1 are in the range of 2.1 to 75.0 milliseconds. There are no data about temperature inactivation of TSE agents for such short exposure times. The tabulated time duration events for combustion are five orders of magnitude less than the recommended 15-minute incineration process time that was used in the incineration studies of Brown *et al* (2004). Based on these considerations and without benefit of experimental data for combustion conditions, exposure of TSE agents to commonly occurring engine combustion temperatures and pressures cannot be considered an adequate means to inactivate these agents.

This large difference between time durations of fuel exposure to high temperatures, for combustion and incineration processes, is a serious drawback to modeling a dynamic combustion engine upon a steady-state incineration process.

5.6 Considerations of Risk in Relation to Route of Infection

Direct introduction of the infectious agent into the central nervous system is the most efficient means to transmit TSE, but this route of infection would not occur in the course of the production or use of biodiesel. Parenteral routes of infection have transmission efficiencies that vary with the route (Brown *et al*, 1999; Diringer, 1994; Kimberlin, 1988, 1989a, 1989b; Pocchiari, 1991; Prusiner, 1985; Scott, Foster and Fraser, 1993; Taylor *et al*, 1994; Taylor, McConnell and Fraser, 1996); for example, intravenous exposure is about 10 times less efficient than intracerebral exposure, and intraperitoneal, intramuscular, subcutaneous and percutaneous exposures are less efficient still. Oral exposure is likely to be around 1 million times less efficient than intracerebral exposure, and disease transmission by respiratory aerosol exposure has never been observed to occur. Thus, in principle, the very low levels of infectivity that can transmit disease by the intracerebral route may not be sufficient to transmit disease following peripheral exposures.

Despite these observations indicating a minimal risk via likely routes of exposure (*e.g.* by inhalation, oral or percutaneous exposure), it is prudent to conduct a risk assessment in the light of the latest knowledge and to determine appropriate risk management practices to minimise the exposure to any risk identified.

5.7 Gaps in Knowledge

No experimental data are available that bear directly upon inactivation of TSE agents in a CI-engine. An appropriate experiment designed to characterise the combustion processes and resulting emissions from the different types of diesel engines, and considering treatment technologies that are becoming commercially available, would be of academic value only. This is because for other reasons of safety there must be a negligible risk in the biodiesel fuel before it is combusted. It would be unsafe to rely upon combustion in an engine to create TSE-safe end products because not all engines work with 100% of a standard efficiency and some uncombusted fuel could pass

through into the exhaust. For academic processes, such an experiment in theory should use a preparation of TSE-infected, tallow-based, biodiesel fuel at different blend levels to run a CI engine under a variety of operating conditions, including cold-start-up, and should collect exhaust emission samples for use in a bioassay. Given that this type of experiment would be a difficult and complex undertaking, a suitable misfolded beta sheet protein should be utilised as a proxy material in order to assess, at a minimum through an immunoassay of exhaust gases, whether protein if present in diesel fuel ends up being combusted or escapes intact in the resulting emissions stream.

5.8 Conclusions

There are significant differences between the dynamic nature of CI engine combustion and the steady-state incineration conditions for which TSE infectivity reduction data exist. Moreover, evolving engine technologies such as exhaust gas recirculation and exhaust after-treatment modify the temperatures to which tallow-based biodiesel fuel and its combustion-related emissions are exposed.

A CI engine in ideal operating conditions has a fuel conversion efficiency of 98%, which results in approximately 2% of the fuel injected for combustion exiting the tailpipe unburned. However, vehicle fleet ‘disrepair’ figures (based on measured emissions representing incomplete fuel combustion) suggest further increases in the mass of unburned fuel emissions exiting the tailpipe, either as gaseous hydrocarbon or as a component of particulate matter emissions. Fuel escaping combustion is also an important consideration in northern regions where diesel engines are started up in cold temperatures. Cold-start conditions would increase the risk of any TSE infectivity escaping in unburned fuel in the exhaust emissions. This potential for exposure to TSE infectivity from unburned fuel indicates that risk management must be undertaken at earlier stages in the fuel production process to ensure that biodiesel fuel is free of any TSE infectivity.

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6 SUMMARY, RECOMMENDATIONS, AND CONCLUSION

6.1 Summary

The potential for BSE contamination of bovine tissues has led government regulatory agencies to designate certain high risk tissues as 'specified risk material', or SRM, and prohibit their inclusion in either human or ruminant food, or in various other products such as biologicals, pharmaceuticals, medical devices, cosmetics and fertiliser. As a consequence, a substantial tonnage of animal tissue that would otherwise have been used in commercial enterprises is destroyed. The use of SRM to produce tallow for biodiesel production is one possible means to recoup at least some of this lost resource.

6.1.1 Source - Material Selection

Risk can be eliminated at the source by avoiding all tissues from any animal susceptible to TSE infection. Risk can be reduced by a selective avoidance of tissues from animals with neurological signs consistent with TSE, animals living in geographic regions in which TSEs exist in nature, or tissues known to be infectious in animals with TSEs, irrespective of geographic origin. These kinds of prohibitions are widely used to ensure safety of the animal and human food chains. Elimination of such tissues from consideration, however, would be inconsistent with the purpose of this appraisal, which is specifically aimed at an evaluation of risk inherent in the use of bovine SRM for the production of biodiesel.

6.1.2 Process - Rendering

Pressure-cooking (particle size 50 mm or less and at least 133 ºC, 3 bar, 20 minutes, or a process giving equivalent inactivation) is conditionally authorised for use in the EU for SRM, and its derived tallow is authorised for use in biodiesel manufacture, provided the insoluble impurity content is 0.15% or lower. In rendering experiments using deliberately infected bovine tissue, tallow processed by a range of different methods has been shown to be non-infectious, even when its rendering co-product (meat and bone meal) from a particular method remains infectious. The rendering process is therefore an effective means of infectivity reduction, either by partitioning infectivity away from tallow or destroying it outright.

In countries that do not use pressure-cooking, and where TSE infectivity is a concern, conventional filtering methods should be able to reduce the level of insoluble protein impurities to less than 0.02% in SRM tallow, as is done in Europe for the majority of food- and feed-grade tallow. This should substantially reduce potential infectivity because TSE agents are closely associated with protein. Improved filtering and the exclusion of tank bottom material should be capable of reducing the risk to negligible levels.

6.1.3 Process - Biodiesel Processing

Hydrolysis of tallow (fat splitting into simpler components using high temperature and pressure) may be an effective further method of inactivation but requires validation. Trans-esterification of filtered tallow may also reduce TSE infectivity but, again, requires validation. The inactivating effect on TSE infectivity of contact with other fuels, blending agents, and fuel additives is unknown.

6.1.4 Use - Biodiesel Combustion

The effect of combustion in a diesel engine at more than 600 ºC could have some TSE inactivating capacity; however, the very short dwell time at high temperature may be insufficient for complete inactivation to occur if the fuel were to be contaminated by a TSE agent. Although all evidence to date indicates that TSE is not transmissible by inhalation, the potential risk of contaminated fuel aerosols cannot be entirely dismissed, particularly in closed environments.

6.2 Recommendations

6.2.1 Inactivation by the Rendering Process

Although experiments already performed indicate that rendered tallow does not contain detectable infectivity, even when present in the co-produced meat and bone meal, the result should be considered in the light of three qualifications:

1. The low level of infectivity in the BSE spike material
2. The limits of the bioassay detection method
3. The small number of relevant experiments.

A study is underway in the UK at the Veterinary Laboratories Agency (VLA) to examine the capability of a laboratory scale continuous vacuum method of rendering to inactivate the TSE infectivity of tallow spiked with a strain of murine BSE.

6.2.2 Study of North American Rendering Procedures

A scientifically sound risk assessment of tallow produced by North American rendering methods will require information about the actual chemical composition of the insoluble impurities in SRM tallow produced by different processes. A number of research institutes have the capability to initiate studies in this area, and could perhaps determine how potential risks might be reduced. If a sufficiently sensitive analysis showed that no residual protein was detectable in SRM tallow, and that any polypeptides present had a M_r of $\leq$ 10 000, then infectivity could be assumed to be absent or at such a low level as not to constitute a significant risk (SSC, 2001b).

The specifics of rendering to produce tallow, and to reduce the insoluble residues in tallow, merit further study. The following issues remain incompletely examined:

- The effect of various conditions (*e.g.*, temperature) on the rate of sedimentation of insolubles in liquid tallow ('tank bottom') during storage;
- The beneficial effects of using protein precipitants before sedimentation or filtration, and at what stage they should be applied;
- The achievable clearance level of the insoluble fraction after a second diatomaceous earth filtration;
- The chemical composition of the insoluble fraction by nitrogen, protein, polypeptide and amino acid content;
- Whether or not infectivity remains within the fat component of tallow (such as in a lipoprotein), as distinct from the insoluble fraction; and

- Methods to determine quickly, accurately and cheaply the protein content (with any accompanying prion protein content) of tallow for use in a quality control system using the Hazard Analysis and Critical Control Point (HACCP) principles.

6.2.3 Studies of Inactivation by Biodiesel Manufacture

Only one experiment has been carried out to examine the TSE inactivation potential of biodiesel production from animal tallow. This study was done as part of the SSC evaluation and subsequent approval of biodiesel as a way to dispose of tallow from Category 1 materials (that include SRM) in the EU. The data from this experiment were based upon immunoassay to detect misfolded protein (PrP^{TSE}) and have not been substantiated by bioassay.

Infectivity reduction from trans-esterification or catalytic hydro-treatment may or may not be significant, and should be subjected to experimental verification.

6.2.4 Studies of Inactivation by Biodiesel Combustion

No experiments have been undertaken to examine diesel engine combustion and inactivation capability under different operating conditions, and data from incineration experiments have questionable relevance to combustion. Distribution and use of biodiesel with any infectious potential, and dependence upon its combustion for inactivation, is unacceptable. Thus, risk management must focus on the earlier stages in the process of production to ensure a satisfactory supply of tallow devoid of any detectable infectivity and thus having a negligible TSE risk.

6.3 Conclusion

Biodiesel produced from animals infected with TSE poses a negligible risk to human and animal health. This conclusion extends even to the use of specified risk material (SRM) as a source of tallow, based on experimental evidence showing that rendered tallow from BSE- and scrapie-infected animal tissues does not transmit disease to inoculated healthy animals. Thus, although the capacity of biodiesel manufacturing steps to reduce infectivity should be irrelevant, any such reduction would still be desirable as an added measure of safety. At present, the potential for infectivity reduction through biodiesel manufacturing and combustion can only be estimated from analogy to methods known to inactivate infectivity in saline suspensions of contaminated tissue. Studies of the actual biodiesel process, using experimentally contaminated input tallow, are the only means by which more definitive, scientifically-based conclusions can be made about the capacity of these processes to reduce or eliminate TSE infectivity.

7 ADDENDUM

Recently Published Information of Significance

This report was largely written in 2004 and the first half of 2005. Where appropriate the report has been updated to reflect new information. In addition, there are some new publications that deserve to be mentioned. These are briefly discussed below.

The EC TSE Roadmap

This was published by the EC on 15 July 2005 (EC, 2005). In the light of an improving situation in the EU, the EC has discussed with Member States and the European Parliament amendments to current BSE measures that will enable the elimination of BSE without endangering the consumer. In the short to medium term (2005 to 2009) and in regard to tallow it is proposed to relax the processing procedures. How this might be done and on what Category of starting material is not discussed.

EFSA Opinion on the Safety of Tallow

The Scientific Panel on Biological Hazards of the EFSA, in answer to question number EFSA-Q-2003-099, have provided an Opinion on the assessment of the human and animal risk posed by tallow with respect to residual BSE risk. This has been adopted on 27-28 April 2005. The Opinion is given in the context of food and feed. However, it is pertinent to note amongst other things that a worst case mean estimate for human exposure due to tallow produced from a mixture of tissues with no SRM removed and sourced from a GBR Category IV country with unreliable surveillance is 1.6×10^{9} bovine oral ID_{50} per week. This is equivalent to 8.3×10^{-8} bovine oral ID_{50} per year. This is stated to be 48k times less than the exposure of humans in the UK at the time of highest exposure. The Opinion did not cover the issue if the tallow was produced entirely from SRM or Category 1 material, and processed by a method that did not inactivate infectivity in the co-produced MBM. Nevertheless, under the current situation pertaining in North America the risk in the starting materials, even if entirely SRM, is likely to be low and to present a low risk when further processed and refined to produce biodiesel.

Cumulative Dose

It is generally recognised that if the dose is large enough a single exposure is sufficient to infect and to initiate the chain of events leading to disease and death, *i.e.*, it is an "all or none" event. What has always been in question is whether or not doses lower than the minimal infectious dose, when administered repeatedly, can similarly infect and induce disease by way of accumulation. The first major study on this using the oral route in hamsters was flawed because the authors, Diringer, Roehmel, and Beekes, (1998) failed to administer the same number of hamster doses to the control animals as to the animals exposed repeatedly. Notwithstanding this criticism the authors showed that there may be a cumulative dose effect when the repeat doses were given close together. Subsequently Gravenor *et al*, (2003) showed that the probability of infection is inconsistent with the hypothesis that each dose acts as a cumulative or independent challenge.

A recent study reports that in mice there is a high incidence of scrapie when induced by repeated injections of sub-infectious doses (Jacquemot *et al*, 2005). The study showed that low prion doses constitute a risk for development of prion disease even if the same

total dose inoculated in a single challenge does not induce disease. Notwithstanding that this study used a parenteral route of administration it seems there is still some doubt about the role that sub-infectious doses may play in the effective exposure of individuals.

Infectivity Detected in Muscle and Nerve in Natural BSE

Buschmann and Groschup (2005) have recently shown that transgenic mice over-expressing bovine *PrP* have much shorter (208 days) incubation periods when inoculated with high titred brain material from cases of BSE than any conventional mice to which BSE has been transmitted. The comparative sensitivity of detection of BSE infection in the transgenic mice and conventional RIII mice was determined by end-point titration of a brainstem homogenate of known titre previously titrated in cattle. The transgenic mice were 104 times more sensitive than RIII mice and 10 times more sensitive than even cattle.

The transgenic mice were used to determine infectivity in several tissues of cattle with natural BSE and confirmed previous reports that infectivity was present only in central and peripheral nervous tissue and not in lymphoreticular tissues other than the Peyer's patches of the distal ileum. This incidentally confirmed that the pathogenesis of BSE in cattle is fundamentally different from the pathogenesis of natural and experimental scrapie in sheep and mice and experimental BSE in sheep, goats and mice. However, the increased sensitivity of the transgenic mice revealed infectivity in optic, sciatic and facial nerves (but not the radial nerve) and a solitary mouse died of TSE infection after inoculation of samples from the M. *semitendinosus*, a major muscle of the hind limb. No infectivity was detected in the Mm. *semimembranosus* or in the *longissimus dorsi*. The authors deduced that the maximum infectivity in the M. *semitendinosus* was 10^6 times lower than that in the brain; in other words it was very low.

Clearly the use of these ultra-sensitive mice to detect BSE infectivity in other derived products might reveal detectable infectivity that was not detected using conventional mice or cattle. This could apply, for example, to tallow derived from BSE-infected SRM and the precipitate removed by filtration of crude tallow. On the other hand, if no infectivity was found in such material inoculated in this way, it would give a greater confidence in the conclusion that tallow, even that derived from SRM, is a safe material to use as a source of biodiesel.

Further Remarks

It is premature to take any firm stance on the proposals put forward in the EC TSE Roadmap.

The EFSA Opinion on tallow is encouraging but not entirely aligned with either the source material or the processing conditions used to make biodiesel. Nevertheless it supports the view of the authors that any risk in the tallow is likely to be negligible.

In regard to a cumulative dose effect this is unlikely, in the context of the present TSE situation in North America and countries with a low prevalence and incidence of BSE, to impinge on the risk of using biodiesel made from tallow. Use of ultra-sensitive transgenic mice to detect infectivity in tallow and any precipitate removed by filtration, using BSE-spiked SRM as a starting material, could all provide even better data than exists for the determination of BSE risk when using such material for the generation of biodiesel.

Further References

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