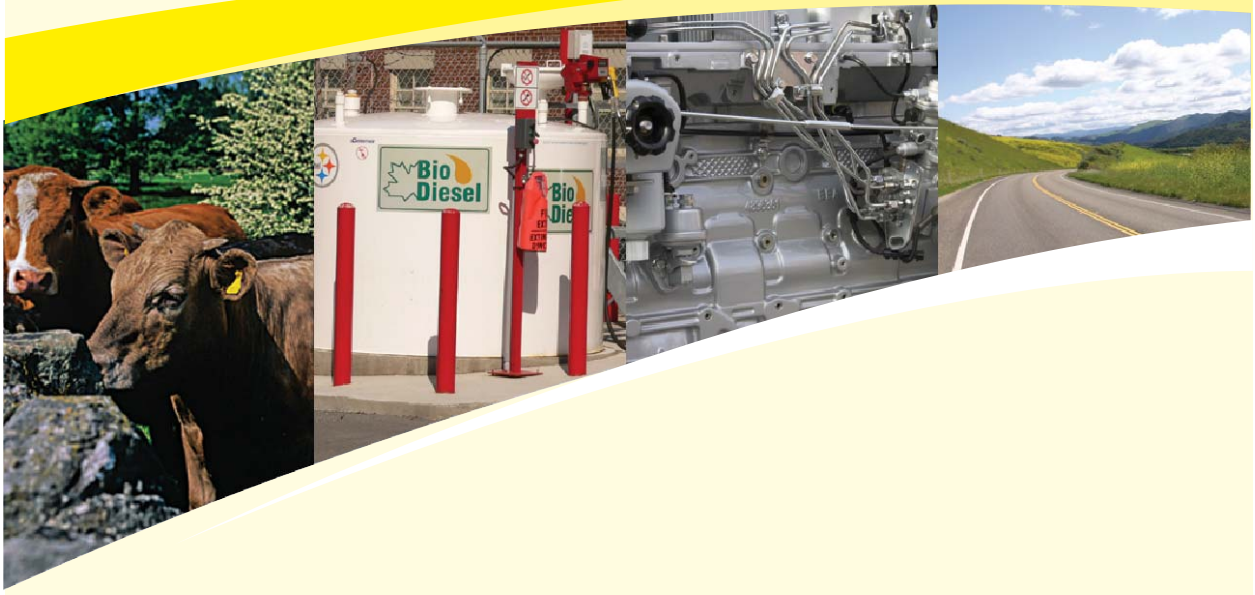
Detection of Prion Proteins and TSE Infectivity in the Rendering and Biodiesel Manufacture Processes



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Acknowledgements

Detection of Prion Proteins and TSE Infectivity in the Rendering and Biodiesel Manufacture Processes was prepared for the International Energy Agency's (IEA's) Executive Committee on Advanced Motor Fuels. The report was supported by agencies in Canada, Finland and the United States through their participation in the IEA's Implementing Agreement on Advanced Motor Fuels. The authors and ATFCAN gratefully acknowledge the Executive Committee on Advanced Motor Fuels, Natural Resources Canada, the United States Department of Energy and the National Technology Agency of Finland.

The views and opinions of the authors expressed in the report do not necessarily state or reflect those of the IEA, nor of any of the participating countries and supporting agencies.

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1 OVERVIEW

This paper addresses emerging issues related to monitoring prion proteins and TSE infectivity in the products and waste streams of rendering and biodiesel manufacture processes. Monitoring is critical to addressing the knowledge gaps identified in "Biodiesel from Specified Risk Material Tallow: An Appraisal of TSE Risks and their Reduction" (IEA's AMF Annex XXX, 2006) that prevent comprehensive risk assessment of TSE infectivity in products and waste. The most important challenge for monitoring TSE risk is the wide variety of sample types, which are generated at different points in the rendering/biodiesel production continuum. Conventional transmissible spongiform encephalopathy (TSE) assays were developed for specified risk material (SRM) and other biological tissues. These, however, are insufficient to address the diverse sample matrices produced in rendering and biodiesel manufacture.

This paper examines the sample types expected in rendering and biodiesel manufacture and the implications of applying TSE assay methods to them. The authors then discuss a sample preparation filtration, which has not yet been applied to these sample types, but which has the potential to provide or significantly improve TSE monitoring. The main improvement will come from transfer of the prion proteins from the sample matrix to a matrix compatible with conventional and emerging bioassays. A second improvement will come from preconcentrating the prion proteins, which means transferring proteins from a larger sample volume into a smaller volume for analysis to provide greater detection sensitivity. This filtration method may also be useful for monitoring other samples, including wash waters and other waste streams, which may contain SRM, including those from abattoirs and on-farm operations. Finally, there is a discussion of emerging mass spectrometric methods, which Prusiner and others have shown to be suitable for detection and characterisation of prion proteins (Stahl *et al.*, 1993). These new methods should be compatible with the variety of sample types expected, and should also work with the proposed filtration methods.

1.1 Monitoring for TSE Infectivity

For purposes of risk assessment, the authors assume that inputs to the rendering process include TSE-contaminated SRM. The fate of TSE infectivity through the rendering and biodiesel manufacturing processes will be a function of two main parameters. The first parameter is the transport of prion protein, which is assumed to be in a sample at some finite level for infectivity to be present. Different process streams designed to isolate various products will contain varying fractions of the prion protein. The authors assume for this section that the maximum TSE titre which could be present in a process stream can be calculated from the fraction of prion protein in that stream. The second parameter is inactivation, which may occur as a result of processing conditions (elevated temperature, elevated pressure, high pH, low pH, etc.).

Monitoring TSE infectivity throughout the rendering and biodiesel production processes requires determination of both the transport and inactivation parameters. It may be useful in some cases to monitor only transport, without assuming inactivation, to arrive at a "worst case" result for TSE infectivity in the various process streams. However, this approach will only work in streams where prion protein detection is possible. TSE infectivity may be present in samples where protein levels are below conventional detection limits. Inactivation can currently be monitored reliably only with bioassays, as will be discussed below.

1.2 Process Streams and Samples

The rendering process generates three major process streams or outputs, namely water, greaves and tallow. Each of these must be considered separately in terms of monitoring, together with sub-samples or further process streams.

The *greaves* from rendering are normally used in preparation of meat and bone meal (MBM), with subsequent use of MBM in further products, including feed, fertiliser and fuel. This stream contains the majority of protein introduced into the rendering process, and monitoring for PrP^{TSE} or TSE infectivity is required for assessing TSE risk.

Tallow produced in rendering is expected to contain low protein levels (BSE Inquiry, 2000); moreover, tests where virulent bovine spongiform encephalopathy (BSE) brain was spiked into the starting material did not detect infectivity in the tallow, even in a sample where the corresponding MBM was highly infective (Taylor, 1998). Nonetheless, monitoring PrP^{TSE} and TSE infectivity is considered here in the context of TSE risk assessment. Generally, residual greaves are removed from tallow by settling, pressing and centrifugation with further removal of insolubles by filtration. When tallow is stored, further solids are deposited into a layer of "tank bottoms," which are cleaned out from time to time. These secondary solids from tallow must be treated as equivalent to greaves, including the risk of TSE infectivity in these materials. Handling and disposal methods must be developed accordingly. After these solids are removed from the tallow, the risk of TSE will be even lower, but still must be considered for risk assessment. Monitoring for prion protein, PrP^{TSE} and TSE infectivity in tallow is required to achieve a complete assessment of TSE risk in tallow and subsequent products.

The *water* produced is normally released as steam or discarded as liquid waste. Depending on the concern for TSE in this stream (*i.e.*, the handling and disposal methods), initial monitoring could use total *PrP* screening. If high *PrP* levels are noted

in some samples, these could be further tested for TSE infectivity. *PrP* spiking experiments would be useful to assess the assumptions of *PrP* and total protein correlations.

Further materials are generated in the rendering and biodiesel manufacturing processes as products and waste (see Figure 1). Table 1 summarises the sample materials expected, along with the sample matrix compatibility for monitoring TSE risk. Generally, a sample matrix will be incompatible with conventional assays if it is non-aqueous. The conventional assays all use either biomolecules or whole animals, and these must be in contact with water-based matrices in order to work properly. Note that the compatibility table does not address the issue of whether detection will be sufficiently sensitive for assessment of that sample.

Figure 1 Source, Process and Use Stream for Biodiesel Manufactured from Tallow

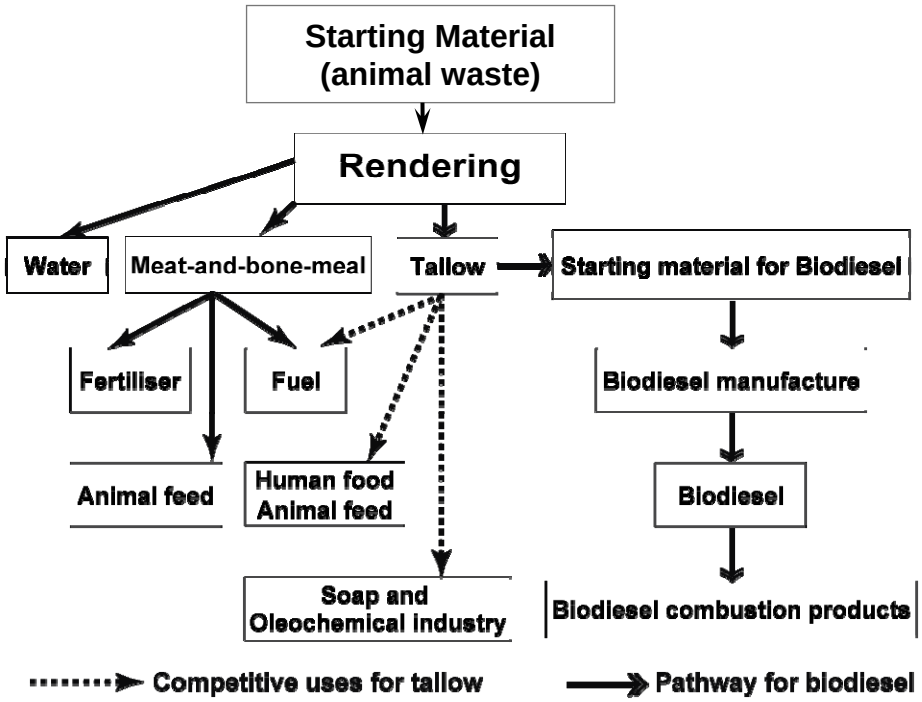


Table 1 Summary of Sample Types from Rendering and Biodiesel Manufacturing Steps and the Potential Use of Standard TSE-related Assays

Sample	Conventional *PrP* Assay (*i.e.* total prion)	PrP^{TSE} Specific Assay (*i.e.* misfolded prion)	TSE Infectivity Assay (*e.g.* mouse bioassay)
Rendering	Compatible with sample matrix?		
Waste Water	Yes	Yes	Yes
Greaves/MBM	Yes	Yes	Yes
Raw Tallow			
- residual greaves	Yes	Yes	Yes
- tallow	No	No	No
- filtered tallow	No	No	No
- filtrate (removed solids)	Unknown	Unknown	Unknown
- stored tallow	No	No	No
- tank bottoms	Unknown	Unknown	Unknown
Biodiesel Production			
Transesterification			
- waste water	Yes	Yes	Unknown
- settling tank bottoms	Unknown	Unknown	Unknown
- glycerine	Yes	Yes	Unknown
- product oils	No	No	No
Distillation			
- biodiesel	No	No	No
- distillation residue (waste oil)	No	No	No

2 PRION PROTEIN TRANSPORT

Analysing prion protein transport requires monitoring of total prion protein as well as specific PrP^{TSE}:

Total prion protein: Analysis of various samples for total prion protein content is the simplest method, but it assumes that the relative prion levels reflect the relative levels of TSE causative proteins, namely PrP^{TSE}. If the total TSE titre in the incoming material is known (*e.g.* spike experiment) or can be assumed (*e.g.* a "worst case" scenario), then a map of the distribution of total protein through the rendering and subsequent manufacturing processes could be used to estimate the maximum TSE titre which could be present in the various process streams.

TSE-causative prion proteins: Monitoring for PrP^{TSE} is more difficult than total prion protein analysis, but is a more reliable indicator of TSE infectivity. Current monitoring methods (described in the following sections) are used to screen SRM and related samples for TSE infectivity; and there is a substantial body of literature correlating TSE infectivity with PrP^{TSE} assay levels. It is unknown whether structural differences between normal *PrP* and PrP^{TSE} proteins could cause different fractionation patterns through the various process streams. Monitoring PrP^{TSE} initially must be done in parallel with total *PrP* monitoring, including PrP^{TSE} spiking experiments. If a strong correlation between *PrP* and PrP^{TSE} can be demonstrated, then routine monitoring may be able to test *PrP* alone as a reliable estimate of the maximum PrP^{TSE} in those samples.

The main limitation of both total protein and *PrP* monitoring using the standard or commercial assay methods is that neither can account for inactivation of TSE infectivity in the various process steps, as will be described below. Research literature reports methods which can directly detect *PrP* and PrP^{TSE} (Paramithiotis *et al.,* 2003), but it is not known when those methods will be available for routine use.

3 MONITORING FOR TSE INFECTIVITY AND INACTIVATION

Monitoring TSE infectivity is the most direct method of determining TSE risk of a sample. The main challenge in assessing the TSE risk of rendering and biodiesel materials is that the currently available bioassay methods are not compatible with most of the relevant sample types. A review of the current testing methods is presented here with the goal of understanding the challenges that currently exist for assessing the TSE risk in biodiesel. In addition, alternative/emerging sample preparation and analytical methods that may provide different approaches to TSE testing in samples with different matrix components are also examined.

The detection of prions is very dependent on the sample and matrix within which the prion is contained. The testing of materials for TSE has been limited to biological tissues, mainly the SRM from cattle and sheep, as well as tissue from mice used in bioassays of SRM. In the human health sector, parallel tests are done for related human samples. Traditional methods to confirm TSE are done on brain material and use histological methods to detect spongiform change or to detect misfolded protein by immunohistochemistry (or both). Detection of scrapie-associated fibrils by electron microscopy is another method used, especially on autolysed material. Also, molecular methods are now commonly used to detect misfolded protein, and these include a number of 'Rapid' tests, Western blotting and associated methods. But these tests can be done only in biological matrices. Immunoassay tests can be done after dilution into a test matrix, but the immuno-recognition (*i.e.* antibody/antigen) interaction is optimised for aqueous systems with well-defined sample constituents (Selby, 1999). This binding is highly dependent on the solution and matrix conditions (*i.e.* pH, ionic strength and polarity) and will not work in a biodiesel matrix. There are currently *no protocols* developed for isolating PrP^{TSE} from biodiesel or other organic solvent-like matrices.

A sample treatment method that dilutes the biodiesel into an aqueous fluid or transfers prion protein into an aqueous medium may permit the use of some conventional tests. Alternatively, new detection techniques such as mass spectrometry may be used to measure PrP^{TSE} directly in a non-aqueous matrix.

3.1 Bioassays for TSE

Bioassays using biomolecular assessment are the method of choice for detecting TSE infectivity (Gizzi, 2003). The assays begin by administering sample material to healthy animals, orally or by injection. After a set period of time, defined by the length of incubation in the host animal or the onset of clinical signs, the animal is euthanised and the brain examined for characteristic pathology. Although this test has been deemed the 'gold standard,' it suffers from long incubation periods. The bioassays using cattle require an incubation period of 30 months (Deslys *et al.*, 2001), which makes this test impractical except for research applications.

Bioassay methods have advanced significantly with the development of transgenic mice *(*Castilla *et al.*, 2003; Weissmann and Flechsig 2003), which are as sensitive as cattle and have shorter bioassay periods. The shorter incubation period of these bioassays, 150 to 196 days (Castilla *et al.*, 2003), allows for research and trending analysis but still prohibits use of the test in routine screening. To date, bioassay tests have been used exclusively on biological fluids, which have been the main focus for TSE contamination. Biological fluids and most tissue homogenates can be injected directly into a host, without modification and without significant deleterious effect on the organism. However, biodiesel cannot be injected directly into an organism's brain due to the acute toxic effects of the diesel. It may be possible to deliver biodiesel orally to mice, since the acute toxicity of similar samples such as crude oil is not severe (oral LD_{50}>4 g/kg, ref. MSDS); however, the oral exposure route is generally regarded as less efficient, and the resulting impact on TSE infectivity assessment is unknown. It may also be possible to use dilution of the diesel sample to lower the toxicity of the injected sample; but this will adversely affect the detection sensitivity of the assay.

3.2 Immunodiagnostic Tests

Several diagnostic tests for TSE/BSE have been developed. All are immunoassay-based tests, using a form of macromolecular binding interaction, a process through which an antibody with a specific affinity and ability to recognise an antigen (*PrP*), or certain epitopes within it, is purified.

Monoclonal antibodies to prions have also been developed and have been shown to enhance the assessment of tissues for indicators of BSE. By using these same antibodies in standard immunoassays, researchers have developed diagnostic tests which can screen for TSE in other samples (Grathwohl *et al.*, 1997; Grassi *et al.*, 2000; MacGregor 2001; Polymenidou *et al.*, 2002; Brun *et al.*, 2004). Although some commercial immunoassays use polyclonal antibodies, most use monoclonal antibodies because they are more sensitive and can selectively indicate prion material in matrices containing many other proteins and related substances. However, neither type of antibody can distinguish 'normal' prions (PrP^{C}) from BSE or TSE causative prions (PrP^{TSE}) by itself. For this reason, most assays include a pretreatment step to detect PrP^{TSE} in the sample by exploiting the resistance of these abnormal prions to degradation by Proteinase K. The sample is first exposed to Proteinase K, which degrades the PrP^{C} much more quickly than the comparatively resistant PrP^{TSE}. After this pretreatment, the sample

is then exposed to the antibody and only PrP^{TSE}, if present, is bound and detected. Some tests, such as the CDI test (Safar *et al.*, 2002), use a different pretreatment process but also enable antibody recognition of PrP^{TSE} distinct from PrP^{C}. Other reports use selective extraction of the *PrP* isoforms followed by immunoassay detection (Barnard *et al.*, 2000), (Paramithiotis *et al.*, 2003).

The European Commission has taken the lead in assessing current diagnostic tests in two comprehensive reports issued in 1999 (European Commission, XXIV, 1999) and 2002 (European Commission, XXIV, 2002). These reports used a common validation approach and common sample material (bovine brains from known BSE-positive animals) as well as a common negative control (bovine brains from New Zealand animals) to examine the performance of various tests. The tests were assessed in terms of sensitivity (proportion of known positive samples which test positive), specificity (proportion of known negative samples which test negative), and detection limit (degree to which a positive sample can be diluted with a negative sample and still test positive). The assay methods described above scored well in all three categories. As a result, they remain the most commonly used testing methods.

3.3 Detection Limits of Diagnostic Tests

The test property of greatest interest to this review is the detection limit. Unfortunately, the detection limit is a parameter which is difficult to define in absolute terms. Care must be taken to ensure that comparisons of detection limits are based on data which is directly related. In the EC studies, for instance, a common source material is used. This has a stated TSE potential, which had already been determined by the transgenic mouse bioassay as $10^{3.1}$ LD_{50}/g (mean lethal doses per gram) (European Commission, XXIV, 1999), meaning that if the material were diluted $10^{3.1}$-fold, then it would have a potency to induce TSE in 50% of exposed test mice. But the reported detection limits for the various assays used in these studies are relevant only for comparison within that group of results. For this reason, when comparing 1999 and 2002 data, EC examiners had to make a special effort to incorporate the 1999 material into the 2002 material and normalise the results for comparison (European Commission, XXIV, 2002). It is useful to note that the best immunoassay detection limits determined were similar to the mouse assay, which was confirmed in a separate study (Deslys *et al.*, 2001).

In principle, the detection sensitivity of the bioassays is very high. For example, one report concluded that a mouse bioassay can register a positive response to as little as 10^5 prion molecules (Prusiner 1991), which corresponds to ~5 femtograms (fg). Another report gives an absolute mass detection limit of 50 picograms (pg) for selective extraction followed by immunoassay detection (Barnard *et al.*, 2000). For tallow and biodiesel samples, however, very low levels of prion protein would be expected and diluting these samples to provide an aqueous matrix will further impede detection at low levels.

3.4 Current Experience in Monitoring of TSE in Tallow and Biodiesel

Before 2003, when industry (Saria Bio-Industries, GmbH) applied to the EC to add biodiesel production to the list of accepted disposal methods for BSE-contaminated materials, their application was only partially successful (Scientific Steering Committee and Directorate-General 2003), with only Category 2 and Category 3 materials admitted to the list. But after submission of further data in 2003, a subsequent review allowed Category 1 materials as well (Scientific Steering Committee and Directorate-General 2004), but with some reservations. The committee presented two main criticisms:

1. "For each of the different steps in the process (rendering, transesterification, hydrolysis) a log reduction of TSE infectivity of at least 10^3 is assumed. Experiments have been done on laboratory scale and as the kinetics of prion reduction are not understood at present it is therefore questionable whether these reductions found in all the steps of the process can be added up. However, since the material at the start of the process has already undergone a treatment of 133 degrees Celsius/20 minutes/3 bar rendering it may be concluded that the resulting biodiesel, as well as the by-products, do not carry a TSE risk."

Note that in North America the typical rendering conditions are not as stringent as described above. Virtually all rendering in North America is done at atmospheric pressure (1 bar) and at about 100 degrees Celsius.

2. The SSC's second criticism focused on testing:

   "A bioassay test, which would normally be the final proof of safety, can not be carried out due to the toxicity of the biodiesel. The conclusion on safety is only valid if the technical process reflects the conditions of the experimental report."

The first criticism stresses the need to characterise reduction of infectivity of the overall process. The assumption that reduction factors for individual process steps can be added up is untenable, as the prions which survive one step may just as easily survive another, or have an inherent resistance and thus have a higher-than-expected survival rate in subsequent processes. The second criticism indicates that standard bioassays for TSE infectivity with mice cannot be done, as was described above.

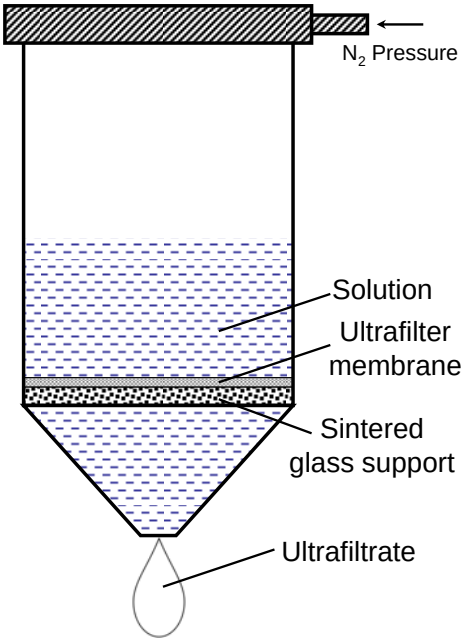
One further caution: the use of immunodiagnostics to screen biological tissue is justified, because the correlation between the assay results and bioassay results is well established. But immunodiagnostics may be used for biodiesel materials only if a similar correlation can be demonstrated. It could be possible that a rendering process does not inactivate the TSE-potential of PrP^{TSE}, but instead just transforms the binding portion of the prion such that it is not recognised by the immunoassay. The immunodiagnostic method might be proposed for routine screening, but the bioassay must be available for confirmation.

4 PROMISING NEW METHODS FOR *PrP* DETECTION

4.1 Isolation and Concentration of *PrP* by Ultrafiltration

The matrix constituents of biodiesel do not permit conventional analysis methods to be used directly. However a combination of a *PrP* isolation method (*e.g.* ultrafiltration, dialysis, precipitation, size exclusion) followed by either immunoassay or bioassay may provide a relatively simple detection methodology. Although isolation protocols such as dialysis and precipitation have been developed exclusively for aqueous samples, ultrafiltration methods may be compatible with organic matrixes.

Figure 2 Schematic Diagram of Ultrafiltration Process using N_2 Gas Pressure



Ultrafiltration is a membrane-based process for filtering water that forces water through a membrane with very small pores (see Figure 2). The structure of ultrafiltration membranes causes molecules larger than the molecular weight cutoff (MWCO) rating of the membrane to be retained on the surface, while allowing smaller species to pass through the membrane.

Ultrafiltration falls between reverse osmosis and microfiltration in terms of the size of particles/molecules removed, with ultrafiltration removing particles in the 5 000 to 100 000 Dalton range. Table 2 shows the molecular weight cutoff required to filter out common impurities. Table 3 shows chemical resistance of some of the most appropriate membranes. Note, however, that membrane behaviour and ultimate performance also depend on the specific characteristics of the sample being processed; and therefore new methods require testing and verification testing (see http://www.vivascience.com/en/faq/membrane_selection.shtml). Isolation of *PrP* requires a membrane which can retain molecules below 5 000 Dalton, which is possible with ultrafiltration as shown in Figure 3.

Table 2 Filtration Capacities of Ultrafiltration Membranes

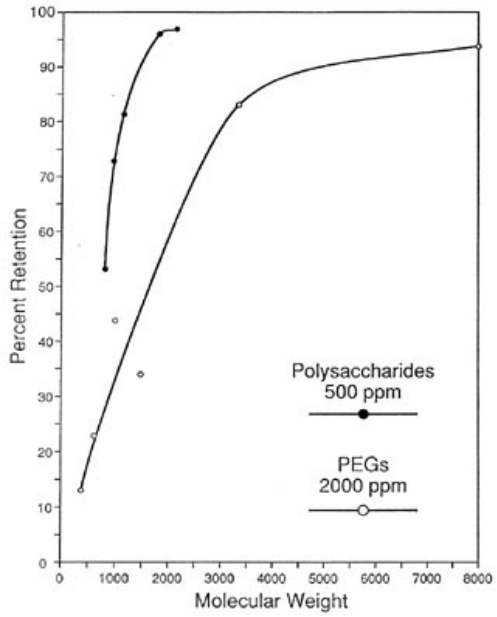
Species Retained	**Molecular Weight Cut Off of Ultrafiltration Membrane to Retain Various Biological Agents (Daltons)**				
	5 000	*10 000*	*30 000*	*50 000*	*100 000*
Bacteria					×
Virus			×	×	×
Proteins	×	×	×	×	×
Peptides	×				

Table 3 Chemical Resistance of Several Membrane Materials

	Composite	CA	PSO	PVDF	PAN	SiO_2	Cellulose
Water (3<pH<8)	✓	✓	✓	✓	✓	✓	✓
Water (pH<3 or pH>8)	✓	×	✓	✓	✓	✓	✓
Temp >35°C	✓	×	✓	✓	✓	✓	✓
Humic acid	(✓)	✓	×	×	(✓)	×	✓
Proteins	✓	(✓)	✓	(✓)	(✓)	✓	✓
Polysaccharides	(✓)	×	✓	×	(✓)	✓	×
Textile waste	✓	×	✓	(✓)	✓	×	×
Aliphatic hydrocarbon	×	×	×	(✓)	✓	✓	✓
Aromatic hydrocarbon	×	×	×	✓	×	✓	(✓)
Oxidizers	×	(✓)	✓	✓	(✓)	✓	(✓)
Ketones, Esters	×	×	×	✓	×	✓	(✓)
Alcohol	✓	×	✓	✓	✓	✓	✓

✓=high resistance/compatible (✓)=questionable resistance ×=low resistance/incompatible
CA=Cellulose acetate
PSO= Polysulfone
PVDF= Polyvinylidenedifluoride
PAN= polyacrylonitrile
SiO_2=silica ceramic
Source: Jorgen Wagner. Membrane Filtration Handbook: Practical Tips and Hints, 2nd Edition. (Nov. 2001). http://www.osmonics.com/library/mfh.htm

Figure 3 Retention of Polysaccharides and Polyethylene Glycols with an Ultrafiltration Membrane



Source: http://www.gewater.com/library/tp/827_Practical_Characterization.jsp

Van Holten *et al.* (2002) have shown that ultrafiltration can remove 2.5 log PrP^{Sc} from a scrapie brain homogenate and isolate it on a membrane filter. Therefore, a biodiesel sample could be ultrafiltered with a solvent-resistant nanofiltration membrane (Bhanushali *et al.*, 2003) to both remove and concentrate the PrP^{Sc} and other large molecular weight compounds. Following isolation, the PrP^{Sc} could be reconstituted in a more immunoassay/bioassay-friendly matrix for conventional detection and screening. As a further benefit, the isolated protein can potentially be analysed using mass spectrometry (as discussed below).

4.2 Testing for *PrP* Using Mass Spectrometry

With the advent of new ionisation techniques during the mid- and late 1980s, especially electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI), mass spectrometry has now become a well-established method for protein and peptide characterisation in all areas of life science research. Mass spectrometry, in particular, is a fast, reliable and relatively inexpensive method for protein analysis. Moreover, the widespread availability of expertise and instrumentation make mass spectrometry an ideal screening tool for disease detection, as in the now well-established newborn screening for metabolic disorders (Enns, 2001).

4.2.1 Sensitivity of Mass Spectrometric Methods

The detection of single atoms has demonstrated that analysing ions in a mass spectrometer can be an extremely sensitive process (Hurst *et al.*, 1977); and although single analyte molecules have not been detected yet in a mass spectrometer, several approaches in recent years have shown that mass spectrometry can detect low attomole or sub-attomole (picogram to femtogram) quantities of peptides or proteins, which is in the range of immunodiagnostic tests (*e.g.* Emmett and Caprioli 1994; Li *et. al*, 1996; Allmaier 1997; Onnerfjord 1998; Keller and Li, 2001). Nevertheless, because of competitive ionisation processes in the source of the mass spectrometer, such low detection limits can only be achieved after thorough sample pretreatment and cleanup, and isolation of the desired species through specific separations, thus substantially adding to the time and infrastructure required for a successful analysis.

4.2.2 Mass Spectrometry and the Prion Protein

The first applications of mass spectrometry in structural investigations of prion proteins compared conventional Edman sequencing with the newly developed peptide mapping to determine the primary protein structure (Stahl *et. al*, 1992 and 1993). The results confirmed that PrP^{C} and PrP^{TSE} are identical in terms of amino acid sequence and glycosylation patterns, which further suggested that conformational and/or other factors must be responsible for the infectivity of PrP^{TSE}. More recently, the same research group reported different extents of glycosylation for PrP^{C} and PrP^{TSE}; nevertheless, due to the complex heterogeneity of the glycosylation even within the same tissue at different locations, and the difficulty of exact quantitation with mass spectrometric methods, this characteristic probably cannot account for unambiguous identification of

the two forms in routine screening (Rudd *et al.*, 1999). Also, the usual partial proteinase digestion step is required to distinguish between PrP^{C} and PrP^{TSE}.

Another study investigated the proton/deuterium (H/D) exchange rate differences of normal and β-amyloid form (*i.e*. TSE form) of prion proteins (Nazabal *et al.*, 2003 and 2005). However, this intriguing but rather difficult approach has not become part of routine analysis. Nevertheless, this methodology has already been used to create an additional characterisation step to distinguish between normal and abnormal prion protein in the patent application by Krebs and Foerster, as indicated in Table 5.

An additional characteristic of prion proteins is their ability to bind metal ions, especially copper ions. As a result, mass spectrometry and other techniques have been used to investigate these prion-metal interactions (Hornshaw *et al.*, 1995, Whittal *et al.*, 2000). For example, recent research indicates that PrP^{C}'s binding affinity to copper differs from that of PrP^{TSE}; moreover, PrP^{TSE} appears to preferably bind to other metals, such as manganese or zinc (Brown 2004). But further investigation is needed to determine whether these characteristics can be effectively used to distinguish between PrP^{C} and PrP^{TSE}.

In addition, a new technology called "FAIMS" (high-**f**ield **a**symmetric waveform **i**on **m**obility **s**pectrometry) might help overcome the inability of mass spectrometry to directly distinguish between the two different forms of *PrP*. Developed at NRC in Ottawa, FAIMS is marketed by Ionalytics (now Thermo), a spin-off company. Employing FAIMS prior to conventional mass spectrometry provides the capability of separating different structural formations or conformers of the same protein before mass spectrometric analysis (Purves *et al.*, 2001, Borysik *et al.*, 2004 and www.faims.com). However, this technology has not yet been applied to prion proteins (Personal communication with Dr. Randy Purves, Ionalytics). Nevertheless, it would be interesting to investigate whether this technology is capable of distinguishing PrP^{C} and PrP^{TSE}. Recently, Ionalytics has collaborated with Dr. Richard Yost from the University of Florida to combine MALDI and FAIMS technology (Guevremont *et al.*, 2005). The combination has promise, since MALDI is a very sensitive ionisation method for proteins and allows sampling directly from a variety of surfaces, a quality which could be advantageous for the determination of prions in biodiesel.

4.2.3 Quantitation and Mass Spectrometry

Since TSE infectivity is likely related to the absolute amount of PrP^{TSE} present in a sample, quantitative analytical methods for prion detection are highly desirable. Because the chemical and physical characteristics of a substance have a tremendous influence on its ionisation efficiency in the source of a mass spectrometer, quantitative analysis by mass spectrometry remains a challenging task. But recent research employing isotopically labeled derivatising reagents for cysteine-containing proteins has been able to compare different protein expression levels in healthy and diseased tissues (Gygi *et al.*, 1999). More recently, improved labeling substances and techniques have made relative quantitation feasible even for low-abundance proteins (Hansen *et al.*, 2003). For prion proteins, developing isotopically labeled standards should be relatively easy and cost-effective, since only one standard compound needs to be developed per species. Since the technique relies on cysteine containing peptides that have been created by enzymatic digestion, it is also feasible to use just a few or even only one quantitation standard for **all** relevant species, if conserved motifs within the amino acid sequence of *PrP* are chosen for analysis. (For example, the peptide with the amino acid sequence (R)VVEQM**C**ITQYQR(E) is located within a conserved domain (www.ncbi.nlm.nih.gov/blast/Blast.cgi) and is present in more than 90 prion protein isoforms or prion proteins from different species. This peptide could be obtained by conventional enzymatic digestion of the prion protein, using trypsin). However, this quantitative approach may not be sufficiently sensitive.

Some current projects or published patents for the mass spectrometric quantitative determination of PrP^{C} and/or PrP^{TSE} are listed below (see Table 4 and Table 5). Several research groups are currently involved in developing mass spectrometric methods for prion detection. This table gives a sample of current ongoing research projects derived from information available from web-based sources:

Table 4 Current Research Projects for Quantitation of *PrP* by Mass Spectrometry

Title of Project	Group and location	Website
Sensitive quantification of PrP^{Sc} using mass spectrometry	Prof. A. Aguzzi ETH Zürich Switzerland	www.research-projects.unizh.ch/a141.htm
Differentiation of prion-strains using ICAT and mass spectrometry	Prof. A. Aguzzi ETH Zürich Switzerland	www.research-projects.unizh.ch/a141.htm
Discovery and quantification of TSE markers using mass spectrometry	Prof. A. Aguzzi ETH Zürich Switzerland	www.research-projects.unizh.ch/a141.htm
Identification of Marker Proteins for Prion Disease in Urine	Prof. A. Aguzzi ETH Zürich Switzerland	www.research-projects.unizh.ch/a141.htm
Development of new technologies for rapid identification of pathogen/pathogenic product	Dr. Stanker Foodborne Contaminants Research Unit, Albany, CA	www.ars.usda.gov/research/projects/projects.htm?ACCN_NO=405257
Molecular Method for Prion Strain Analysis	Prof. J. Requena Foodborne Contaminants Research Unit, Albany, CA	www.ars.usda.gov/research/projects/projects.htm?ACCN_NO=408803

Note that these projects are not yet completed, and it remains uncertain if these methods will become useful or accepted by the scientific community for early diagnosis for TSE infectivity.

Over the past few years, many new methods have been patented for the diagnosis or treatment of TSE related diseases, many of them employing mass spectrometry as an analytical tool. The following table lists a few examples where mass spectrometry is being used to analyse the pathogenic prion. Due to the urgency and importance of the subject, the number of patents and reports is expected to grow over the next years; nevertheless, whether some or all of these methods become accepted by the scientific community remains uncertain.

Table 5 Current Patents for Mass Spectrometric Detection of *PrP*

Year	Patent Title	Authors	Patent# and Source
2002	Mass spectrometric detection of abnormal prion protein in the diagnosis of transmissible spongiform encephalopathies	Krebs, S. Foerster, M.	EP1229331 ep.espacenet.com
2002	Detection and quantification of prion isoforms in neurodegenerative diseases using mass spectrometry	Everett, N. Petell, J.	WO02082919 ep.espacenet.com
2003	Method for detecting pathogenic prion proteins by means of mass spectroscopy	Lengsfeld, T.	20030134340 appft1.uspto.gov/netahtml/PTO/srchnum.html
2005	Determination of protease-resistant prion protein following a spontaneous transformation reaction	Gassner, D. Golla, R.	WO2005001481 ep.espacenet.com
2006	Methods to differentiate protein conformers	Onisko, B. C. Silva, C. Requena, J.	WO2006044551 ep.espacenet.com

5 FUTURE OPPORTUNITIES FOR THE DETECTION OF *PrP* AND *PrP*^{TSE}

The main challenge of using mass spectrometry to detect prions in biodiesel is successful isolation of adequate amounts of protein material from this non-polar matrix. Once a successful protocol for isolating protein from biodiesel has been developed, available mass spectrometric tests for prions should be easy to adopt into routine screening schemes. For example, MALDI time-of-flight mass spectrometry (TOF MS) could readily adopt ultrafiltration for protein detection. Several researchers have already shown that it is possible to use many kinds of porous and non-porous membranes as MALDI TOF MS sample supports (*e.g.* Blackledge and Alexander 1995; Blais *et al.*, 1996, McComb *et al.*, 1997). So membranes utilised for the ultrafiltration of biodiesel could be investigated for the presence of proteins in the same way. Such a methodology could then be expanded into a screening method for the presence of prions in biodiesel and/or the effectiveness of employed filtration steps for removal of prions from biodiesel. Preliminary results from simple biodiesel filtration employing specialized filtration disks indicate the feasibility of the above described approach. A poster regarding these initial research efforts was recently presented at the annual meeting of the American Society for Mass Spectrometry in Seattle (Douma *et al.*, 2006).

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ISBN 0-9781163-1-3