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SCIENTIFIC OPINION

Effect of incineration, co-incineration and combustion on TSE hazards in category 1 animal by-products

EFSA Panel on Biological Hazards (BIOHAZ) | Ana Allende | Avelino Alvarez-Ordóñez | Valeria Bortolaia | Sara Bover-Cid | Alessandra De Cesare | Wietske Dohmen | Laurent Guillier | Lieve Maria Herman | Liesbeth Jacxsens | Lapo Mughini-Gras | Maarten Nauta | Jakob Ottoson | Luisa Peixe | Fernando Perez-Rodriguez | Panagiotis Skandamis | Elisabetta Suffredini | Olivier Andreoletti | Vincent Béringue | John Griffin | Marion Simmons | Kamela Kryemadhi | Barbara Lanfranchi | Angel Ortiz-Pelaez | Romolo Nonno

Correspondence: biohaw@efsa.europa.eu

The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts

Abstract

The European Commission requested EFSA to assess the effect of incineration, co-incineration and combustion of Category 1 animal by-products (ABP) on the BSE/TSE hazards in ash resulting from these treatments. The presence of residual TSE hazards is assessed by detection of prion infectivity or seeding activity. TSE agents or prions are challenging to inactivate completely using heat-based methods. Different TSE strains exhibit varying degrees of thermoresistance. Based on available studies at temperatures 120–134°C, the C-BSE strain is more thermoresistant than other evaluated strains. The vast majority of Category 1 ABP is rendered into 'meat and bone meal' prior to incineration/co-incineration/combustion. Scenarios involving co-incineration for cement production do not need to be considered because all ash is incorporated into the cement. It is not possible to generalise the time/temperature combinations to which Category 1 ABP are subjected across all processes. Due to the challenges in precisely measuring the temperature and residence time in industrial systems, and the wide range of system designs and operating conditions, it can only be assumed that Category 1 ABP are exposed to at least the legal requirements as determined by the conditions of the gas produced or injected into the process: 850°C for 2 s or 1100°C for 0.2 s. The limited sensitivity of the method used in a study involving C-BSE at 1000°C for 20 min prevented a conclusive exclusion of residual C-BSE prions.. Therefore, it is not possible to exclude – with high certainty (> 99%) – the presence of residual BSE/TSE hazards in ash produced from the incineration, co-incineration or combustion of Category 1 ABP. It is recommended to generate data on the actual reduction of infectivity in 'meat and bone meal' spiked with thermoresistant TSE field strains after treatment with the time/temperature combinations required by the legislation or specific industry processes.

KEYWORDS

ABP, animal by-products, ash, combustion, incineration, prions, TSE

CONTENTS

SUMMARY

The European Food Safety Authority (EFSA) was asked by the European Commission to deliver a scientific opinion by 30 April 2025 on the effect of incineration, co-incineration and combustion of Category 1 material referred to in Article 8 Regulation (EC) No 1069/2009 on the BSE/TSE hazards in the ash resulting from these treatments (ToR1). If the outcome of ToR1 is that there is no residual TSE/BSE infectivity, then EFSA will have to assess the effect of incineration, co-incineration and combustion on the biological hazards other than the BSE/TSE and on the chemical hazards in the ash resulting from these treatments (ToR2).

The requestor elucidated the necessity for a high level of certainty that no residual TSE hazards remain following the processes, and that the threshold applied to applications on alternative processing methods of Category 1 animal by-product (ABP) (6 $\log_{10}$ reduction of TSE agents) is not relevant for this mandate. The presence of residual TSE hazards was assessed by the detection of TSE agent (or prion) infectivity or seeding activity and for the most thermoresistant animal TSE field strain as a worst-case scenario. Multiple data sources were used. Relevant literature was selected on the thermal inactivation of TSE agents. Different stakeholders from the industry were approached to gain insight into the practices for rendering, processing and disposal of Category 1 ABP in Europe. Previous EFSA scientific opinions and Scientific Steering Committee (SSC) minutes and opinions were reviewed, as well as a number of documents submitted by a stakeholder (European Sustainable Phosphorus platform (ESPP)), including scientific papers, old and ad hoc recent risk assessments.

In order to answer the ToR1, the mandate was translated into four assessment questions (AQ): AQ1: What is the most thermoresistant animal TSE field strain identified?; AQ2: What are the relevant/actual scenarios used by the industry in the EU for the processing and/or disposal of Category 1 material?; AQ3: What are the overall heat treatment (time/temperature) profiles of incineration, co-incineration or combustion processes before and after the gas is raised to the minimum legal requirement of 850°C for at least 2 s or 1100°C for 0.2 s?; AQ4: Can the presence of prions be excluded with more than 99% certainty in ash produced from Category 1 ABP after applying the time/temperature combinations of the relevant/actual scenarios identified in AQ2?

TSE agents are difficult to fully inactivate using heat-based methods. Different TSE strains exhibit varying degrees of thermoresistance. The matrix in which the prions are found and pretreatments (e.g. fixation or drying) can significantly influence their resistance to heat. Such variability compromises the possibility of extrapolating findings from one specific set of conditions to another.

Studies on the thermoresistance of EU TSE field strains are limited. Most research has concentrated on wet heat conditions at low temperatures relevant to cleaning and sterilisation, rather than the extreme dry heat conditions used in incineration. Evidence suggests that, among the evaluated strains at these lower temperatures, the C-BSE strain is more thermoresistant than other evaluated strains.

For practical reasons, the vast majority of ABP waste is rendered into 'meat and bone meal' (MBM) prior to incineration, co-incineration or combustion. It is acknowledged that the rendering of Category 1 ABP with method 1 provides a reduction of infectivity in the order of 3 $\log_{10}$, while method 4 (both methods as defined in Chapter III Annex IV of Commission Regulation (EU) No 142/2011), the second most applied method to Category 1 material in Europe, would provide an unknown reduction of infectivity, assumed to be lower than that achieved by method 1.

It has not been possible to determine accurately the relative amounts of Cat 1 ABP entering each scenario. It appears that rendering followed by co-incineration in cement plants and rendering followed by incineration in rotary kilns are currently the most common methods for the disposal of Category 1 ABP. During the process of co-incineration in cement plants, all the ash is incorporated into cement. Any scenario involving co-incineration results in cement production, and it does not need to be considered further because it does not result in ash as a by-product.

There are many different types of incineration, co-incineration and combustion systems in place in installations across Europe. These systems vary in design, size, capacity, structure and methodology. Consequently, the time/temperature combinations – and therefore the level of TSE agent reduction achieved – can vary significantly between systems. Given the wide range of system designs and operational conditions, it is not possible to generalise the time/temperature combinations to which Category 1 ABP are subjected across all processes.

Due to the challenges in precisely measuring the temperature and residence time in industrial systems, it can only be assumed that Category 1 ABP is exposed, during ash production, to at least the minimum legal requirements as determined by the conditions of the gas produced or injected into the process, namely, 850°C for 2 s or 1100°C for 0.2 s.

Only four TSE inactivation studies have examined conditions approaching those used in incineration, and none precisely replicate the time/temperature profiles specified in the regulation or achieved in industrial processes. While a treatment at 1000°C for 15 min has been demonstrated to completely inactivate the 263K hamster strain in tissues, no evidence is available for treatment durations of 0.2s or 2s. In the only study involving C-BSE at this temperature (1000°C) for 20 min, the limited sensitivity of the method used prevented a conclusive exclusion of residual C-BSE prions.

There is not sufficient relevant experimental data on the actual thermoresistance of TSE agents and on industrial operating conditions. Therefore, it is not possible to exclude, with high certainty (> 99%), the presence of residual BSE/TSE hazards in ash produced from the incineration, co-incineration or combustion of Category 1 ABP material.

It is recommended to collate and summarise actual time/temperature combinations to which MBM derived from Category 1 ABP is subjected during industrial processes; to conduct experimental studies comparing the thermoresistance of all animal TSE field strains identified in the EU under temperatures and processing conditions that reflect industry practices; to carry out experimental studies in which MBM is spiked with a thermoresistant TSE field strain (using C-BSE

in the absence of any alternative data) and then treated at the minimal time/temperature combinations required by the legislation or specific industry processes; and to evaluate the extent of the reduction of TSE agent infectivity achieved by processing methods other than method 1 (pressure sterilisation).

1 | INTRODUCTION

1.1 | Background and Terms of Reference as provided by the requestor

Organic fertilisers and soil improvers (OF/SI) are considered to be at the beginning of the feed chain. Therefore, Category 1 material referred to in Article 8 of Regulation (EC) No 1069/2009[1] are prohibited in the manufacturing of organic fertilisers and soil improvers (OF/SI).

Only derived products of Category 2 and 3 materials referred to in Article 32 of Regulation (EC) No 1069/2009 may be used in the manufacturing chain of OF/SI. Indeed, several scientific assessments by the European Commission's Scientific Steering Committee (SSC) and the European Food Safety Authority (EFSA) considered ash derived from incineration, co-incineration and combustion, carried out in accordance with methods laid down in Annex III to Regulation (EU) No 142/2011, of Category 2 and 3 materials as safe materials for the manufacturing of OF/SI. Ash from Category 3 and Category 2 materials may be used directly as fertiliser, mixed into compound fertilisers or used as starting material for the extraction of phosphorus for further use in fertilisers.

The prohibition of use of Category 1 material in the manufacturing chain of OF/SI aims to prevent, control and eradicate certain transmissible spongiform encephalopathies (TSE) as referred to in Article 3(1)(a) of Regulation (EC) No 999/2001,[2] in particular to prevent the BSE prion from being recycled in the feed chain through the fertilisers and soil improvers. Another key measure in the prevention and eradication of TSE is the safe collection and disposal of specified risk materials (SRM) as defined in Article 3(1)(g) of the afore-mentioned Regulation. The prohibition of use of Category 1 material, and in particular SRM, in the feed chain is based on science and it has been in application since the first feed ban in 1994 introduced by Decision 94/381/EC.[3]

In accordance with Article 12 of Regulation (EC) No 1069/2009, Category 1 material collected at slaughterhouses and other establishments should be disposed as waste by incineration or, when used for manufacturing of renewable fuels, processed into meat-and-bone meal (MBM).

However, none of the following scientific opinions considered the revalorisation and recycling of ash from Category 1 material in the manufacturing chain of OF/SI as a safe method for the prevention and eradication of TSE:

- Overview of the BSE risk assessments of the European Commission's Scientific Steering Committee (SSC) and its TSE/BSE ad hoc Group[4];
- Opinion on open burning of potentially TSE-infected animal materials adopted by the Scientific Steering Committee at its meeting of 16–17 January 2003[5];
- Opinion on the use of small incinerators for BSE risk reduction by the Scientific Steering Committee meeting of 16–17 January 2003[6]; and
- Opinion of the Scientific Panel on Biological Hazards of the European Food Safety Authority on the 'Quantitative risk assessment of the animal BSE risk posed by meat and bone meal with respect to the residual BSE risk'.[7]

Furthermore, the Commission is currently not aware of any new scientific data, evidence, publication, assessment or technological solution that would require revision of the existing legislation on animal by-products and TSE in view of revalorisation of Category 1 ash for uses in the manufacturing chain of OF/SI.

This is why, following several requests from the fertilisers industry, in particular from European Sustainable Phosphorous Platform (ESPP), to explore the valorisation of currently prohibited resources for the recycling of phosphorus as new resources for the manufacturing of fertilisers, the Commission seeks for a review of the existing scientific literature in order to explore the possible presence of biological and chemical hazards in ash from Category 1 materials after incineration, co-incineration and combustion.

Terms of reference

In the light of the above, in accordance with Article 29 of Regulation (EC) No 178/2002,[8] the Commission requests EFSA to provide a scientific opinion considering the following terms of reference:

[1]Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21 October 2009 laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (Animal by-products Regulation, OJ L 300, 14.11.2009, p. 1.

[2]Regulation (EC) No 999/2001 of the European Parliament and of the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (OJ L 147 31.5.2001, p. 1).

[3]Commission Decision 94/381/EC of 27 June 1994 concerning certain protection measures with regard to bovine spongiform encephalopathy and the feeding of mammalian derived protein (OJ L 172, 7.7.1994, p. 23).

[4]https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_ssc_out364_en.pdf.

[5]https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_ssc_out310_en.pdf.

[6]https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_ssc_out311_en.pdf.

[7]EFSA Journal (2005) 257, 1–30.

[8]Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety (OJ L 31, 1.2.2002, p. 1).

1. to assess the effect of incineration, co-incineration, and combustion of Category 1 material referred to in Article 8 Regulation (EC) No 1069/2009 on the BSE/TSE hazards in the ash resulting from these treatments.

   If the outcome of ToR1 is that there is residual TSE/BSE infectivity, then there is no need to proceed to ToR2.

2. to assess the effect of incineration, co-incineration, and combustion of Category 1 material referred to in Article 8 Regulation (EC) No 1069/2009 on the biological hazards other than the BSE/TSE and on the chemical hazards in the ash resulting from these treatments.

1.2 | Interpretation of the Terms of Reference (if appropriate)

- The requestor elucidated the necessity for a high level of certainty that no residual BSE/TSE hazards remain following the processes, as this would result in a revision of Regulation (EC) No 1069/2009 allowing the use of Category 1 material for applications that are presently prohibited.
- ToR1 will be addressed by defining the effect as the absence of residual BSE/TSE hazards (prions) with a high level of certainty, which is interpreted as a probability > 99% (almost certain) that no residual TSE hazard remains following the processes.
- The requestor also clarified that the threshold applied to animal by-products (ABP) applications on alternative processing methods of Category 1 ABP is not relevant for this mandate because, in this case, there is no comparison to an approved method that achieves or is assumed to achieve at least a reduction of 6 $\log_{10}$ of TSE in the matrix of interest, as it is currently applied by EFSA in the evaluation of alternative methods for processing or disposal of Category 1 ABP (EFSA BIOHAZ Panel, 2015, 2020, 2021). This is the case regardless of the potential infectivity load before the treatment and regardless of the pre-treatment (method 1 or any other) applied to the raw material.
- The effect of incineration, co-incineration and combustion on TSE agents is assessed by considering the available evidence on the effect of heat treatment on reducing/eliminating prions.
- The presence of prions will be assessed by detection of TSE agent infectivity or seeding activity. The opinion will assess the residual infectivity for the most thermoresistant animal TSE field strain as a worst-case scenario (WCS).

The ToR1 of the mandate was translated into four assessment question(s) (AQ):

- AQ1: What is the most thermoresistant animal TSE field strain identified?
- AQ2: What are the relevant/actual scenarios used by the industry in the EU for the processing and/or disposal of Category 1 material?
- AQ3: What are the overall heat treatment (time/temperature) profiles of incineration, co-incineration or combustion processes before and after the gas resulting from the processes is raised to the minimum legal requirement of 850°C for at least 2 s or 1100°C for 0.2 s?
- AQ4: Can the presence of prions be excluded with more than 99% certainty in ash produced from Category 1 ABP after applying the time/temperature combinations of the relevant/actual scenarios identified in AQ2?

2 | DATA AND METHODOLOGIES

2.1 | Data

2.1.1 | Thermal inactivation of TSE agents

Relevant literature was selected by the working group. No extensive literature search was conducted since it was acknowledged that key papers are limited, and it would be unlikely that any relevant paper would be missed. The papers were reviewed in full, and data were extracted for the descriptive analysis of the experiments in which field and/or laboratory TSE strains were subject to heat treatments in different matrices.

2.1.2 | Data related to industry practices

The European Fat Processors and Renderers Association (EFPRA) was contacted to gain insight into the practices for the rendering and disposal of Category 1 ABP in Europe. In particular, the enquiry focused on the amount and disposal of Category 1 ABP and mixes with other categories; the standard processing methods applied for rendering at European and national level, and the treatment applied to non-rendered wet material such as carcasses from positive or suspect TSE cases.

Three stakeholders, two of them selected from the list of EU countries' approved establishments in the ABP field[9] and one European association, were invited as hearing experts to provide data on the three disposal methods: incineration, co-incineration and combustion. Generic operational questions were put to the hearing experts, including: the type of raw or fuel material and the processing steps until the end product and by-products are produced; the conditions in terms of time, temperature, pressure and mode under which incineration, co-incineration and combustion are applied to Category 1 material (or ABP in general); treatment, management and analyses conducted on the ashes (bottom ash, slag and fly ash); application, if any, of incineration, co-incineration and combustion to fresh (wet) material such as carcasses from positive or suspect TSE cases directly without rendering, or to rendered material (meat and bone meal – MBM). The data provided by the hearing experts were considered by the working group. Whenever the information provided by industry, in the form of ad hoc consultation or participation in working group meetings as hearing experts, is reflected in the scientific opinion, it has been referenced as a personal communication.

2.1.3 | Other data sources

The previous EFSA scientific opinions and Scientific Steering Committee (SSC) minutes and opinions dealing with incineration, co-incineration and combustion of ABP were reviewed. Information relevant for this risk assessment is described in Section 3.2.

A number of documents were submitted by a stakeholder (European Sustainable Phosphorus platform (ESPP)), including: scientific papers, risk assessments from the late 80s, SSC opinions, as well as a legal opinion on 'End of Waste' and use of Cat 1 ABP incineration ash as fertiliser commissioned by ESSP to Environmental law Chambers Ltd., a new risk appraisal of use of Category 1 ABP ash as fertiliser commissioned by ESPP to SAFOSO and an assessment on the sanitary safety of ABP ash commissioned by ESPP to bio.e Biosystems Engineering Ltd.

Whenever any information provided by the stakeholder is reflected in the scientific opinion, it has been referenced accordingly.

2.2 | Methodologies

The approach to answer the ToR was defined in advance and is described in the protocol (Annex A). Protocol development followed the draft framework for protocol development for EFSA's scientific assessments (EFSA, 2020). It covers both the problem formulation (i.e. what the assessment aims to address) and which methods will be used for addressing the problem. The problem formulation ('what') includes the clarification of the mandate (see Section 1.2) and consists of the steps (1) translation of the mandate into scientifically answerable assessment questions (AQ), (2) definition of the subquestions (SQ) of each AQ, if needed, and their relationship (conceptual model) and (3) the selection of the approach for the assessment. The planning of the methods for conducting the assessment ('how') consists of (1) specifying the evidence needs and the methods for answering each AQ, including uncertainty analysis and (2) the methods for integrating evidence across AQ/SQ and addressing the remaining and overall uncertainty.

TSE strains other than those identified in AQ1 and laboratory strains were considered in the narrative of the answer, acknowledging that they may experience a higher level of reduction, without concluding on the residual infectivity.

The answer to AQ4 was provided by consensus judgement within the working group, considering the impact on the most thermo-resistant animal TSE field strains (AQ1) of the actual and total exposure times to temperatures contributing to the reduction of infectivity (AQ3) in the scenarios used by the industry in the EU (AQ2). For this purpose, evidence on inactivation dynamics of animal TSE field strains, processing parameters, and the specific characteristics of the standard processes applied were collected. When not available at the temperature ranges of the relevant scenarios, all data extracted from the relevant literature on the effect of heat on prions at temperature and time combinations the closest to the minimum requirement of the legislation were assessed.

The sources of uncertainty were identified while describing the data from relevant studies on heat inactivation of prions at low and high temperatures using animal TSE field and laboratory strains.

The available data have been reviewed together with the uncertainty, and whenever it is concluded that there are no data available for the conditions under assessment and no conclusions can be drawn under conditions other than those reported in the studies, then the answer to AQ4 will be NO, even if a reduction in prion infectivity in the final material can be ascertained and acknowledged.

The answer to AQ4 will be YES only if, based on the evidence, the experts judge that there is more than 99% certainty that the ashes produced under the conditions of a selected scenario do not contain any prion infectivity.

The approaches and methodology applied for answering some AQ can be found in full in the protocol (Annex A), while more details for methods used for other AQ are provided in the following sections. AQ3 was slightly amended after approval of the protocol, to the following: What are the overall heat treatment (time/temperature) profiles of incineration, co-incineration or combustion processes before and after the gas is raised to the minimum legal requirement of 850°C for at least 2 s or 1100°C for 0.2 s?

[9] https://food.ec.europa.eu/safety/animal-products/approved-establishments-abp_en.

3 | ASSESSMENT

3.1 | Legal framework

3.1.1 | Category 1 animal by-products

The main legislation relating to ABP and derived products not intended for human consumption is set out in Regulation (EC) No 1069/2009 and its implementing regulation, Commission Regulation (EU) No 142/2011. Category 1 animal by-products are defined in Article 8 of Regulation (EC) No 1069/2009. They consist of the highest risk animal by-products, including the following:

a. entire bodies and all body parts, including hides and skins, of the following animals[10]

- *(i) animals suspected of being infected by a TSE in accordance with Regulation (EC) No 999/2001 or in which the presence of a TSE has been officially confirmed;*
- *(ii) animals killed in the context of TSE eradication measures;*
- *(iii) animals other than farmed and wild animals, including in particular pet animals, zoo animals and circus animals;*
- *(iv) animals used in a procedure or procedures defined in Article 3 of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes), in cases where the competent authority decides that such animals or any of their body parts have the potential to pose serious health risks to humans or to other animals, as a result of that procedure or those procedures without prejudice to Article 3(2) of Regulation (EC) No 1831/2003;*
- *(v) wild animals, when suspected of being infected with diseases communicable to humans or animals;*

b. the following material:

- *(i) Specified risk material*
- *(ii) entire bodies or parts of dead animals containing specified risk material at the time of disposal;*

c. animal by-products derived from animals which have been submitted to illegal treatment as defined in Article 1(2)(d) of Directive 96/22/EC or Article 2(b) of Directive 96/23/EC;

d. animal by-products containing residues of other substances and environmental contaminants listed in Group B(3) of Annex I *to Directive 96/23/EC, if such residues exceed the permitted level laid down by Community legislation or, in the absence thereof, by national legislation;*

e. animal by-products collected during the treatment of waste water required by implementing rules adopted under point (c) of the first paragraph of Article 27:

- *(i) from establishments or plants processing Category 1 material; or*
- *(ii) from other establishments or plants where specified risk material is being removed;*

f. catering waste from means of transport operating internationally;

g. mixtures of Category 1 material with either Category 2 material or Category 3 material or both.

The methods for disposal or use of Category 1 materials are set out in Article 12 of Regulation (EC) No 1069/2009. According to that article, *Category 1 material shall be:*

(a) disposed of as waste by incineration:

- (i) *directly without prior processing; or*
- (ii) *following processing, by pressure sterilisation if the competent authority so requires, and permanent marking of the resulting material;*

(b) recovered or disposed of by co-incineration, if the Category 1 material is waste:

- *(i) directly without prior processing; or*
- *(ii) following processing, by pressure sterilisation if the competent authority so requires, and permanent marking of the resulting material;*

Under Article 12, Category 1 animal by-products can also be used as a fuel for combustion with or without prior processing. In addition to incineration, co-incineration and combustion, Category 1 material can be disposed of or used in other

[10] Legal text included verbatim in the opinion appears in italics.

ways. For example, Category 1 material, other than animal by-products from TSE suspects, TSE confirmed animals and animals killed in the context of TSE eradication measures, can be disposed of an authorised landfill following pressure sterilisation. Category 1 animal by-products can also be used in the manufacture of derived products under certain conditions.

Method 1 (pressure sterilisation) is the standard method for processing Category 1 material. According to Chapter III Annex IV of Commission Regulation (EU) No 142/2011, the competent authority of a member state can allow the application of methods other than method 1 for the rendering of Category 1 ABP. There are four other standard approved methods for Category 1 ABP: methods 2,3,4 and 5. The most frequently applied methods to process Category 1 ABP in order to produce MBM are method 1 and method 4 (see Section 3.5.1.1).

According to point A.2 Chapter III Annex IV of Commission Regulation (EU) No 142/2011:

A. Processing method 1 (pressure sterilisation)

The animal by-products with a particle size of no greater than 50 millimetres must be heated to a core temperature of more than 133°C for at least 20 min without interruption at a pressure (absolute) of at least 3 bars. The pressure must be produced by the evacuation of all air in the sterilisation chamber and the replacement of the air by steam ('saturated steam'); the heat treatment may be applied as the sole process or as a pre- or post-process sterilisation phase.

According to point D.2 Chapter III Annex IV of Commission Regulation (EU) No 142/2011:

D. Processing method 4

After reduction, the animal by-products must be placed in a vessel with added fat and heated in a manner which ensures that a core temperature greater than 100°C is achieved for at least 16 min, a core temperature greater than 110°C is achieved for at least 13 min, a core temperature greater than 120°C is achieved for at least 8 min and a core temperature greater than 130°C is achieved for at least 3 min.

The core temperatures may be achieved consecutively or through a coincidental combination of the time periods indicated.

3.1.2 | Incineration, co-incineration and combustion

Annex I of Commission Regulation (EU) No 142/2011 introduces the following definitions:

- *'**Co-incineration**' means the recovery or disposal of animal by-products or derived products, if they are waste, in a co-incineration plant;*
- *'**Combustion**' means a process involving the oxidisation of fuel in order to use the energy value of the animal by-products or derived products, if they are not waste;*
- *'**Incineration**' means the disposal of animal by-products or derived products as waste, in an incineration plant, as defined in point 4 of Article 3 of Directive 2000/76/EC;*
- *'Incineration and co-incineration residues' means any residues as defined in point 13 of Article 3 of Directive 2000/76/EC, which are generated by incineration or co-incineration plants treating animal by-products or derived products*. Residues include bottom ash, slag and fly ash;
- *'**Co-incineration plant**' means any stationary or mobile plant whose main purpose is the generation of energy or the production of material products as defined in point 5 of Article 3 of Directive 2000/76/EC;* According to this article, a plant can only be designated as a co-incineration plant if it uses waste as a regular or additional fuel or where waste is thermally treated for the purpose of disposal;
- *'**Incineration plant**' means any stationary or mobile technical unit and equipment dedicated to the thermal treatment of waste as defined in point 4 of Article 3 of Directive 2000/76/EC*.

Under Article 24 of Regulation (EC) No 1069/2009, operators of establishments or plants that carry out disposal of waste by incineration, co-incineration (if they are waste) or combustion of animal by-products and derived products must be approved, excluding establishments or plants which have a permit to operate in accordance with Directive 2000/76/EC (now superseded by Directive 2010/75/EU).

3.1.2.1 | *Incineration and co-incineration*

Generally, plants that are involved in the incineration or co-incineration of Category 1 animal by-products operate under the provisions of Directive 2000/76/EC (now superseded by Directive 2010/75/EU) rather than Regulation (EC) No 1069/2009 unless they are incinerating animal carcasses. This is based on exclusion provisions set out in Article 2 of Directive 2008/98/EC (Waste Framework Directive). This Directive sets the basic concepts and definitions related to waste management, including definitions of waste,[11] recycling and recovery.

[11]Waste' means any substance or object which the holder discards or intends or is required to discard; (Point 1 of Article 3 of Directive 2008/98/EC on waste ("the Waste Framework Directive").

Under Article 43 of Regulation (EC) No 1069/2009, the export of animal by-products and derived products destined for incineration or landfill is prohibited.

Incineration, co-incineration and combustion plants are widely distributed throughout the Member States of the EU. Article 47 of Regulation (EC) No 1069/2009 requires each member state of the EU to draw up a list of establishments, plants and operators which have been approved or registered in accordance with the Regulation within its territory.[12] These, including incineration, co-incineration and combustion plants, are listed in the animal by-products section of the official website of the European Union.

Annex III of Commission Regulation (EU) No 142/2011 sets out the requirements for incineration, co-incineration and combustion plants. Section 2 of Chapter I of the annex requires that '*Incineration or co-incineration plants shall be designed, equipped, built and operated in such a way that the gas resulting from the process is raised in a controlled and homogeneous fashion, even under the most unfavourable conditions, to a temperature of 850°C for at least 2s or to a temperature of 1100°C for 0.2s, as measured near the inner wall or at another representative point of the chamber where the incineration or the co-incineration is carried out, as authorised by the competent authority.*'

The addition of 1100°C for 0.2s was the result of an evaluation of the combustion of tallow in a thermal boiler process for safe disposal of Category 1 ABP (EFSA BIOHAZ Panel, 2004), in which it was concluded that the burning process at 1100°C and 0.2s is more complete than at 850°C and 2s. With an estimated 0.13% C in ash from the total carbon flow, and 99.7% as CO_2, 0.12% as CO and 0.02% as total organic carbon (TOC) the C-compounds are utilised up to 99.97% when these conditions are applied.

Point 6, Section 1, Chapter 1 states that '*incompletely incinerated animal by-products must be reincinerated or disposed of by other means, other than by disposal in an authorised landfill, in accordance with Articles 12, 13 and 14, as applicable, of Regulation (EC) No 1069/2009*'. Section 3, Chapter 1 states that '*Incineration and co-incineration residues shall be minimised in their amount and harmfulness. Such residues must be recovered, where appropriate, directly in the plant or outside it in accordance with relevant Union legislation or disposed of in an authorised landfill.*' '*Transport and intermediate storage of dry residues, including dust, shall take place in such a way as to prevent dispersal in the environment, such as in closed containers.*'

Annex III Chapter II Section I of Commission Regulation (EU) No 142/2011 establishes the specific operating conditions for high-capacity incineration and co-incineration plants. '*Incineration or co-incineration plants treating only animal by-products and derived products with a capacity of more than 50 kg per hour (high-capacity plants) and which are not required to have a permit to operate in accordance with Directive 2000/76/EC shall comply with the following conditions:*

a. *The plants must be equipped for each line with at least one auxiliary burner. This burner shall be switched on automatically when the temperature of the combustion gases after the last injection of combustion air falls below 850°C or 1100°C, as applicable. It must also be used during plant start-up and shut-down operations to ensure that the temperature of 850°C or of 1100°C, as applicable, is maintained at all times during these operations and as long as unburned material is in the chamber where the incineration or co-incineration is carried out.*
b. *When animal by-products or derived products are introduced into the chamber where the incineration or co-incineration is carried out by a continuous process, the plant must operate an automatic system to prevent the introduction of animal by-products or derived products at start-up, until the temperature of 850°C or of 1100°C, as applicable, has been reached, and whenever the temperature is not maintained.*
c. *The operator must operate the incineration plant in such manner that a level of incineration is achieved such that the slag and bottom ashes total organic carbon content is less than 3% or their loss on ignition is less than 5% of the dry weight of the material. If necessary, appropriate techniques of pre-treatment shall be used'.*

Annex III Chapter III Section I of the same Regulation establishes the specific operating conditions for low-capacity incineration and co-incineration plants. '*Incineration and co-incineration plants treating only animal by-products and derived products with a maximum capacity of less than 50 kg of animal by-products per hour or per batch (low-capacity plants) and which are not required to have a permit to operate in accordance with Directive 2000/76/EC shall:*

a. *only be used for the disposal of:*

   (i) *dead pet animals referred to in Article 8(a)(iii) of Regulation (EC) No 1069/2009;*
   (ii) *Category 1 materials referred to in Article 8(b), (e) and (f), Category 2 materials referred to in Article 9 or Category 3 materials referred to in Article 10 of that Regulation; and.*
   (iii) *dead individually identified equine animals from holdings not subject to health restrictions in accordance with Article 4(5) or 5 of Directive 2009/156/EC, if authorised by the Member State;*

b. *when Category 1 materials referred to in Article 8(b) of Regulation (EC) No 1069/2009 are introduced into the low-capacity plant, be equipped with an auxiliary burner;*
c. *operate in such way that the animal by-products are completely reduced to ash'.*

[12] https://food.ec.europa.eu/safety/animal-products/approved-establishments-abp_en.

3.1.2.2 | *Combustion*

Specific rules for the operation of combustion plants were introduced in 2014 through the amendment of Commission Regulation (EU) No 142/2011 by Commission Regulation (EU) No 592/2014. Commission Regulation (EU) No 142/2011 was amended by Commission Regulation (EU) 2020/735 to permit the combustion of meat-and-bone meal as a fuel. Specific requirements for meat-and-bone meal as a fuel for combustion relating to issues such as meat-and-bone meal storage, fuel systems and emission requirements are set out in Chapter V, Point D of Annex III.

Annex III Chapter IV Section 2 '*Operating conditions of combustion plants*' of Commission Regulation (EU) No 142/2011 establishes that combustion plants must be designed, built, equipped and operated in such a way that even under the most unfavourable conditions the animal by-products and derived products are treated for at least 2 s at a temperature of 850°C or for at least 0.2 s at a temperature of 1100°C.

The gas resulting from the process is raised in a controlled and homogeneous fashion for 2 s to a temperature of 850°C or for 0.2 s to a temperature of 1100°C. The temperature must be measured near the inner wall or at another representative point of the combustion chamber, as authorised by the competent authority.

Annex III Chapter V establishes types of plants and fuels that may be used for combustion and specific requirements for particular types of plants. Two of them are allowed to process Category 1 material:

Stationary internal combustion engines

1. *Starting material*:

For this process, a fat fraction derived from animal by-products of all categories may be used provided it meets the following conditions:

a. *unless fish oil or rendered fat is used which has been produced in accordance with Section VIII or XII of* Annex III *to Regulation (EC) No 853/2004, respectively, the fat fraction derived from animal by-products must first be processed using:*

   (i) in the case of a fat fraction of Category 1 and 2 materials, any of the processing methods 1 to 5 as set out in Chapter III of Annex IV.
   (ii) Where this fat is moved by a closed conveyer system, which may not be by-passed, and provided such a system has been authorised by the competent authority, from the processing plant for immediate direct combustion, the permanent marking with glyceroltriheptanoate (GTH) referred to in point 1 of Chapter V of Annex VIII shall not be required;
   (iii) in the case of a fat fraction of Category 3 material, any of the processing methods 1 to 5 or processing method 7 as set out in Chapter III of Annex IV;
   (iv) in the case of the materials derived from fish, any of the processing methods 1 to 7 as set out in Chapter III of Annex IV;

b. *the fat fraction must be separated from the protein, and in the case of fat from ruminant origin which is intended to be combusted in another plant, insoluble impurities in excess of 0.15% by weight must be removed.*

Combustion plants in which meat-and-bone meal is used as a fuel for combustion

1. *Type of plant: Combustion plants with a total rated thermal input not exceeding 50 MW.*
2. *Starting material: Meat-and-bone meal of Category 1 and Category 2 materials, to be used as a fuel for combustion in accordance with the requirements set out in point 3 alone or in a mixture of meat-and-bone meal, rendered fat and manure.*
3. *Specific requirements for meat-and-bone meal used as a fuel for combustion:*

   a. meat-and-bone meal shall be stored in the combustion plant securely in a closed storage protected from access by animals and shall not be sent to another destination unless authorised by the competent authority in case of breakdown or abnormal operating conditions;
   b. the combustion plant must be equipped with:

   (i) an automatic or continuous fuel management system to place the fuel directly in the combustion chamber without fur-ther handling;
   (ii) an auxiliary burner which must be used during start-up and shutdown operations to ensure that the temperature require-ments set out in Section 2(2) of Chapter IV are met at all times during those operations and as long as unburned material is in the combustion chamber.

3.2 | Previous risk assessments on the impact of incineration on TSE agent infectivity

Earlier risk assessments attempted to estimate Classical BSE (C-BSE) prion infectivity in ashes produced by the incineration of carcases of BSE cases. For example, in the 'Risks from disposing of BSE infected cattle in animal carcase incinerators for

the Environment Agency', UK (DNV Technica, 1997a), several methods were applied. Using the total protein content and the total amino acid content as a surrogate, it was assumed that the reduction of infectivity would be in proportion to the reduction in protein content. Extrapolating from acid hydrolysis, it was speculated that the average protein content in the ash would be 3 mg of protein/100 g of ash. The protein content of an animal carcass was assumed to be 25% resulting in an estimated reduction of protein in the incinerator of $0.25/3 \times 10^{-5}$, i.e. 8000-fold. However, based on advice from the UK's Spongiform Encephalopathy Advisory Committee (SEAC), the authors used 6 $\log_{10}$ as the best estimate of overall reduction of infectivity in an incinerator operating under normal conditions. Assuming that after rendering and incineration of a BSE-infected carcase of 500 kg, including the head, 75 kg of ash would be produced and taking into account the abnormal operation that could occasionally occur in the operation of an incinerator, the authors concluded that overall the results of the study showed the presence of protein and infectivity in the final ash were not zero, even if it was argued that some amino acids present in the prion protein could not be detected in the ash samples.

A more targeted risk assessment of the use of MBM as fuel for the production of cement was conducted, focusing on a specific cement production company in Ireland (Bradley, 1997). This qualitative assessment considered that the small size of the MBM particles, similar to coal dust, would assist in achieving immediate combustion in the flame at temperatures in excess of 2000°C in the kiln and more than 1400°C during the preheating of the raw mineral material. Despite stating that a zero risk cannot be proved, the conclusion was that the burning of Irish MBM as a fuel for the manufacture of cement under general conditions presented a 'negligible risk' for humans, animals or the environment.

EFSA and its predecessors have conducted limited assessments on the impact of incineration on prion infectivity. In 2003, the Scientific Steering Committee (SSC) produced an assessment on the level of reduction of prion infectivity by incineration in small incinerators (SSC, 2003a). This assessment included evaluating a risk assessment prepared for SEAC by De Nork Veritas (DNV, 2001). Small incinerators, defined as those processing less than 50 kg/hour, were typically used for processing ABP at locations such as abattoirs, knackeries, hunt kennels or laboratories.

The SSCs recommended a framework for assessing the risks associated with incinerating meat and bone meal potentially contaminated with TSE, which included five components:

- *Identification and characterisation: determining the risk materials involved, possible means of transmission and potential at-risk groups*.
- *Risk reduction: assessing the risk reduction achieved by the specific process*.
- *Containment of risks: evaluating the containment of risks under normal and emergency operating conditions, including the effectiveness of control measures*.
- *Interdependent processes: considering related processes such as transport, storage and loading of TSE-related risk materials*.
- *End use of products: identifying the intended end use of the products, such as disposal or recycling*.

The SSC highlighted significant variability in the design, nature of use, performance characteristics and management quality of incinerators, leading to many uncertainties in identifying the risks posed by small incinerators used to treat SRM. Therefore, the SSC recommended that each type of incinerator should receive its own assessment on the safety of the ash.

Additionally, the SSC concluded that the protein content of ash from incineration is not a reliable measure for the degree of risk reduction of TSEs. In the absence of reliable data on the potential residual infectivity of the ash, the SSC recommended that it should be disposed of in controlled landfills.

In 2003, the SSC also produced an assessment on the open burning of potentially TSE-infected animal materials (SSC, 2003c). The committee expressed serious concerns about using open burning to destroy pathogen-contaminated animal waste, especially waste possibly contaminated with heat-stable pathogens. Key issues included: the potentially very high variability of the pathogen inactivation, the nature of the gaseous and particulate emissions, and the risks from the residual ash.

The SSC recommended that open burning should only be considered for pathogen destruction under exceptional circumstances following a specific risk assessment. Specifically, for animal waste possibly contaminated with BSE/TSE prions, the committee concluded that open burning should be considered a risk, due to the uncertainty about the degree of inactivation. The SSC highlighted the need for suitable monitoring methods for TSE contamination of both air and ash. It also stressed the importance of establishing protocols for safe burning in emergency situations.

In an overview of the BSE risk assessments of the SSC and its TSE/BSE ad hoc Group, adopted between September 1997 and April 2003, the SSC concluded that the direct incineration of carcasses and incineration or burning under appropriate controlled conditions of rendered material were economically feasible technologies for safely disposing of TSE risk materials (SSC, 2003d).

In 2004, the EFSA Panel on Biological Hazards produced an opinion on the combustion of tallow in a thermal boiler process as a method for safe disposal of Category 1 Animal by-products (EFSA BIOHAZ Panel, 2004). The process involved the incineration process in which tallow in a liquid form (70°C) was vaporised to form particles with an average size of 20 microns and burned at a temperature of 1305°C (residence time at conditions of at least 1100°C for 0.2 s). The Panel concluded that the process was at least equally as efficient as the reference method (i.e. 850°C during 2 s) in terms of carbon destruction and that this process did not present an additional risk in the treatment of Category 1 ABP. However, the level of inactivation of prions achievable by the proposed method or the reference method was not considered in the opinion.

In 2021, the EFSA Panel on Biological Hazards evaluated the effectiveness of various processing methods for inactivating harmful microorganisms in Category 2 and 3 ABP, which are used as organic fertilisers or soil improvers (EFSA BIOHAZ

Panel, 2021). They specifically looked at whether thermal processes could achieve a 5 $\log_{10}$ reduction in two types of bacteria, *Enterococcus faecalis* and *Salmonella* Senftenberg (775W). The panel included ash derived from incineration, co-incineration and combustion in their assessment. The opinion concluded that the required reduction of these resistant microorganisms would be achieved with 99%–100% certainty in ash. The risk from using ash derived from Category 1 materials was not considered in this evaluation. Despite this, the opinion cited the conclusion of the Spongiform Encephalopathy Advisory Committee (SEAC) (2003) that the risk of TSE agent infectivity from ash was extremely small if incineration was conducted at 850°C, the temperature recognised by the EU as the standard method for waste disposal by incineration. Therefore, ash from incineration, co-incineration or combustion was generally considered safe and could be disposed of in landfills. However, it was also highlighted in the opinion that there is some uncertainty left considering that the duration may not be sufficient, the starting concentration of prions is unknown and there may be some protecting material.

Paisley and Hostrup-Pedersen (2005) estimated the BSE risk associated with fly ash and slag from the incineration of MBM in a power plant in Denmark. The authors assumed that all SRM and MBM produced in Denmark would be incinerated in such a plant with an average of 6 (range: 0–15) clinical BSE cases rendered into MBM and a median of 10 sets of SRM or carcasses (0–31) infected but not detected would also be incinerated. They estimated a total infectivity in fly ash of 8.7×10^{-10} oral cattle ID_{50} per week, after assuming a 6 $\log_{10}$ reduction during incineration. One ton of fly ash would contain $\leq 1.8 \times 10^{-7}$ oral cattle ID_{50} 95% of the time, which the authors considered to pose a 'negligible risk' for the phosphate or fertiliser industry.

Cummins et al. (2002) estimated the human exposure to TSE from the MBM derived from SRM treated in a combustion facility. The authors concluded that the human exposure resulting from the combustion of SRM derived MBM was extremely small, significantly less than the background societal risk of approximately 2.5 cases of sporadic Creutzfeldt–Jakob disease (CJD) in Ireland each year, at the time of conducting the assessment. However, the authors argued that the species barrier had a large impact on the exposure calculations.

More recent studies commissioned by the industry (ESPP) have addressed the safety of the use of ashes produced by Category 1 ABP. Alquinta González and McDonnell (2022) assessed the sanitary safety of ABP ash through a literature review using 6 $\log_{10}$ reduction as the target reduction parameter. It was acknowledged by the authors that there are no data available that evaluate the infectivity reduction achieved by applying incineration and co-incineration conditions, as in the legislation. The variable thermoresistance of different prion strains was also acknowledged by the authors, with some of them, e.g. 22L, C-BSE and H-BSE, not reaching a 6 $\log_{10}$ reduction at temperatures of 200°C or below. Finally, the authors concluded that it is not possible to determine with certainty that incineration and co-incineration processes inactivate the most thermostable prions at the operational conditions indicated in the legislation.

SAFOSO carried out a risk appraisal of the use of Category 1 animal by-product ash as a fertiliser (SAFOSO for ESPP, 2024). This involved documenting and evaluating the steps contributing to minimising the potential risk associated with the presence of prions throughout the processing operations for Category 1 ABP until the production of ash. The appraisal also reviewed current practices of using Category 1 ABP ash as fertiliser in Europe and North America in the light of prion disease trends. The report was based on a combination of official publicly available information, scientific publications, grey literature and personal communications from diverse industry groups. The appraisal included a quantitative risk assessment based on two rendering scenarios (method 1 and any of methods 2–5), and the incineration of material from five cows at the clinical stage of C-BSE, processed in a single batch at a single processing plant. The key parameter, the reduction of C-BSE infectivity under normal operating conditions of incineration, was sourced from the above-mentioned risk assessment by DNV Technica (1997b) and a complementary one (DNV Technica, 1997c). Based on these reports, a 6 $\log_{10}$ reduction in C-BSE infectivity was assumed, with a further 3 $\log_{10}$ reduction achievable by rendering with method 1, based on scientific literature. The overall infectivity reduction factor was estimated in the order of 30–100 thousand for scenarios with rendering with methods 2–5 and 30–100 million for method 1. As reported previously by DNV (1997b), '*the societal risk results showed that the total ingestion of infectivity by all the population of England and Wales through environmental pathways in 1996 was approximately 3 ID_{50} units and that the maximum individual exposure was less than one millionth of an infective dose per year*'. Thus, the authors of the SAFOSO study concluded that cattle would need to consume significantly large volumes of ash to become infected. The authors further concluded that there is no evidence to suggest that ash produced from Category 1 ABP, treated according to EU regulations and used as an approved fertiliser, poses a risk of C-BSE transmission. However, given the infectivity risk dilution and the uncertainty and multiplicity of environmental and management factors, the authors decided not to pursue an estimate of the C-BSE risk exposure to cattle, which renders the previous statement on the safety of ash as fertiliser unjustified.

3.3 | Methodologies to measure prions

3.3.1 | Direct methods for measuring biological activity of prions

The gold standard method for the detection and quantification (generally by endpoint titration) of prion infectivity is bioassay, i.e. its capacity to infect animal models. Most of the seminal studies demonstrating the strong resistance of prions to inactivation procedures have been conducted through the titration of infectivity in bioassay experiments utilising TSE strains that have been adapted in laboratory animal models, mainly mice and hamsters.

A range of inoculation routes can be used (intracerebral, intravenous, oral, intraperitoneal), with the bioassay of a sample $\log_{10}$ dilution series by the intracerebral (i.c.) route being the most common and sensitive approach, given that it is generally the most efficient and fastest. The attack rates observed in the various challenge groups permit extrapolation to calculate the dose that causes infection in 50% of recipient animals (the ID_{50}), thus enabling estimation of the amount of infectivity present in the tested tissue in terms of the ID_{50}. This approach has been shown to detect up to 8–9 $\log_{10}$ ID_{50}/g prion infectivity in the brain of humans and animals with terminal disease, depending on the prion strain and bio-indicator animal (Andréoletti et al., 2011; Brown et al., 2000; Buschmann & Groschup, 2005; Douet et al., 2017; Taylor et al., 2002; Vanni et al., 2020).

Alternatively, cell-free prion amplification assays such as protein misfolding cyclic amplification (PMCA) (Saborio et al., 2001) or real-time quaking-induced conversion (RT-QuIC) (Atarashi et al., 2011) estimate prion concentration by measuring self-converting activity. Both methods detect PrP structural conversion and polymerisation upon adding PrP^{Sc} 'seeds' from infected samples (for a review, see Krauss & Vorberg, 2013). These fast, reliable techniques have broad applications, including prion inactivation studies and diagnostics (Caughey et al., 2017; Cazzaniga et al., 2021). They have emerged as sensitive and efficient methodologies for the detection and quantification of prions. These assays can be applied on sample $\log_{10}$ dilution series similar to end point titration by bioassay, which permits extrapolation to calculate the dose that causes seeding in 50% of recipient tubes (the SD_{50}) and enables estimation of the amount of seeding activity present in the tested tissue in terms of the SD_{50}. The sensitivity of detection achieved by PMCA and RT-QuiC equals or exceeds the sensitivity of the reference bioassay models.

In their most technically advanced formats, these assays correlate strongly with animal bioassays in their ability to detect prions. RT-QuIC has shown sensitivity comparable to animal bioassays for detecting 263K prion seeding activity (Wilham et al., 2010). PMCA assays demonstrate significantly higher analytical sensitivity than ad hoc bioassays (Douet et al., 2017; Makarava et al., 2012; Moudjou et al., 2014). A recent study found that RT-QuIC and PMCA are equally sensitive in detecting sheep C-BSE prions, both exceeding bioassays using transgenic mice expressing ovine PrP by > 1000-fold (Thomas et al., 2023). Additionally, both assays reliably quantify prion concentrations in fluids and tissues from infected animals or patients (Wilham et al., 2010, Lacroux et al., 2014; Thomas et al., 2023; Douet et al., 2017).

In prion inactivation studies using steel-wire-bound prions, RT-QuIC seeding activity correlates well with infectivity but is not always eliminated alongside it (Hughson et al., 2016; Mori et al., 2016). This may reflect RT-QuIC's higher sensitivity and its ability to generate amyloid fibres that are off-pathway to prion infectivity. Some treatments may selectively target infectious prion species while sparing those involved in RT-QuIC conversion. Inactivation methods targeting vCJD or 263K prions on steel wires (e.g. disinfectants or physical processes) showed good correlation between residual prion infectivity/activity as measured by bioassays and PMCA (Bélondrade et al., 2020; Herzog et al., 2024; Igel et al., 2024; Matsuura, Ishikawa, Bo, et al., 2013; Pritzkow et al., 2011).

All these methods (bioassay, PMCA and RT-QuIC) estimate infectivity or seeding activity by measuring their persistence to the point of extinction in a dilution series. The resulting values depend on the specific strain/host combination used in each assay and do not provide an absolute quantitative measurement of 'prion content'. Instead, they reflect a $\log_{10}$ reduction in these parameters relative to the starting material.

3.3.2 | Direct methods measuring PrP^{Sc} content

According to the protein-only hypothesis, prion infectious particles are mainly or exclusively composed of PrP^{Sc}, the aggregated and protease-resistant form of cellular prion protein (PrP^{C}). Indeed, PrP^{Sc} is the hallmark of TSE and the only pathognomonic biomarker identified so far. The immunodetection of PrP^{Sc} is thus utilised for the diagnosis of prion diseases, as well as to track the presence of prions in different tissues. The methods described in this section are capable of detecting and quantifying PrP^{Sc} after denaturation, i.e. PrP^{Sc} monomers derived from prion particles, rather than the prion particles themselves.

As the detection of PrP^{Sc} equates to the detection of prions, PrP^{Sc}-based immunological assays, such as western blot (WB), have been used as surrogate markers for the quantification of prion reduction (see, e.g. Müller et al., 2007). Nevertheless, the molecular mechanisms of prion replication and the ultrastructure of infectious particles remain to be fully elucidated. This limitation compromises the reliability of PrP^{Sc} as a proxy for the reduction of prion infectivity. In vivo studies have evidenced a broad size spectrum of infectious PrP^{Sc} aggregates, varying from a few to hundreds of PrP^{Sc} molecules (Silveira et al., 2005) and which endow structural heterogeneity (Igel et al., 2023) and variation of the size distribution of the PrP^{Sc} aggregates according to the strain and host species (Tixador et al., 2010; Laferrière et al., 2013; Cortez et al., 2021). The hypothesis that small particles possess greater infectious potency than large PrP^{Sc} multimers has been postulated (Silveira et al., 2005; Tixador et al., 2010), and upon sonication, large particles have been shown to fragment into smaller infectious particles, thereby enhancing infectivity (Terry et al., 2016).

Lack of correlation between reduction of infectivity and reduction of PrP^{Sc} was also revealed in a study by Langeveld et al. (2021), whereby bovine C-BSE infected brain heated under wet conditions at 115°C for 40 min retained a high level of infectivity when inoculated in Tgbov XV mice despite > 1000-fold reduction of PrP^{Sc} detectable by WB.

In the EFSA's scientific opinion on the evaluation of an alternative method for production of biodiesel from processed fats derived from Category 1, 2 and 3 ABP (submitted by College Proteins) (EFSA BIOHAZ Panel, 2020), the limitation of immunological assays was highlighted: '*Inactivation studies …using WB to assess the presence of detectable PrP^{Sc} following*

treatment, are increasingly being considered suboptimal as predictors of the resistance of natural TSEs when they are subjected to such inactivating protocols… due to the inherently limited analytical sensitivity and biological relevance of WB for the detection of prion infectivity. However, similar methods have been used in previous inactivation studies and official guidelines (EMA, 2019)'.

Mass spectrometry (MS)-based methods have been documented as capable of absolute quantification of prion protein and exhibiting high sensitivity in detecting tryptic peptides derived from recombinant PrP (Onisko, Silva, et al., 2007; Silva et al., 2011; Sturm et al., 2012). Evidence for the capacity of these methods to detect and quantify PrP^{Sc} from hamster brains has been provided (Onisko, Silva, et al., 2007). MS-based methods have demonstrated possessing the potential for enhanced sensitivity in comparison to immunological-based methods of detecting PrP^{Sc} (Onisko, Dynin, et al., 2007; Silva et al., 2011), although a direct comparison of their respective sensitivities remains to be conducted. These methods have been employed for absolute quantification of PrP^{Sc} in different prion strains (Gielbert et al., 2013; Onisko, Dynin, et al., 2007; Silva et al., 2011) but have not been widely adopted for the detection of prions, in general, or for residual prions after decontamination with heat or other treatments, in particular.

3.3.3 | Indirect methods

Other methods have been applied to indirectly evaluate the presence of residual prions in the aftermath of thermal treatments. These methods are predicated on the premise that the total protein or amino acid content of the material post-thermal treatment can be employed as a surrogate for prions, given that the infective agent is a protein (DNV Technica, 1997a; Gulyurtlu et al., 2005). In previous EFSA evaluations of alternative thermal treatments for the processing for disposal of Category 1 material, it was highlighted that amino acid content may be a poor indicator of prion destruction since these data are insufficient to indicate effective destruction of the TSE agents during the very short residence time of (0.2 s) (SSC, 2003b).

According to the SSC opinion on the use of small incinerators for BSE risk reduction (SSC, 2003a), *'protein content is a useful indicator of the general performance of an incinerator. However it is much more problematic whether it is also a valid marker for possible BSE/TSE contamination as it known that BSE/TSE are relatively heat resistant as compared to other proteins. Failure to detect certain amino acids present in prions is encouraging but the sensitivity limits for amino acids are relatively poor for reassurance purposes'*.

Consequently, these methods have not been pursued for the detection of TSE in ashes produced by incineration, etc., of animal by-products.

3.4 | Thermoresistance of animal TSE agents

The vast majority of experiments investigating the resistance of prions to thermal treatment have been conducted in the range of 100–200°C, with few experiments using temperatures relevant for this assessment, i.e. in the range of 850–1100°C. As noted previously (EFSA BIOHAZ Panel, 2015), there is not enough evidence to justify a linear relationship between increasing temperature and decreasing PrP^{Sc} infectivity, which prevents extrapolation of the effect of very severe thermal treatments from experiments at relatively low temperatures.

The following sections summarise the limited available experimental data on prion thermoresistance. This is not intended as an exhaustive review of all data relating to the thermoresistance of prions, but instead is focused on those studies that allow comparison of the thermoresistance of animal TSE field strains (low temperature range) and to those that most closely represent the conditions related to the heat treatment or incineration of animal waste (i.e. the application of heat in the high temperature range to tissues or homogenates as the principal treatment), with a focus, where possible, on animal TSE field strains. It does not, for example, include papers related to the data on decontamination/inactivation of prions already bound to the surface of surgical equipment, or the early reports on optimising autoclaving protocols for scrapie decontamination. Table A.1 in Appendix A summarises data from the publications discussed below, to illustrate the great diversity of experimental parameters and methods to be found in this small body of literature, none of which replicates either the legislative requirements or the industry practices associated with the incineration processes under assessment in this Opinion.

3.4.1 | Thermoresistance of TSE strains at low temperatures

Given the paucity of experimental studies conducted at temperatures pertinent to incineration, it has been necessary to examine studies employing lower temperatures. The objective of this approach is twofold: firstly, to provide a comprehensive summary of the knowledge on prion strains' thermoresistance under differing experimental conditions, which is primarily based on studies using rodent-adapted TSE strains, and secondly, to compare the thermoresistance of animal TSE field strains to select the most thermoresistant.

3.4.1.1 | *Laboratory rodent-adapted TSE strains*

The robust resistance of TSE to inactivation by a range of chemical and physical decontamination treatments, including standard autoclaving at 121°C, has been demonstrated in studies with rodent-adapted strains (Taylor, 2000). Significant inter-strain variations in heat resistance have been revealed (Kimberlin et al., 1983; Somerville et al., 2002; Taylor et al., 2002), although all the tested strains were difficult to inactivate by wet heat treatments. The disparities in heat resistance observed among TSE strains have been attributed to the intrinsic, structural attributes of the strain-specific agents, rather than the PrP genotype of the host, based on experimental studies utilising mice with two different PrP genotypes. This phenomenon accounts for an observed difference in resistance to gravity-displacement autoclaving at 126°C for 30 min of greater than 3 $\log_{10}$ units (Taylor et al., 2002). Furthermore, the C-BSE-derived mouse-adapted strains were observed to exhibit greater resistance to heat inactivation than mouse-adapted scrapie strains (Somerville & Gentles, 2011; Taylor et al., 2002).

Studies with rodent-adapted TSE strains have also allowed the assessment of the effect of different experimental and pretreatment conditions on prion resistance to heat inactivation. Dry heat was shown to be less efficient than wet heat in decontaminating C-BSE and scrapie-derived strains (Fernie et al., 2007; Taylor et al., 1996). When the hamster-adapted 263K scrapie agent was incompletely inactivated by autoclaving, the inactivation curve showed a thermoresistant 'subpopulation' that persisted over time (Rohwer, 1984). Indeed, it was later shown that after a 3.3 $\log_{10}$ reduction by one autoclave cycle at 134°C, the infectivity titre was only reduced by a further 1.3 $\log_{10}$ after a second autoclave cycle (Taylor et al., 1998). Drying of scrapie-infected tissue on surfaces is known to enhance its thermoresistance (Taylor 2000). Therefore, stabilisation of a thermoresistant subpopulation may be achieved by heat fixation before or during autoclaving. Other studies have demonstrated that protein fixation with formol also enhances the thermoresistance of scrapie (Taylor & McConnell, 1988). Additionally, research has indicated that, in conjunction with water content, fat and glycerol may influence the mechanism of thermal inactivation of prions. Inactivation of prion infectivity after heat treatment at temperatures of up to 200°C in the presence of different fat/glycerol/water mixtures was studied by Muller et al. (Müller et al., 2007). Compared with untreated samples, the residual infectivity of heat-treated samples varied considerably, with the different compositional environments inducing differences in inactivation of up to 5 $\log_{10}$. As an example of the matrix effect, after heat treatment for 20 min at 90°C, the infectivity titre was reduced by 3 $\log_{10}$, while reductions in infectivity titre of up to 5 $\log_{10}$ were achieved in the presence of different amounts of fat. When combining the inactivating effect of fat with denaturants, it was reported that an aqueous mixture of fat, urea and dithiothreitol reduced the infectivity titre by up to 7 $\log_{10}$.

Studies by Grobben et al. (2005, 2006) used mouse brain containing 301V prions (derived from C-BSE) to spike bovine vertebral column (Grobben et al., 2005) or dried bone chips (Grobben et al., 2006), in order to simulate the primary treatment steps of gelatine extraction, namely degreasing and autoclaving (for 20 min at 132–134.8°C) for the raw material, or biphasic autoclaving (up to 115°C, then back to 92°C before being raised again for 20 min at 133°C under 3 bar pressure) for the dried bone chips. These studies resulted in a reduction of the titre of > 6.5 $\log_{10}$ and 4 $\log_{10}$ respectively.

These findings were summarised in the EFSA's scientific opinion on the evaluation of an alternative method for production of biodiesel from processed fats derived from Category 1, 2 and 3 ABP (submitted by College Proteins) (EFSA BIOHAZ Panel, 2020): *'Seminal studies on prion decontamination/inactivation relied on the use of laboratory-adapted rodent prion strains derived from naturally occurring scrapie (such as ME7 or 263K), or classical BSE (301V) (Brown et al., 1990; Taylor, 1991, 1999). Over the last 30 years, however, it has become clear that the strains involved in naturally occurring prion diseases (scrapie, BSE, CWD or CJD) display very different resistance/sensitivity to decontaminating treatments (autoclaving or chemical inactivation) (Taylor, 1986, 2000; Taylor et al., 1994)'*.

Overall, the accumulated data with laboratory rodent-adapted strains demonstrate the difficulty of fully inactivating TSE agent infectivity using heat-based methods and suggest that, in order to reliably evaluate a decontamination process, it must be reproduced in the laboratory using experimental conditions and TSE strains appropriate to the process being evaluated.

3.4.1.2 | *Animal TSE field strains (AQ1)*

As a result of the increased surveillance in the EU after the BSE crisis, several new strains of animal TSE have been identified in addition to the already known classical scrapie (CS) and C-BSE: Nor 98 or atypical scrapie (AS) in sheep and goats (Benestad et al., 2008), BASE or low-type BSE (L-BSE) and high-type BSE (H-BSE) in cattle (Biacabe et al., 2007; Casalone et al., 2004), and more recently new chronic wasting disease (CWD) strains in Scandinavian cervids (Benestad et al., 2016; Tranulis et al., 2021). It has also been clearly demonstrated that CS is not a unique entity. Naturally occurring CS isolates can have a wide biological variability, with evidence supporting the circulation of at least six to seven different strains (Bohl et al., 2023; Igel et al., 2023; Marín-Moreno et al., 2021; Barrio et al., 2020), although to date there is no standardised classification and nomenclature for these strains. This opinion will focus on animal TSE field strains identified in the EU so far.

Despite the species barrier, C-BSE and some CS isolates are transmissible to inbred lines of wild type (wt) mice, albeit mostly at the highest concentration. Although these limitations result in a limited measurement range, i.e. 2–3 $\log_{10}$ infectivity, some initial studies have been carried out with C-BSE and CS to evaluate the efficacy of rendering procedures in pilot scale facsimiles (Taylor et al., 1995, 1996, 1997). One such study aimed to evaluate the efficacy of rendering by pressure sterilisation on C-BSE and CS brain homogenates (Schreuder et al., 1998). Infected homogenates added to homogenised raw material (abattoir waste) were treated by autoclaving in closed vials at various pressures/temperatures, including those required by the 1996 EU Rendering Directive (133°C, 3 bar pressure, 20 min). After 20 min at 133°C, some residual C-BSE

infectivity was detected, with a reduction of between 2 and 3 $\log_{10}$, whereas no residual CS infectivity was detected (reduction of scrapie infectivity was > 2.2 $\log_{10}$). In this study, the C-BSE agent consistently appeared to be more resistant to heat inactivation procedures than the scrapie agent, particularly at lower temperatures and shorter times.

The development of transgenic mice overexpressing the normal PrP^C of the species of interest has overcome the species barrier for measuring infectivity in laboratory rodents (Defranco & Telling, 2025), allowing sensitive and relatively rapid measurement of infectivity levels in samples of natural strains, even after treatments that reduce their infectivity by several $\log_{10}$. These transgenic mouse models allowed, for the first time, a reliable comparison of the heat resistance of natural versus rodent-adapted TSE strains. A large study investigated the heat resistance of cattle C-BSE in comparison with the C-BSE derived mouse-adapted strain 301V, the hamster-adapted scrapie strain, Sc237 and human sporadic CJD (Giles et al., 2008). Inactivation was assessed by exposure of brain homogenates to sodium dodecyl sulfate, variations in pH and different time/temperature regimes. Bioassays demonstrated that cattle C-BSE prions are up to 1000-fold more resistant to inactivation than 301V prions, arguing that despite being derived from C-BSE prions, mouse 301V prions are not necessarily a reliable model for cattle C-BSE prions. CJD and hamster Sc237 prions were 1 to > 6 $\log_{10}$ less resistant to inactivation than cattle C-BSE, respectively. Treatment of C-BSE for 15 or 30 min at 134°C decreased infectivity by 2.9 and 3.2 $\log_{10}$, respectively. These estimates are consistent with those previously obtained in wt mice following pressure sterilisation (133°C, 3 bar pressure, 20 min) of C-BSE infectivity spiked into abattoir waste (Schreuder et al., 1998).

Another study using a different bovine transgenic mouse model and untreated brain macerates of C-BSE as starting material showed a ~2 $\log_{10}$ reduction in C-BSE infectivity after heat treatment at 133°C for 20 min (Matsuura, Ishikawa, Bo, et al., 2013). This study also confirms previous evidence that drying increases prion thermoresistance, as pretreatment of C-BSE at 65°C for 24 h reduced the effect of wet heat treatment by several $\log_{10}$.

Studies comparing the heat resistance of natural animal strains circulating in the EU are still very limited and incomplete. For example, there are no experiments using European CWD, or comparing different field CS strains. Two recent studies (Chapman et al., 2020; Spiropoulos et al., 2019) assessed the effect of heat treatment at EU-recommended pressure sterilisation conditions (133°C, 3 bar pressure, 20 min) on naturally occurring animal TSE strains in the EU. The studies used brain macerates from diseased animals as starting material and measured infectivity in relevant transgenic mouse models (overexpressing ovine or bovine PrP) before and after heat treatment. They found that atypical scrapie (AS), CS and C-BSE were still able to infect some of the inoculated mice after treatment, with $\log_{10}$ reductions (95% CI) of 8.1–10.2, 5.9–7.3 and 4.3–7.6, respectively. Conversely, L-BSE and H-BSE did not contain detectable infectivity after treatment, with the minimum $\log_{10}$ reduction estimated depending on the sensitivity limit of the bioassay for the two inocula (> 9.4 $\log_{10}$ for L-BSE and > 3.9 $\log_{10}$ for H-BSE). Therefore, under the experimental conditions used in these two studies, C-BSE was the animal TSE strain with the highest resistance to wet heat treatment at 133°C. It is important to emphasise that the $\log_{10}$ reductions obtained in these experimental studies provide the best estimates for ranking animal TSE strains, but cannot be directly applied to industrial processes, as additional factors (dilution of infectious material, heat transfer time, exposure to organic solvents or other chemicals) may affect TSE thermoresistance (Chapman et al., 2020).

The survival of C-BSE prions to long-term heat treatment (up to 3 h) was confirmed in another study (Yoshioka et al., 2013), where C-BSE was mixed with grease to simulate embedding in fat during rendering of animal by-products. In this context, heating at 140°C, 160°C or 180°C for 1 or 3 h always resulted in detectable residual seeding activity by PMCA. This study also showed that the reduction of C-BSE prions by heat is time-dependent even after long-term treatments, as samples treated for 3 h had lower residual prion levels than those treated for 1 h.

In conclusion, only a limited number of studies have been conducted to compare the resistance of animal TSE strains to heat inactivation. The available evidence suggests that C-BSE is the most thermoresistant of the strains investigated. However, in contrast to the inactivation methods of interest in this opinion, the majority of these studies focused on wet heat treatment conditions for the purpose of exploring the efficacy of prion inactivation procedures for laboratory or surgical equipment/waste.

3.4.2 | Thermoresistance of TSE strains at high temperatures

Only a few studies have explored the thermoresistance of prions at temperatures approaching those reached during incineration, and none of them reproduce the minimum time–temperature profiles required by the regulation (850°C for 2 s or 1100°C for 0.2 s), or those achieved in practice by incineration facilities processing large quantities of ABP waste. In addition, only one of them directly studied the resistance of C-BSE prions. On the one hand, they are informative, as the range of temperature tested was between 400°C and 1000°C. On the other hand, the duration of heat treatment is much longer than that mandated in the regulation.

The most relevant studies in terms of temperatures applied and sensitivity of prion detection by bioassay were conducted using hamster-adapted scrapie strains, 263K or Sc237. As previously discussed, the resistance of prion strains to heat may vary substantially between laboratory and field strains. Notably, studies conducted at temperatures between 130°C and 140°C suggest that C-BSE is consistently more thermoresistant than 263K or Sc237, but the range of differences is variable (Cardone et al., 2006; Fernie et al., 2007; Giles et al., 2008), which precludes direct extrapolation from hamster-adapted strains to C-BSE.

In seminal experiments, Brown et al. (2000) studied the resistance of hamster 263K scrapie brain tissue, fresh or formalin-fixed, to heating at 600°C (a temperature that completely reduces tissue to ash) and 1000°C for different durations. Total inactivation

of fresh tissue was achieved after 1000°C for 5 min. After 600°C for 5 min, no transmission occurred in the inoculated hamsters using fresh material. However, after a 15 min treatment at 600°C, five out of 15 animals developed disease, with prolonged and variable incubation periods. According to the authors, this finding suggested residual infectivity near extinction. Transmission was also observed in one out of 21 animals inoculated with fixed brain tissue treated at 600°C for 5 min.

To address the apparent discrepancies at 600°C between 5 and 15 min of treatment and to rule out possible cross-contamination, the experiments were reproduced a few years later (Brown et al., 2004). Thermal treatment at 1000°C for 15 min consistently ensured sterility. Heating at 600°C for 15 min reduced infectivity to less than one ID_{50} (2 positive transmissions out of 21). By comparison, in untreated tissues, infectivity levels were approximately 10^9 ID_{50}/g. These data suggest that at temperatures approaching 1000°C, under the air conditions and incineration times (15 min) used in these experiments, tissues contaminated with the 263K scrapie strain can be completely inactivated. After 15 min treatment, the infectivity extinction point was somewhere between 600°C and 1000°C, approaching the operating temperature of some incinerators.

Another study used a different hamster-adapted scrapie strain, Sc237, and a longer duration of dry heat treatment (> 1 h) at temperatures in the range of 200–1000°C (Murayama et al., 2006). Residual infectivity or seeding activity was examined in transgenic mice expressing hamster PrP or using PMCA and showed extinction of detectable infectivity or seeding activity at temperature > 200°C. Although different animal models and hamster-adapted scrapie strains were used, the outcomes of the Murayama et al. (2006) and Brown et al. (2004) studies are consistent with the time-dependent inactivation of prions by heat at temperatures ranging from 300°C to 600°C.

Only one study specifically addressed the fate of C-BSE infected brain at temperatures approaching those legally required by the regulation (Matsuura et al., 2020). A macerate of pooled C-BSE-infected bovine spinal cord was dry-heated in a ceramic crucible for 20 min in an electric heating oven at varying temperatures between 150°C and 1000°C. The exposure time was counted after the tissues had reached the target temperature, i.e. the total heat treatment time was more than 20 min. The times taken to reach the different target temperatures were not reported. Residual infectivity was examined in transgenic mice expressing bovine PrP by using the follicular dendritic cell (FDC) assay, which is a bioassay methodology developed for the rapid detection of BSE transmissibility (Matsuura, Ishikawa, Somerville, et al., 2013). The FDC assay is based on the detection of PrP^{Sc} in the spleen of mice at 150 days after intraperitoneal (i.p.) inoculation. Residual seeding activity was assessed using a PMCA method for which the limit of detection was not reported; it was however considered to be potentially less sensitive than bioassay (Matsuura et al., 2020). A complete loss of transmissibility was observed in tissues heated at 600°C or higher for 20 min. In contrast, residual activity persisted in tissues treated at ≤ 400°C for 20 min. Given the relatively low infectious titre detectable by i.p. challenge in the FDC assay in the starting bovine tissues, the maximum reduction in infectivity that could be assessed in this experiment was between 3 and 3.5 $\log_{10}$, as derived by the EFSA experts from the data available in the article.

3.4.3 | Concluding remarks on the thermoresistance of TSE agents

- TSE agents are difficult to fully inactivate using heat-based methods.
- Different TSE strains exhibit varying degrees of thermoresistance. The matrix in which the prions are found and pre-treatments (e.g. fixation or drying) can significantly influence their resistance to heat. Such variability compromises the possibility of extrapolating findings from one specific set of conditions to another.
- Studies on the thermoresistance of EU TSE field strains are limited. Most research has concentrated on wet heat conditions at low temperatures relevant to cleaning and sterilisation, rather than the extreme dry heat conditions used in incineration. Evidence suggests that at these lower temperatures the C-BSE strain is more thermoresistant than other evaluated strains.
- Only four studies have examined conditions approaching those used in incineration, and none precisely replicate the time/temperature profiles specified in the regulation or achieved in industrial processes. One study using a laboratory scrapie strain (263K) demonstrated prion survival at very high temperatures (at least 600°C for 15 min); complete inactivation of 263K hamster strain was observed at 1000°C for 15 min.
- C-BSE prions have been shown to survive at: 400°C for 20 min and at 180°C for 3 h.
- A complete loss of C-BSE transmissibility was observed in tissues heated at 600°C or higher for 20 min. However, due to the limited sensitivity of the detection methods used in the available study, infectivity cannot be ruled out after this treatment.

3.5 | Current industry practices to process category 1 animal by-products

3.5.1 | Rendering of ABP

3.5.1.1 | *Rendering practices*

The text in this section is extracted from the written communication from hearing experts (Alm and Dobbelaere (EFPRA) 19 September, 2024), except where alternative citations are referenced.

During the period 2014–2023, 4.3 million tonnes of Category 1 raw material was rendered annually in the EU, resulting in one million tonnes of Cat. 1 MBM.

Virtually all Category 1 ABP in the EU are rendered in approved plants using one of the standard rendering methods (methods 1–5 in the regulation) and the resulting MBM is then incinerated in approved incineration, co-incineration or combustion plants. A small amount of raw Category 1 ABP is incinerated directly in the Canary Islands. There are also a number of small incineration plants in the UK that directly incinerate Category 1 ABP. Carcass waste from TSE positive or suspected animals and Category 1 ABP are rendered without any additional requirement for prion inactivation or disinfection, and the fat can be used for biofuel production, provided it has undergone method 1 treatment.

Eight EU Member States (Germany, Hungary, Croatia, Ireland, Slovakia, Slovenia, Denmark, Sweden) require method 1 for the processing of Category 1 material. In Austria, the Netherlands, Belgium, Finland and Poland, method 1 is applied, but it is not mandatory. Spain, Portugal, France and Italy apply method 1 and other methods. Information was not available for the other Member States.

The percentage of Category 1 ABP processed by the standard methods is as follows:

- Method 1 = 57%
- Method 2 = unknown, possibly not used
- Method 3 = negligible (only one plant known)
- Method 4 = 43%
- Method 5 = unknown, possibly not used

The voluntary use of method 1 for Category 1 can be based on the fact that Category 1 fats can only be used in biofuels if they have been treated by method 1. The biofuel sector is the key market for Category 1 and 2 fats. Rendering plants using a different method often apply specific post-treatment with Method 1 for the fat (fat sterilisation).

Category 2 and 3 materials are often processed in Category 1 lines, and it is difficult to estimate the exact proportion of Category 1 animal by-products in the materials to be rendered and incinerated/co-incinerated/combusted. In 2023, there were 25 Category 2 processing lines spread across 10 EFPRA member states, with around 80 Category 1 lines in total. Larger member states, such as Spain, France, Germany, Hungary and Italy, tend to have more than one Category 2 line due to the economic dynamics between the use of Category 1 and 2 materials. The cost of maintaining Category 2 lines is high, although the revenue from Category 2 MBM (used as fertiliser) is slightly higher than that of Category 1 MBM (used as an energy substitute). In many cases, the additional revenue does not justify the cost of maintaining separate Category 1 and Category 2 lines.

The other main factor which gives rise to the mixing of different categories of ABP is the cost of collection and storage. Separate collection of different categories is only profitable in areas with a high density of animals and slaughterhouses, such as parts of France, Spain, Germany and Italy. In contrast, in areas with low-density farming areas, Category 2 materials and some Category 3 materials may be collected with Category 1 to simplify logistics and minimise costs. Under the EU legislation, mixtures of different ABP categories must be given the designation of the highest risk category. Given these factors, EFPRA estimates that only 40%–50% of the Category 1 products are likely to be from actual Category 1 materials as per Regulation (EC) No 1069/2009. The destination of Category 1 MBM is market driven and will vary between incineration plants, co-incineration plants and combustion plants depending on market conditions.

3.5.1.2 | *Inactivation of prions by the standard processing methods*

It was assumed that Category 1 BSE-infected material treated with method 1 gives a reduction of about 2.8 $\log_{10}$, under optimal conditions (EFSA BIOHAZ Panel, 2017). However, new data relating specifically to C-BSE strains (Chapman et al., 2020) indicate that the reduction in infectivity achievable by method 1 (pressure sterilisation) is greater than the previously reported 3 $\log_{10}$ by at least one $\log_{10}$ cycle (EFSA BIOHAZ Panel, 2020). The level of reduction of TSE agent infectivity by the application of Method 4 is unknown. According to EFSA BIOHAZ Panel (2017), it is less effective than method 1.

3.5.2 | Operational aspects of incineration plants

Incineration is used to dispose of Category 1 animal by-products when they are considered as a waste material under environmental legislation. During this process, animal by-products are converted into ash, water vapour and carbon dioxide. Incineration provides a means of reducing the volume of the by-products and eliminating pathogens, and offers several environmental benefits, such as reducing the need for landfills, handling all types of waste, reducing air pollution by avoiding open fires, breaking down certain chemical hazards and preventing leachate. However, the process still emits toxic gases, though large-scale incinerators typically have efficient flue gas cleaning systems, and the residual ash requires proper disposal.

High-capacity incineration plants (processing more than 50 kg/hour) achieve a large reduction of the weight (up to 75%) and volume (up to 90%) of the waste. They require large investment and high operation and maintenance costs. All the waste must be transported to the incinerator site.

Low-capacity incineration plants, processing less than 50 kg/h, include small-scale units like medical waste incinerators. These typically handle 10–12 kg/h and may require waste to be shredded or reduced in size before incineration. They can be constructed from readily available materials, and many are portable, allowing easy transport to waste storage locations. While they share advantages with large-scale incineration plants, such as waste volume reduction, they lack flue gas cleaning systems.

In a study of large-scale batch incineration of medical waste, the temperature/time (T°/t) profiles were measured over a number of cycles (Matee & Manyele, 2015). The average cycle time based on intervals between loadings was observed to be 32.7 min and 28.97 min for the data based on time to drop to 550°C after the maximum temperature, which was between 850°C and 950°C. The researchers observed varying speeds of flow, or duration, and maximum temperatures, as well as variations in the time taken to reach maximum temperature, depending on the amount of waste loaded in the preheated chamber, waste composition and moisture content. In any case, to reach the target temperature there was a minimum time required that depended on multiple factors, but that was always higher than the minimum time of exposure to the specified temperature required by the EU legislation.

Incineration plants can operate on a batch or continuous basis. According to EFPRA, the incineration of Category 1 MBM in the EU is always carried out in incineration plants that operate continuous systems. The flux of incoming waste is controlled so that the system operates at maximum efficiency. The same applies to co-incineration and combustion plants.

Various types of incinerators are used for the disposal of animal by-products.

Information on the operation of high-capacity incinerators was provided by a hearing expert (G. Tooremans, personal oral communication, 11 October 2024). The following text describes three types of incinerators: rotary kilns, grate incinerators and fluidised-bed incinerators. These descriptions relate to the incineration plants run by the expert's company, and operating conditions might differ at other companies. At the end of each section, drawings of the relevant type of incinerators are provided. These are taken from other sources for illustration purposes and do not specifically relate to the incinerators operated by the hearing expert's company.

Rotary kilns

This type of incinerator is used for treating hazardous waste. It can be divided into three parts: the feeding system, the rotary kiln and the gas abatement system, including the post-combustion chamber (PCC). It runs on a continuous basis.

a. Feeding system. Different feeding systems are used in this type of incinerator:

   - Solid waste bunker: stores solid waste which is fed into the kiln using a crane.
   - Liquid waste tank farm: stores liquids which are then fed into the kiln.
   - Drum storage: waste stored in drums is inserted directly into the kiln.
   - Direct injection system: material is injected directly into the kiln and PCC through a closed system. Additionally, waste of high calorific value can be fed in order to get the correct temperature. The feeding system is continuous, ensuring the waste composition is as homogeneous as possible, which maintains consistent temperatures and residence times in the kiln and PCC.

b. Rotary kiln: The kiln is a long rotating cylinder, usually 10 metres in length. The residence time is 30 min to 1 h. It is not possible to measure the temperature in the kiln, but it is estimated to be above 1200°C at the back of the kiln, with the flame temperatures being even higher. The temperature is a bit lower during the initial drying of the waste material at the front of the kiln and then gradually increases. The incineration process occurs in three stages: the drying of the waste at the beginning of the kiln, the combustion of the waste in the middle of the kiln, and the burn-out of the waste at the end of the kiln.

   Incinerator ash, commonly known as bottom ash, is produced during this part of the incineration process. The ash produced is like lava, providing evidence of the very high temperatures that the incinerated material reaches. The bottom ash must have a total organic carbon content of less than 3%. A quality control system for the ash is in place. This consists mainly of a visual system. Any ash not meeting the visual requirements is returned to the kiln. A laboratory analysis is also carried out at least monthly. This analysis includes the bottom ash, the boiler ash and the fly ash. The ash is landfilled on-site. There is no attempt to recover the inorganic or other materials because the ash is heavily chemically loaded.

   Three key factors, the three Ts, define the process in the kiln: the temperature (more than 1000°C), the turbulence (a few rotations every minute) and the time (30–60 min). While the material that is incinerated in a rotary kiln may not be exposed to 1000°C for a full 30–60 min, it will be 'for quite a long period'.

c. The flue gas cleaning system: consists of a post-combustion chamber (PCC), a steam boiler to recover energy and a flue gas cleaning system. The PCC ensures the complete combustion of the flue gases. In the steam boiler, the hot flue gases are cooled and the energy is recovered as steam. Boiler ash is produced by the steam boiler. The flue gas cleaning system contains a number of sections, including an electrostatic filter, a wet flue gas cleaning system and a dioxin filter that are used for removing contaminants such as mercury. Fly ash is produced in the electrostatic filter. Following the cleaning process, the flue gases exit through a chimney.

The temperature to comply with the legal requirement is measured in the PCC. On average, the temperature ranges from 1040°C to 1050°C at the end of the PCC, which can be considered the coldest part of the PCC. The temperature is more than 1100°C in the hottest part.

Rotary kilns are only used for the processing of waste which is not suitable for treatment by other techniques like waste-to-energy (WtE), cement kilns, etc. Only a limited amount of the waste input of the rotary kilns consists of ABP. They are used to process a few hundred tonnes of ABP per year, mainly food containing contaminants and laboratory animals. At present, no MBM is processed in rotary kilns (Figure 1).

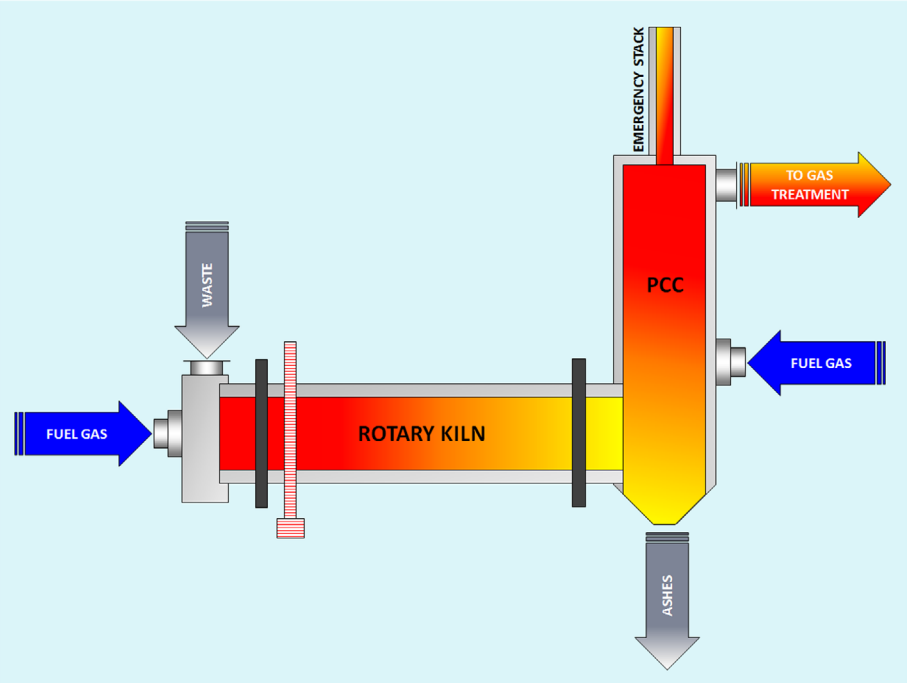

FIGURE 1 Example of rotary kiln incinerator (*Source*: IDRECO S.r.l., https://www.idreco.com/rotary-incinerators-for-industrial-wastes/).

Grate incinerator

Grate kilns are less suitable for the processing of chemically heavily contaminated waste than rotary kilns. They are set to run at 1000°C, but the actual temperature may be lower (around 800–850°C) due to poorer turbulence and air circulation being achieved. Residence time is 30–60 min. It is more difficult to get full burn out, but this design of kiln still meets the legal obligations for time–temperature. Residues are removed from the system at various points.

This type of incinerator is used for treating non-hazardous waste such as municipal waste or low-hazardous waste such as paint. High-hazardous waste is generally not incinerated in this type of incinerator because the flue gas system is not equipped to remove the hazards that are present.

Similar to the rotary kiln, the incineration process (Figure 2) can be divided into three parts: the feeding system, the kiln and the flue gas cleaning system. Due to the aim to process different types of waste, the design of these three parts is different in comparison with the rotary kiln, focusing on an efficient processing of municipal solid waste and similar industrial waste. For example, the feeding system is simpler, consisting only of feed from a solid waste bunker similar to that described for the rotary kiln.

The combustion chamber contains inclined moving grates, and the material for incineration gradually moves along these. The temperature in the chamber can reach 1000°C but is generally around 850°C. The temperature gradually increases after the waste material is added, and it peaks in the middle of the chamber. Then, it gradually decreases towards the back of the chamber, where the ash is removed. There is an after-burner in the post-combustion chamber to ensure that the legal requirement of 850°C for 2 s is met. The residency time is 30–60 min.

A major difference between this type of plant and the rotary kiln is that the grate incinerator does not provide good turbulence, and because of this, the quality of the incineration process can be less effective than in a rotary kiln. Air is used to generate combustion, and this can also cause the temperature to decrease.

The flue gas cleaning system in this type of incinerator consists of a PCC, steam boiler and a flue gas cleaning system. Bottom ash, boiler ash and fly ash are produced and collected. The bottom ash is about 20% of the input material in volume. A quality control system for the ash, similar to the rotary kiln, is in place. As much material as possible is recovered from the bottom ash. It is processed in a separately operated ash treatment plant where the bottom ash is washed, and ferrous and non-ferrous metals are removed. The metals are then cleaned and recovered. Inorganic material is also treated and, in some cases, recovered.

In the grate incinerator, both contaminated food and international catering waste are incinerated.

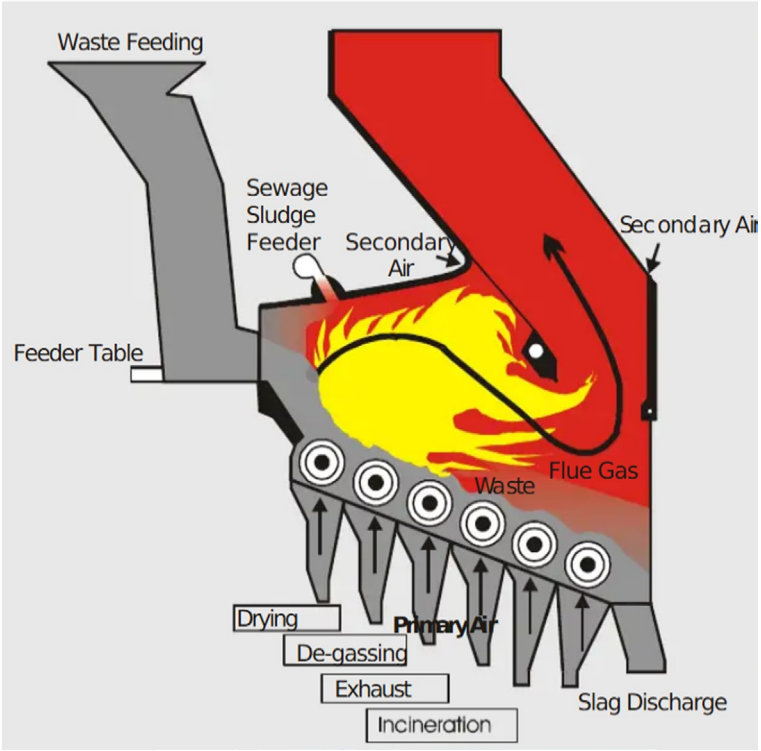

FIGURE 2 Example of grate incinerator. https://wiki.monksleigh.com/index.php?title=Grate&mobileaction=toggle_view_desktop; https://www.scribd.com/document/788168887/UBA-2001.

Fluidised-bed incinerator

Fluid bed kilns are mostly used for sludge or sewage treatment and are heated from below, reaching temperatures of around 650–950°C, with a short residence time of 1–2 min for the solid phase and 2 seconds for the air phase.

This type of incinerator is usually used to process waste-water treatment sludge combined with non-recyclable waste of high calorific value. The combustion area consists of a bed of sand. The sand is fluidised by injection of air via the bottom of the bed. The waste material is fed into this chamber and incinerated. The material to be incinerated must be quite small in size or it will not be held in suspension in the chamber. The temperature ranges from 650°C to 850°C in the combustion chamber and 950°C in the PCC. The reason why the temperature in the combustion chamber cannot be higher than this is that higher temperatures would cause the sand to turn into glass, reducing the system's efficiency. The flue gas cleaning system is similar to the flue gas cleaning system of the grate incinerator. The quantity of bottom ash produced in this type of incinerator is lower compared to the other types of incinerators described (Figure 3).

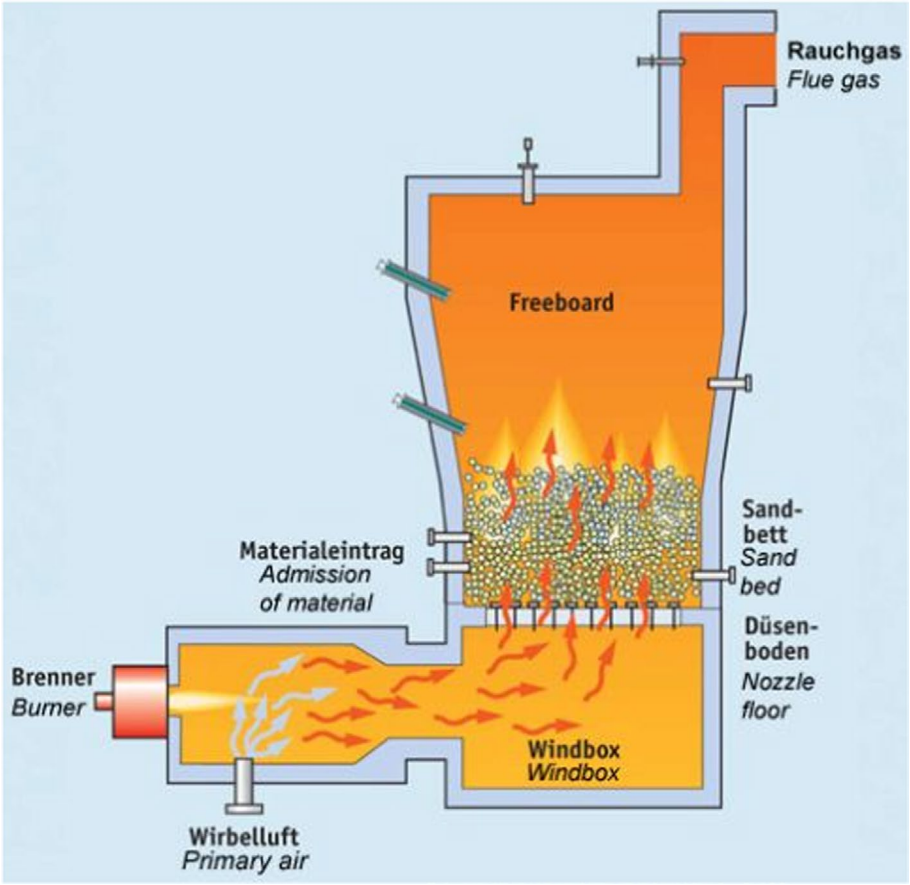

FIGURE 3 Example of fluidised-bed incinerator (*Source*: https://marinersgalaxy.com/incinerator-construction-and-working-procedures-of/ (original source unknown)).

The company only incinerates ABP that cannot be processed by other methods, such as composting and anaerobic digestion. In the rotary kilns, a few hundred tonnes of carcasses of small-sized laboratory animals are incinerated. In the early 2000s, fluidised bed incineration was used to process large amounts of preprocessed ABP as a result of the BSE crisis.

They have two types of landfills for landfilling inorganic materials only, like the (physico-chemically pretreated) residues from the incinerators. They have operated landfill sites since the 1980s.

3.5.3 | Operational aspects of co-incineration plants

Co-incineration involves burning waste and conventional fuel simultaneously in industrial processes. Category 1 animal by-products are mainly co-incinerated in cement kilns and power generation plants, promoting efficient energy recovery and reducing fossil fuel consumption. MBM is used in the following ways: (a) as a biomass fuel in combined heat and power (CHP) plants, producing heat energy for on-site facilities or nearby sites; (b) as a low-carbon biomass fuel in power plants, serving as an alternative to fossil fuels and helping to decarbonise the power supply; (c) as a supplementary biomass fuel in cement kilns to replace fossil fuels and enhance other waste materials used as cement fuels.

The following text describes the operation of co-incineration plants involved in the production of cement. Information was provided by a hearing expert (N. Nikolakakos, CEMBUREAU, personal oral communication, 20 February 2025). Please note that this description pertains to the general operation in Europe of co-incineration plants used for the production of cement. A drawing of the relevant type of co-incinerator was provided by the hearing expert.

The cement manufacturing process involves the extraction of raw materials like limestone and clay, typically sourced from quarries near cement plants to minimise transportation costs. These materials are crushed, ground into a fine powder, preheated and then heated in a rotary kiln at high temperatures to produce clinker, which is rapidly cooled. When ground with gypsum, this clinker forms cement.

Heat is required to produce clinker. In the past (ca. 40 years ago) fossil fuels were the heat source. To reduce reliance on fossil fuels and to lower carbon emissions, the industry increasingly uses alternative fuels, including biomass and waste-derived fuels, such as Category 1 MBM (in authorised installations). The raw materials enter the kiln and the temperature typically reaches 800°C–900°C in the preheater. The MBM is added in the main burner just before entering the rotary kiln, where all materials reach 1400°C, with gas temperatures around 2000°C. Alternative fuels typically remain in the kiln for over 10 s, ensuring full combustion and incorporation of the resulting ash into the clinker. The ash from the MBM is inorganic in nature and replaces virgin raw materials from the raw mix recipe which comes from the quarry. This is an example

of a circular economy in action. The final temperature is measured after the clinker exits the rotary kiln, as it is impossible to measure during the process due to the kiln's rotation. The temperatures of the clinker and of the flame can be measured in the kiln. Cameras are used to monitor the flame shape and temperature, and the operator can adjust the fuel accordingly. Temperature is monitored by thermocouple in all other production stages. Regarding ash residues, the cement production process is a closed system with no solid waste produced. All MBM entering the rotary kiln is fully incinerated, and the ash is incorporated into clinker and thereafter into the cement. This applies to all cement plants in the EU, ensuring no ash waste. All categories of MBM could in principle be utilised in co-incineration plants, depending on market demand and business feasibility.

If the clinker does not meet quality standards, it is separated from good quality clinker at the cement plant. Depending on the quality, clinker can be blended back in small quantities in cement production without affecting the final quality of the cement product. If the clinker quality is very poor, it might be reintroduced into the production process, but this is a last resort due to high energy and time costs. Operators follow specific quality instructions and preventive measures to avoid such issues. There is quality control for MBM (and its associated ash) to ensure its chemistry is compatible with clinker production. While it is unlikely that the material would be rejected outright, it could happen on a case-by-case basis. Cement operators must check the quality of the material upon receipt, as it will impact the clinker quality once introduced into the system. Quality control is crucial before the material enters the production process (Figure 4).

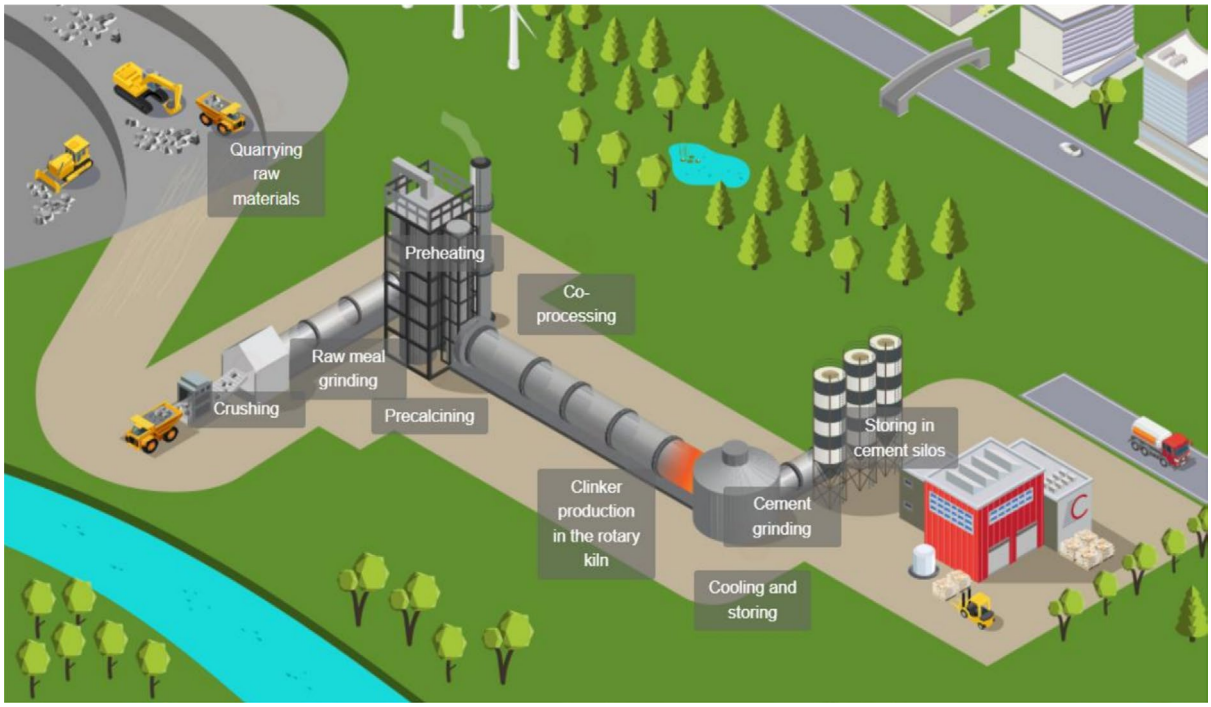

FIGURE 4 Steps of cement production in a kiln co-incinerator (source: CEMBUREAU).

3.5.4 | Operational aspects of combustion plants

Combustion plants offer a means of converting animal by-products not considered as waste under environmental legislation into energy, while minimising the environmental impact and reducing the volume of the products. The use of MBM, including Category 1 MBM, as a fuel for combustion has been permitted since the enactment of Commission Regulation (EU) No 735/2020 in 2020.

The following text describing the operation of combustion plants was provided by hearing experts (N. Rodrigues and M. Barbedo, personal oral communication, 25 November 2024). Please note that these descriptions pertain to combustion plants run by the experts' company, and operating conditions might differ at other companies.

The company operates two combustion units, one for processed fat (Categories 1 and 3) and the other for Category 1 MBM. Method 4 is used in the processing of Category 1 ABP.

The Category 1 MBM produces thermal energy that is used for various purposes in the plant. The combustion unit consists of a feeding system, a fluidised bed gasifier, and a boiler for recovering thermal energy in the form of steam.

MBM, with a particle size of 3–4 mm, is gasified by injecting air from the bottom. The reaction is exothermic and the temperature inside the reactor rises to around 750°C. It generates synthesis gas (biogas), which is sent through a sealed pipeline to the thermal boiler, and bottom ash and fly ash.

At start-up, the afterburner chamber is heated by natural gas until the temperature read at the end of the chamber is ≥ 850°C. From that moment on, the natural gas supply is cut off, and the synthesis gas generated in the gasifier begins to be consumed. In the start-up phase, with natural gas consumption, it takes about 1 h to reach the required temperature of

850°C in the post-combustion chamber on Monday, due to the weekend shutdown, and on the remaining days this temperature is reached after 15–30 min.

Using thermodynamic calculations, the MBM is estimated to reach a temperature higher than 850°C in the fluidised bed gasifier with an overall residence time of 11.48 s. However, it is not possible to determine through calculations how long the particles are effectively at a temperature above 850°C in the fluidised bed unit. In the boiler, the fly ash and bottom ash generated during gasification are exposed to temperatures of at least 850°C for at least 2 s. The PCC is designed so that, in the worst-case conditions, it guarantees a minimum time of 2 s. Temperature probes measure the temperature at the end of the chamber. Bottom ash and fly ash are generated in the boiler: The fly ash (lighter) passes through the entire post-combustion chamber, thus completing the time of at least 2 s, and the bottom ash, which is deposited along the PCC, will remain there until the unloading and cleaning operation is carried out, normally every week. Whenever the temperature drops below 850°C, the supply of synthesis gas and ash is cut off until the minimum temperature of 850°C is restored.

The ash is tested twice a year to monitor whether or not it meets the legal requirement of no more than 3% total organic carbon content. Ashes are used as a soil amendment for forests. MBM is the only ABP used in the combustion unit. In the combustion unit described by the hearing experts, the MBM is initially subjected to gasification in a fluidised bed reactor and the generated synthesis gas is used as fuel in the thermal boiler for the production of steam. The ash generated as a result of MBM gasification is also injected into the boiler combustion chamber. Direct combustion systems are also used in other companies, where the MBM goes directly to the boiler unit, which is not the case with this company.

3.5.5 | Production and uses of ash

It has been shown that between 100 and 310 kg of ash is produced for each ton of MBM that is incinerated (Coutand et al., 2008). Ash is made up of various inorganic compounds that are present in the original material being burned. Ca and P are the main constituents of ash produced by the incineration of MBM. In one study, industrial ash produced by the incineration of MBM had Ca and P contents, up to 47% of the total ash quantity (Coutand et al., 2008). Other elements (Si, Al, Mg, Fe, Na, K, $SO3^{2-}$ and Cl^{-}) made up between 8% and 16% of MBM ash. Ash derived from MBM was mainly composed of whitlockite (βTCP) $Ca_3(PO_4)_2$ and hydroxyapatite $Ca_5(PO_4)_3(OH)$. This is consistent with the high content of Ca and P in ash. Hydroxyapatite is the principal mineral in bone. In another study, the major elements of biomass ash from animal residue incineration were: Ca as the most abundant element comprising 16%–32% of the total ash, followed by P ranging from 8.3 to 13%, Al, Fe, K, Mg, Mn and Na (Leng et al., 2019).

In an ideal incineration process, all organic material should be completely combusted into carbon dioxide, water vapour and other gases. The resulting ash would primarily consist of inorganic residues, such as minerals and metals. Charcoal-like residues or carbon-rich particles may remain in the ash if the combustion process is not fully efficient. These residues can be indicative of incomplete combustion. Some complex organic compounds might be more resistant to complete combustion and could end up in the ash in trace amounts. These include certain proteins and lipids that are harder to fully oxidise.

To address any potential issues with residual organic material, some facilities may employ additional processing or treatment of the ash. This might include further heating, chemical treatments, or mixing with other materials. Ash from incineration is periodically tested to ensure it meets regulatory standards for safety and environmental impact. This includes testing for the presence of hazardous substances and ensuring that any residual organic material does not pose a risk.

The phosphorous component of ash is potentially a valuable fertiliser. Currently, in the EU, only products derived from Category 2 and 3 materials may be used in the manufacturing chain of organic fertilisers/soil improvers as set out in Article 32 of Regulation (EC) No 1069/2009.

The Commission Delegated Regulation (EU) 2023/1605[13] establishes in its Article 3 that ash obtained from Category 2 and 3 materials produced in the Union and manufactured in a fertiliser plant approved in accordance with Article 24(1), point (f), of Regulation (EC) No 1069/2009 is declared the end point for certain organic fertilisers and soil improvers. This was the result of the EFSA's scientific opinion on the inactivation of indicator microorganisms and biological hazards by standard and/or alternative processing methods in Category 2 and 3 animal by-products and derived products to be used as organic fertilisers and/or soil improvers' (EFSA BIOHAZ Panel, 2021). Ash from Category 2 and Category 3 materials may be used directly as fertiliser, mixed into compound fertilisers, or used as starting material for the extraction of phosphorus for further use in fertilisers.

Currently, in the EU, ash derived from Category 1 material must be disposed of by landfill. Category 1 materials are currently prohibited in the manufacture of organic fertilisers and soil improvers in the EU, but they are used as a fertiliser in some countries. Ash derived from Category 1 material is used as an organic fertiliser in the UK on the basis that *'ash resulting from the incineration of category 1 specified risk material is not under the control of the ABP regulations. This comes under the control of Environmental Controls (Waste Framework Directive)'*.[14] Five plants in the UK produce around 70,000 tons of ash as a result of incinerating Category 1 MBM, most of them used as arable fertiliser under an *End of Waste* status or in fertilising

[13]The Commission Delegated Regulation (EU) 2023/1605 of 22 May 2023 supplementing Regulation (EC) No 1069/2009 of the European Parliament and of the Council as regards the determination of end points in the manufacturing chain of certain organic fertilisers and soil improvers https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32023R1605.

[14]Animal by-products: how to burn them at an incinerator site - GOV.UK (www.gov.uk).

products (SAFOSO for ESSP, 2024). This provision was introduced prior to the UK departure from the European Union and is still in operation. In Switzerland, there is a legal prohibition on the use of ash derived from Category 1 ABP as fertiliser, unless the presence of certain pollutants (heavy metals, polycyclic aromatic hydrocarbons) is kept under certain limits. Since 2016, between 2000 and 2500 tonnes per year of bottom ash from Category 1 MBM produced in a rotary kiln have been used as a fertiliser in eucalyptus woods in Portugal. Similarly, other non-EU countries allow the use of Category 1 ABP as fertiliser after rendering, e.g. the USA (SAFOSO for ESSP, 2024).

3.6 | Scenarios considered in this assessment (AQ2)

The scenarios listed below are representative of different process combinations under which ashes relevant to this mandate could be produced. They are listed for illustration purposes and are not intended to be an exhaustive list of potential industrial scenarios, given the many variables identified in scale and operating practices. It has been deemed unnecessary and unrealistic to determine the minimum temperature and shortest time that the material can be subjected to in each of the combinations, due to the impossibility of collecting comprehensive data relevant to previous or current practices.

Although it is possible to burn fresh ABP as a means of waste disposal, for practical reasons the vast majority of ABP waste is rendered into MBM prior to use/disposal. While fresh materials are rarely used, the following scenarios focus on approaches that include the primary rendering step,

1. Rendering (method 1 or method 4) followed by incineration (rotary kiln, grate or fluidised bed incinerators)
2. Rendering (method 1 or method 4) followed by co-incineration (in cement or in thermal plants)
3. Rendering (method 1 or method 4) followed by combustion

It has not been possible to determine accurately the relative amounts of Category 1 ABP treated under each scenario. It appears that rendering followed by co-incineration in cement plants and rendering followed by incineration in rotary kilns are currently the most common methods for the disposal of Category 1 ABP. During the process of co-incineration in cement plants (Scenario 2), the resultant residue (clinker) is incorporated into the cement, or re-incinerated if it is of poor quality. Any scenario involving co-incineration resulting in cement production, therefore, does not need to be considered further because it does not result in stand-alone ash as a by-product.

3.7 | Heat treatment (time/temperature) profiles of incineration, co-incineration or combustion processes (AQ3)

The minimum temperature and time requirements set out in the legislation do not represent the full range of temperatures and residence times that the material undergoing incineration actually experiences. Data collected for this opinion indicate that compliance with the legal requirements typically involves exposing the material to high temperatures (ranging between 700°C and 2000°C) for variable residence times. In many cases, the actual exposure time may exceed the minimum legislative requirements. The three different types of incinerators – rotary kiln, grate and fluidised bed incinerators – provide variable efficiency of incineration. Rotary kilns seem to generate the highest temperatures for the longest residency times (in the example provided in Section 3.5.2, at least 1000°C for several minutes), while fluidised bed incinerators generate the lowest (from 800°C to 850°C for seconds to 1 min). In a combustion plant, finely ground MBM can be subjected to time/temperature profiles in line with the minimum requirements of 850°C for at least 2 s.

These observations are based on consultations to a limited number of companies and publicly available data. While the estimated time/temperature combinations are representative of various process types, they should be viewed as general indications rather than precise measurements. Variations are expected due to the inherent complexity of the technologies and differing operational contexts.

Experts have highlighted the difficulty of accurately measuring the actual temperature of the material during the process. Although the legislation requires that the '*gas resulting from the process is raised in a controlled and homogeneous fashion*' at the specified temperature and time, the temperature of the combusting gas or the fuel used may differ from the maximum temperature achieved by the material itself. This discrepancy complicates the ability to ascertain and generalise the conditions under which ashes from incineration, co-incineration or combustion of Category 1 ABP are produced.

There are many different types of incineration, co-incineration and combustion systems. These systems vary in design, size, capacity, structure and methodology. Consequently, the time/temperature combinations – and therefore the potential level of prion reduction achieved – can vary significantly between systems.

Given the wide range of system designs and operational conditions, it is not possible to generalise the time/temperature combinations to which Category 1 ABP are subjected across all processes. Due to the challenges in precisely measuring the temperature and residence time in industrial systems, it must be assumed that Category 1 ABP are exposed, during ash production, to at least the minimum legal requirements as determined by the conditions of the gas produced or injected into the process, namely, 850°C for 2 s or 1100°C for 0.2 s.

4 | UNCERTAINTY ANALYSIS

TABLE 1 sources and causes of uncertainty, and impact on the conclusions.

Source of uncertainty	Cause of the uncertainty	Impact of the uncertainty on the conclusions
The most thermoresistant animal field TSE strain	Thermoresistance of classical scrapie (CS) has been assessed with uncharacterised isolates. Given that the total number of field strains of CS is unknown, the data cannot be exhaustive, and we cannot exclude the existence of CS strains that are more thermoresistant than Classical-BSE (C-BSE). The same applies to European Chronic wasting disease (CWD) strains, whose thermoresistance is still uncharacterised.	This could result in the overestimation of the level of reduction of TSE agents by incineration, co-incineration and combustion, if there was an animal field TSE strain more thermoresistant than C-BSE
	The ranking of animal TSE strains is based on limited studies using wet-heat treatment at 133°C rather than dry-heat treatments in the range of 800–1050°C provided by incineration/co-incineration/combustion	This could result in an over- or underestimation of the level of reduction of TSE agents by incineration, co-incineration and combustion
Thermoresistance of animal TSE field strains, higher temperature range (> 400°C)	A very limited number of studies are available on the resistance of prions to incineration temperatures and only one study is available for animal TSE field strains. None of these studies reproduces the time/temperature profiles stipulated in the regulation or achieved by industrial processes	This could result in an over- or underestimation of the level of reduction of TSE agents by incineration, co-incineration and combustion
	The few experimental studies conducted at incineration temperatures have used fresh brain tissue or homogenates as starting material instead of meat and bone meal (MBM). Preheating and drying of infected brain tissue has been shown to increase the thermoresistance of rodent-adapted prion strains to further heat treatment.	This could result in an overestimation of the level of reduction of TSE agents by incineration, co-incineration and combustion
	Many effects of the high temperature have not been assessed: formation of protective residues/material at high temperatures (e.g. ash insulation), oxidative conditions, combustion dynamics, MBM size and composition, etc.	This could result in an over- or underestimation of the level of reduction of TSE agents by incineration, co-incineration and combustion
Time–temperature profiles in the compartments producing ash	There is considerable variability in the design, nature of use, performance characteristics of incinerators, co-incinerators and combustion plants, leading to variability in time/temperature profiles in the compartments where ash is produced. Due to the limited industry data collected and the difficulty of measuring the temperature reached by the material, there is also uncertainty around the actual values.	If the actual time–temperature profile that is applied in the production of ash is higher or lower than those set out in the regulations – the gas produced or injected into the process, this could result in an over- or underestimation of the level of reduction of TSE agents by incineration, co-incineration and combustion

5 | CONCLUSIONS

- TSE agents are challenging to inactivate completely using heat-based methods. The thermoresistance of TSE strains varies considerably.
- Limited studies on the thermoresistance of EU TSE field strains indicate that the C-BSE strain appears to be more thermoresistant than other strains evaluated.
- The vast majority of Category 1 ABP is rendered into MBM prior to use/disposal. The scenarios considered include rendering (method 1 or method 4, as defined in Chapter III Annex IV of Commission Regulation (EU) No 142/2011) followed by incineration or co-incineration or combustion.
- Although accurate quantification of Category 1 ABP entering each scenario is not possible, rendering combined with co-incineration in cement plants and rendering with incineration in rotary kilns currently represent the most common disposal routes.
- In co-incineration processes used for cement production, the ash produced is fully incorporated into the cement. Consequently, scenarios involving co-incineration for cement production do not need to be considered because ash is incorporated into cement.
- Due to the wide range of system designs and operating conditions, it is not possible to generalise the time/temperature combinations for Category 1 ABP across all processes. In practice, residency times may be considerably longer, and the actual temperatures reached by the material during the processes may be higher than those required by the legislation, varying between treatment and industrial settings.
- Since the minimum temperature and time requirements set out in the legislation do not represent the range of temperatures and residence times the actual material is exposed to, it can only be assumed that Category 1 ABP is exposed, during incineration, co-incineration, and combustion, to at least the minimum legal requirements as determined by the conditions of the gas produced or injected into the process, namely, 850°C for 2 s or 1100°C for 0.2 s.
- While a treatment at 1000°C for 5 min has been demonstrated to completely inactivate the 263K hamster strain in tissues, no evidence is available for treatment durations of 0.2 s or 2 s. In the only study involving C-BSE at this temperature (1000°C) for 20 min, the limited sensitivity of the method used prevented a conclusive exclusion of residual C-BSE prions.
- There is not sufficient relevant experimental data on the actual thermoresistance of TSE agents and on industrial operating conditions.
- Therefore, it is not possible to exclude – with high certainty (> 99%) – the presence of residual BSE/TSE hazards in ash produced from the incineration, co-incineration or combustion of Category 1 ABP.

6 | RECOMMENDATIONS

- To promote the implementation of studies and/or surveys aimed at collating and summarising actual time/temperature combinations to which MBM derived from Category 1 ABP is subjected during industrial incineration/co-incineration/combustion processes authorised by the current legislation.
- To conduct experimental studies comparing the thermoresistance of all animal TSE field strains identified in the EU under temperatures and processing conditions that reflect industry practices.
- To carry out experimental studies in which MBM is spiked, as a first priority, with a thermoresistant TSE field strain (using C-BSE in the absence of any alternative data) and then treated at the minimal time/temperature combinations required by the legislation or specific industry processes. These studies should use spiked material with sufficient titre (infectivity/seeding activity) and employ highly sensitive detection methods (e.g. bioassay, PMCA) to quantify infectivity reductions and look for any residual infectivity. In addition, such studies could provide additional information, e.g. the role of ash formation in prion persistence and/or inactivation at high temperature, the potential impact of different oxygen environments (open air vs. controlled atmosphere), the molecular/structural changes of the prion protein at high temperature and the influence of strain PrP^{Sc} diversity and prion PrP^{Sc} assembly composition on its heat resistance.
- To evaluate the extent of the reduction of TSE agent infectivity achieved by processing methods other than method 1 (pressure sterilisation).

ABBREVIATIONS

ABP	Animal by-products
AQ	Assessment Question
AS	Atypical scrapie
BIOHAZ	EFSA Panel on Biological Hazards
BSE	Bovine Spongiform Encephalopathy
CJD	Creutzfeldt–Jakob disease
CS	Classical scrapie
CWD	Chronic Wasting Disease
EFPRA	European Fat Processors and Renderers Association
ESPP	European Sustainable Phosphorous Platform
FDC	Follicular Dendritic Cell

i.c.	Intracerebral
ID_{50}	infectious dose 50%
MBM	Meat-and-Bone Meal
PCC	Post-combustion chamber
PMCA	Protein Misfolding Cyclic Amplification
PrP	Prion Protein
PrP^{C}	Cellular Prion Protein
PrP^{Sc}	Abnormal Isoform of the Cellular Prion Protein
RA	Risk Assessment
RF	Reduction Factor
RT-QuiC	Real-Time Quaking-induced Conversion
SA_{50}	Seeding Activity
SEAC	Spongiform Encephalopathy Advisory Committee
SRM	Specified Risk Material
SSC	Steering Scientific Committee
SQ	Sub-Questions
ToR	Terms of Reference
TSE	Transmissible Spongiform Encephalopathy
UK	United Kingdom
USA	United States of America
WB	Western blot
WCS	Worst Case Scenario
WG	Working Group
wt	wild type

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PANEL MEMBERS

Ana Allende, Avelino Alvarez-Ordóñez, Valeria Bortolaia, Sara Bover-Cid, Alessandra De Cesare, Wietske Dohmen, Laurent Guillier, Lieve Maria Herman, Liesbeth Jacxsens, Lapo Mughini-Gras, Maarten Nauta, Jakob Ottoson, Luisa Peixe, Fernando Perez-Rodriguez, Panagiotis Skandamis, and Elisabetta Suffredini.

GLOSSARY

Isolate	Biological material that has been obtained through the sampling of an individual infected animal. An isolate may contain a single prion strain or a mixture of strains. Its properties can be studied through bioassays or biochemical methods to determine strain identity and transmissibility.
MBM	Meat-and-bone meal (MBM). Product of the rendering industry with high protein content produced from Category 1 and 2 animal by-products, which are not fit for human consumption, in accordance with one of the processing methods set out in Chapter III of Annex IV (Commission Regulation (EU) No 142/2011).
Prion/TSE agent	Prions are the infectious agents responsible for TSE. Prions lack nucleic acids and propagate solely through prion protein (PrP) conformational changes.
PrP^{Sc}	Abnormal, misfolded form of the prion protein
PrP^{C}	Normal cellular prion protein
Rendering	A process of using high temperature and pressure to convert whole animal and poultry carcasses or their by-products with no or very low value to safe, nutritional, and economically valuable products. It is a combination of mixing, cooking, pressurising, fat melting, water evaporation, microbial and enzyme inactivation (Romans, J.R., Costello, W.J., Carlson, C.W., Greaser, M.L., & Jones, K.W. (2001). Packing house by-products. In The Meat We Eat. Danville, Illinois: Interstate Publishers, Inc.).

TSE	A group of fatal, neurodegenerative diseases caused by misfolded prion proteins. TSE affect humans and animals, leading to neurodegeneration, spongiform changes, and progressive neurological symptoms. Examples include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, chronic wasting disease in cervids.
TSE Strain/prion strain	A distinct and host-specific variant with reproducible pathological, biochemical and molecular characteristics in the original host/s and which, when serially passaged through congenic or transgenic mouse lines, produces consistent characteristics of relative incubation period, spongiform lesion profile, molecular profile and immunopathology. Strains are different conformations of PrP^{Sc}. Adapted from the definition of strain as in the 'TSE strain characterisation in small ruminants. A technical handbook for national reference laboratories in the EU': https://www.eurl-tse.eu/wp-content/uploads/2024/09/EURL_smallruminants_discriminatory_guidance_v3_revised.pdf.
WtE	Waste-to-energy

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

Summary of the studies on thermal inactivation of prions included in the opinion

Table A.1 summarises data from some of the publications discussed in the opinion to illustrate the great diversity of experimental parameters and methods to be found in this small body of literature.

TABLE A.1 Summary of the studies described in the text on thermal inactivation of prions.

Reference	Strain	Tissue type and matrix (if any)	Type of treatment	Duration	Temperature	Model and measurement	Outcome
Taylor et al. (1995)	C-BSE $10^{1.7}$ ID_{50}/g	Bovine brain tissue with natural fat content	Batch. 150 mm diameter under atmospheric pressure	150 min	Up to 121°C	i.c and i.p bioassay in RIII/FaDK-ro mice	No infectivity
		Bovine brain tissue with natural fat content	Continuous atmospheric 30 mm diameter	50 min	Up to 112°C		Infectivity still detectable in MBM. Not titrated
				125 min	123°C		No infectivity
				50 min	122°C		Infectivity still detectable in MBM. Not titrated
				125 min	139°C		No infectivity
		Bovine brain tissue with high fat content	Continuous atmospheric 30 mm diameter	30 min	136°C		No infectivity
				120 min	137°C		No infectivity
			Continuous vacuum 10 mm diameter	20 min	120°C		Infectivity still detectable in MBM. Not titrated
				57 min	121°C		Infectivity still detectable in MBM. $10^{1.6}$ ID_{50}/ml
		Bovine brain tissue with natural fat content	Continuous wet rendering 20 mm diameter	120 min	101°C		No infectivity
				240 min	119°C		No infectivity
				240 min	72°C		No infectivity
			Batch pressure 50 mm diameter	30 min	133°C		No infectivity
			Batch pressure 30 mm diameter	28 min	135°C		No infectivity
				28 min	145°C		No infectivity

(Continues)

TABLE A.1 (Continued)

Reference	Strain	Tissue type and matrix (if any)	Type of treatment	Duration	Temperature	Model and measurement	Outcome
Fernie et al. (2007)	263K	LVG hamster brain macerate	Dry heat	20 min	200°C	i.c. LVG hamsters	2.97 $\log_{10}$ reduction
	263k	LVG hamsters brain macerate	Dry heat	60 min	200°C	i.c. LVG hamsters	3.36 $\log_{10}$ reduction
	ME7	SV mice brain macerate	Dry heat	20 min	200°C	i.c. in C57BL mice	3.02 $\log_{10}$ reduction
	ME7	SV mice brain macerate	Dry heat	60 min	200°C	i.c. in C57BL mice	3.06 $\log_{10}$ reduction
	301v	VM mice brain macerate	Dry heat	20 min	200°C	i.c. VM mice	2.3 $\log_{10}$ reduction
	301v	VM mice brain macerate	Dry heat	60 min	200°C	i.c. VM mice	3.03 $\log_{10}$ reduction
	22A	VM mice brain macerate (7.2 ID_{50}/g)	Dry heat + porous load autoclave	60 min + 18 min	160°C + 134°C	i.c. VM mice	Detectable infectivity. Not titrated
	22A	VM mice brain macerate 50 and 375 mg	Porous load autoclave	9 min-18 min-30 min-60 min	134–138°C	i.c. VM mice	Infectivity was measured at a single dilution, not titrated.
	301V	VM mice brain macerate 50 and 375 mg	Porous load autoclave	9 min-18 min-30 min-60 min	134–138°C	i.c. VM mice	Some infectivity was detected after autoclaving for all strains. The 3 strains had different titre reduction. Most infectivity was detected in the 301V samples. Significantly more infectivity survived in the 375 mg samples than in the 50 mg samples. There was a trend towards a small but inconsistent reduction in residual infectivity with increasing time of exposure. There was no significant effect owing to increasing temperature.
	263k	LVG hamster brain macerate 30 and 375 mg	Porous load autoclave	9 min-18 min-30 min-60 min	134–138°C	i.c. LVG hamsters	
	C-BSE	Bovine brain macerates	Gravity displacement autoclave	18 min (1 or 2 rounds)	134°C		2.5 (1 round) and 1.9 (2 rounds) $\log_{10}$ reduction. The first exposure to porous load autoclaving appeared to enhance survival of infectivity during the second run.
	C-BSE	Bovine brain macerates	Gravity displacement autoclave	4 h (1 or 2 rounds)	72°C		0.5 (1 round) or 0.6 (2 rounds) $\log_{10}$ reduction.

TABLE A.1 (Continued)

Reference	Strain	Tissue type and matrix (if any)	Type of treatment	Duration	Temperature	Model and measurement	Outcome
Taylor et al. (1997)	Classical scrapie (pool of brain macerates from natural cases) Titre ≥3.1 log_{10} ID_{50}/g	Ovine brain tissue with natural fat content	Batch. 150 mm diameter under atmospheric pressure	150 min	114°C	i.c and i.p bioassay in C57BL mice	Because the weight of the solid material remaining after rendering was 30% of the weight of the starting material, the maximum titre in the resulting MBM would be > 3.1 log_{10} ID_{50}/0.3 g (or per ml of 30% suspension). The MBM samples all contained detectable scrapie infectivity, apart from those produced by protocols involving the exposure of either spiked raw materials or infected MBM to steam under pressure.
		Ovine brain tissue with natural fat content	Continuous atmospheric 30 mm diameter	50 min	102°C		
				125 min	121°C		
				50 min	100°C		
				125 min	138°C		
		Ovine brain tissue with high fat content mixed with other tissues (pork intestine and bone)	Continuous atmospheric 30 mm diameter	30 min	134°C		
				120 min	138°C		
			Continuous vacuum 10 mm diameter	27 min	117°C		
				61 min	126°C		
		Ovine brain tissue with natural fat content	Continuous wet rendering 20 mm diameter	120 min	103°C		
				240 min	120°C		
				240 min	72°C		
		Material from continuous wet rendering	Solvent extraction of greaves 20 mm diameter	10 min	80°C		
		Greaves from the previous treatment	Steam treatment 20 mm diameter	20 min	100°C		
		Greaves from the previous treatment	Steam treatment 20 mm diameter	40 min	100°C		
		Ovine brain tissue with natural fat content	Batch pressure raw material 50 mm diameter	30 min	134°C		
			Batch pressure raw material 30 mm diameter	18 min	136°C		
				18 min	145°C		
		Meal with natural fat content	Batch pressure meal 2.2 mm diameter	20 min	136°C		
				20 min	145°C		

(Continues)

TABLE A.1 (Continued)

Reference	Strain	Tissue type and matrix (if any)	Type of treatment	Duration	Temperature	Model and measurement	Outcome
Rohwer (1984)	263K	Hamster brain homogenate	Heat	1 h	121°C	i.c. bioassay in golden Syrian hamsters	At 121°C, 99.999% of infectivity (5 $\log_{10}$) was destroyed within 1 m exposure. At 100°C, 97% (< 2 $\log_{10}$) was destroyed within 2 m of exposure. At both temperatures small subpopulations of infectivity survived for longer periods.
					100°C		
Schreuder et al. (1998)	C-BSE	Abattoir waste (mainly porcine) spiked with bovine brainstems (pool of 6)	Autoclaving (hyperbaric, pressure not reported)	3 min	105°C	i.c and i.p bioassay in RIII mice	Reduction factor (RF):0
				3 min	121°C		RF ($\log_{10}$): 0.2
				15 min	125°C		RF ($\log_{10}$): 1.6
				3 min	134°C		Not calculated (low number of mice)
				20 min	133°C		RF ($\log_{10}$): 2.2
	Classical scrapie	Abattoir waste (mainly porcine) spiked with ovine brainstems (pool of 60)		3 min	105°C	i.c and i.p bioassays in RIII /C57BL6 or VM mice	RF ($\log_{10}$): 0
				3 min	121°C		RF ($\log_{10}$): 1
				15 min	125°C		RF ($\log_{10}$): > 1.7 or 1.9[a]
				3 min	134°C		RF ($\log_{10}$): > 1.7 or > 2.1
				20 min	133°C		RF ($\log_{10}$): > 1.3 or > 1.7
Sommerville and Gentles (2011)	ME7 87A	Mice brain homogenates (10%) from 18 combinations of TSE strain and mouse strain (SV, VM or C57Bl)	Dry heat	10 min	65–110°C	ME7, 22A, 22C, 79A, 139A 301C and 301V: i.c. bioassay (incubation time) in SV and VM mice 87A and 22F i.c. bioassay (incubation time) in C57BL	ME7 and 87A: ~4–5 $\log_{10}$ reduction at 110°C
	22C 22A 22F						22A, 22C and 22F: ~2–5 $\log_{10}$ reduction at 90°C–110°C
	79A 139A						79A and 139A: ~4–5 $\log_{10}$ reduction 90°C–110°C
	301C 301V/VM						301V and 301C: ~3–4.5 $\log_{10}$ reduction at 110°C
Grobben et al. (2006)	301V	Dried bone chips spiked with infected mouse brain	Two phase autoclaving. 3 bar	20 min	Up to 115°C, then down to 92°C, followed by 133°C for 20 m	i.c. bioassay in VM mice	4 $\log_{10}$ reduction

TABLE A.1 (Continued)

Reference	Strain	Tissue type and matrix (if any)	Type of treatment	Duration	Temperature	Model and measurement	Outcome
Cardone et al. (2006)	263K	Food spiked with Syrian hamster brain.	Autoclaving with UHP (690 MPa)	3–10 min	120–125°C	WB/Bioassay	1.5/3 $\log_{10}$ reduction
			Autoclaving with UHP (1000 MPa)		135–137°C		> 3/5.7 $\log_{10}$ reduction
			Autoclaving with UHP (1200 MPa)		135–142°C		> 3.5/6.3 $\log_{10}$ reduction
	C-BSE	Cattle brain tissue	Autoclaving with UHP (600 MPa)	5 min	130°C	WB	0.5 $\log_{10}$ reduction
	Mouse C-BSE	Mouse brain tissue					1 $\log_{10}$ reduction
	Mouse vCJD						1.5 $\log_{10}$ reduction
	263K	Hamster brain tissue					1.5 $\log_{10}$ reduction
Taylor et al. (2002)	ME7	SV or VM mouse brain macerates	Gravity-displacement autoclaving	30 min	126°C	i.c. bioassay in SV mice/i.c. bioassay in VM mice	≥ 5.7/≥ 5.5 $\log_{10}$ reduction
	22C						≥ 5.2/≥ 5.2 $\log_{10}$ reduction
	22A						4.2/≥ 4 $\log_{10}$ reduction
	139A						≥ 5.4/≥ 5.4 $\log_{10}$ reduction
	301V						1.9/2.7 $\log_{10}$ reduction
Grobben et al. (2005)	301V	Raw bovine vertebral column spiked with mouse brain	Degreasing followed by autoclaving	20 min	132–134.8°C	i.c. bioassay in VM mice	≥ 6.5 $\log_{10}$ reduction
Matsuura, Ishikawa, Bo, et al. (2013)	C-BSE	WET macerates/semidry macerates/dry macerates of spinal cord (pool of 4 cows)	Wet heat 0.21–0.68 MPa	20 min	133–170°C	i.c. bioassay in tg mice (incubation time assay)	Bioassay (~4 $\log_{10}$ maximum reduction detectable) showed that infectivity was not detected in the wet macerates treated > 155°C, but was detected in dry macerates even at 170°C.
						Quantitative end-point titration by PMCA	Seeding activity was still detectable in wet macerates treated at 170°C (7.1–7.7 $\log_{10}$ reduction)
Spiropoulos et al. (2019)	AS/Nor 98	Sheep brain tissue macerates	Autoclaving, 3 bar	20 min	133°C	i.c. bioassay in transgenic mice	10 $\log_{10}$ reduction (95% CI: 8.1–11.2)
	CS (PG127 or DAW isolate)						6 $\log_{10}$ reduction (95% CI: 5.9–7.3)

(Continues)

TABLE A.1 (Continued)

Reference	Strain	Tissue type and matrix (if any)	Type of treatment	Duration	Temperature	Model and measurement	Outcome
Chapman et al. (2020)	C-BSE	Bovine brain tissue macerates	Autoclaving, 3 bar	20 min	133°C	i.c. bioassay in transgenic mice	5.78 $\log_{10}$ reduction (95% CI: 4.28–7.62)
	L-BSE						Lower 95% CI: 9.40 $\log_{10}$ reduction (infectivity not detected after treatment)
	H-BSE						Lower 95% CI: 3.94 $\log_{10}$ reduction (infectivity not detected after treatment)
Taylor et al. (1998)	263K	Pooled macerate of hamster brains (0.34 g)	Porous-load autoclave	18 min (1 or 2 cycles)	134°C	i.c. bioassay in Syrian hamsters	3.3 $\log_{10}$ reduction after 1 cycle. A further 1.3 $\log_{10}$ reduction after the second cycle
Taylor and McConnell (1988)	22A (titre 6 $\log_{10}$ ID_{50}/g)	Mice brain (50 mg fragments or whole brain). Sterile saline solution 48h	Porous-load autoclaving	18 min	134°C	i.c. bioassay in VM mice	No detectable infectivity
		Fixed in 10% formol saline					1.5 $\log_{10}$ reduction
Giles et al. (2008)	C-BSE	Bovine brain homogenate (50 µL 10%)	Autoclaving	15 min	134°C	i.c. bioassay in transgenic mice (tgBov)	2.9 $\log_{10}$ reduction (2.3–3.7)
				30 min			3.2 $\log_{10}$ reduction (2.5–4.3)
				2 h			> 4 $\log_{10}$ reduction (3.1, > 4)
	301V	B6.I mouse brain homogenate (50 µL 10%)		15 min		i.c. bioassay in transgenic mice (tgMoPrP-B)	6.2 $\log_{10}$ reduction (5.2–7.8)
				30 min			> 8.1 $\log_{10}$ reduction (6.5– > 8.1)
				2 h			> 8.1 $\log_{10}$ reduction (6.6– > 8.1)
Peretz et al. 2006) combined with Giles et al. (2008)	SCJD (MM1)	Human brain homogenate (10%)	Autoclaving	15 min, 30 min, 2 h	134°C	i.c. bioassay in transgenic mice	> 7.2 $\log_{10}$ reduction (4.3– > 7.2)
	Sc237	Hamster brain homogenate (10%)		30 min, 2 h	154°C	i.c. bioassay in transgenic mice (tg-hamster)	>7.2 $\log_{10}$ reduction (6.4– > 7.2)

TABLE A.1 (Continued)

Reference	Strain	Tissue type and matrix (if any)	Type of treatment	Duration	Temperature	Model and measurement	Outcome
Murayama et al. (2006)	Sc237	Hamster brain macerate	Dry heating	1 h (+ time necessary to achieve the desired temperature (20 to 85 min)	200°C, 600°C, 800°C, 1000°C	i.c. bioassay in tg mice expressing hamster PrP	No disease (RF >>8 $\log_{10}$)
						PMCA (3 rounds) with hamster brain as substrate	No positive PMCA reactions (control positive up to the 10^{-9} dilution)
			Autoclaving (0.23 up to 2 MPa)		135–200°C	i.c. bioassay in tg mice expressing hamster PrP	100% AR at 135°C, 0.23 MPa, 30 m 20% AR at 142°C, 0.3 MPa, 30 min No disease for treatments starting from 152°C- 0.4 MPa-30m to 200°C-2 MPa-0 m.
						PMCA (3 rounds) with hamster brain as substrate	As for the bioassay, positive PMCA reactions observed at 135°C and 142°C. No positive reactions at 152°C and over. like bioassay, no positive from temp over 152°C
Yoshioka et al. (2013)	C-BSE	Macerate of bovine spinal cords spiked in pre-heated yellow grease at the final treatment temperature	Dry heat	1 h	140°C	Yellow grease eliminated before performing bioassay or PMCA i.c. bioassay in bovine PrP mice PMCA using bovine PrP mice brain as substrate	1.54 $\log_{10}$ reduction by bioasssay, 2.75 $\log_{10}$ by PMCA
				3 h	140°C		> 3 $\log_{10}$ (bioassay, 83% AR) or 4 $\log_{10}$ (PMCA)
				1 h	160°C		> 3 $\log_{10}$ (bioassay, 67% AR) or 4 $\log_{10}$ (PMCA))
				3 h	160°C		> 3 $\log_{10}$ (bioassay, 17% AR) or 6 $\log_{10}$ (PMCA)
				1 h	180°C		> 3 $\log_{10}$ (bioassay, 100% AR) or 3.25 $\log_{10}$ (PMCA)
				3 h	180°C		> 3 $\log_{10}$ (bioassay, 0% AR) or 6.75 $\log_{10}$ (PMCA, low dilutions still positive at round 4)

(Continues)

TABLE A.1 (Continued)

Reference	Strain	Tissue type and matrix (if any)	Type of treatment	Duration	Temperature	Model and measurement	Outcome
Matsuura et al. (2020)	C-BSE	Macerate of bovine spinal cords	Dry heat (electric furnace)	20 min	150°C	PMCA assay using bovine PrP mouse brain substrate and 'FDC' bioassay based on detection of PrP^{Sc} and PMCA seeding activity in the spleen at day 150 post-infection (ip route, 500 μL)	PMCA and bioassay positive (100% AR), no quantitative assessment
					200°C		PMCA and bioassay positive (100% AR)
					400°C		Bioassay positive (40% AR after PMCA assay on the spleen tissue), no PMCA seeding
					600°C		Bioassay negative, no PMCA seeding
					800°C		Bioassay negative, no PMCA seeding
					900°C		Bioassay negative, no PMCA seeding
					1000°C		Bioassay negative, no PMCA seeding
Brown et al. (2000)	263K	Hamster brain pool fresh or fixed thawed	Dry electric heat in pressure reactor	5 min to 15 min	150°C	i.c. bioassay in golden Syrian hamster	100% AR 2–3 log reduction at 15 m
					300°C		100% AR 6 log_{10} reduction at 15 m
					600°C		AR 0/15 in 5 m and 5/18 in 15 m 425
					1000°C		No transmission at 5 m
Muller et al. (2007)	263K	Purified and protease K-treated PrP^{Sc} (PrP 27–30) in different fat/water mixtures	Pressure reactor with external electric heating	Between 11 min and 47 min	Up to 200°C	WB/SDS-PAGE immunoblot (3F4) sensitivity 1 ng. Bioassay in Syrian golden hamsters	Fat increases backbone resistance by 1 log_{10}. Glycerol protects by 2 log_{10} 100% fat, 20 m 200°C: > 6 log ID_{50} reduction 90% fat/10% water, 20 m 200°C: > 4.6 log ID_{50} reduction 100% glycerol, 20 m 200°C: > 6 log ID_{50} reduction
Taylor et al. (1996)	ME7	Mice brain macerate	Hot air fan oven	20 min	200°C	i.c. bioassay in mice	100% AR. 6log ID_{50}/gr reduction
				60 min			0% AR. No infectivity detectable

TABLE A.1 (Continued)

Reference	Strain	Tissue type and matrix (if any)	Type of treatment	Duration	Temperature	Model and measurement	Outcome
Brown et al. (2004)	263K	hamster brain pool Titre 9.2–9.7 log ID_{50}/g	Dry heat in air	15 min	600°C	i.c. bioassay in hamsters	2/21 AR in quark crucible
			Dry heat in N_2				No infectivity detected
			Dry heat in air		1000°C		No infectivity detected
			Dry heat in N_2				No infectivity detected

Abbreviations: AR, attack rate; i.c., intra cerebral; MBM, meat and bone meal; MPa, megapascal; PMCA, protein misfolding cyclic amplification; RF, reduction factor; UHP, ultra-high pressure; WB, western blot; WB, western blot.

[a]Range according to experimental conditions and the different bioassays.

ANNEX A

Protocol

Annex A is available under the Supporting Information section on the online version of the scientific output.