

**Validation of the clearance of TSE infectivity by the initial  
steps of the acid bone gelatine manufacturing process  
with an additional short NaOH treatment**

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## SUMMARY REPORT.

**The industrial process for production of acid bone gelatine was accurately downscaled to allow laboratory scale production of gelatine from bovine bone raw material experimentally contaminated with brain tissue infected with the 301V strain of mouse-passaged bovine spongiform encephalopathy (BSE). A novel, additional step was introduced to the process in which the bone material was for a short time treated with sodium hydroxide. Samples of the infective brain and of a crude gelatine extracted from the process were inoculated intracerebrally into experimental mice to determine the amount of infectivity present in each. The infectious brain had an infectivity titre of  $10^{7.7}$  ID<sub>50</sub>/g. No animals succumbed following inoculation with the gelatine extract. The calculated clearance factor for this modified acid bone manufacturing process was  $\geq 10^{5.4}$  ID<sub>50</sub>.**

### Introduction.

The Gelatin Manufacturers of Europe (GME) commissioned a validation study on the inactivation and removal effect of the gelatine manufacturing process on Transmissible Spongiform Encephalopathies (TSE). The background, aims, approach and planned execution of this study are extensively described in the protocol of the study, titled *Evaluation of the inactivation/removal effect of the gelatin manufacturing process on TSE infectivity*, prepared by GME on 7 May 1999, amended version of 18 November 1999. The study is a demonstration project supported by the European Commission Quality of Life program (KA1 Evaluation of the inactivation/removal effect of the gelatin manufacturing process on TSE infectivity, QLK1-CT-2000-00009) and is coordinated by Delft Gelatin BV.

This report contains the result of the inactivation and removal of TSE infectivity by the initial steps of the acid bone process to which a short treatment with sodium hydroxide was added. A description of the experiments done, and all other data associated with this experiment are also reported.

The results of the downscaled standard acid bone process were reported in *Validation of the clearance of TSE agent by the acid gelatine manufacturing process* of 20 August 2002. In that experiment, the initial process steps up to extraction demonstrated a clearance of  $10^{2.6}$  ID<sub>50</sub>. Various experiments reported in the scientific literature have shown that treatment of Transmissible Spongiform Encephalopathy (TSE) infected brain tissue with sodium hydroxide can considerably reduce infectivity levels. It was therefore decided to add an experimental short treatment with sodium hydroxide to the experimental acid bone process to further investigate the clearance of TSE infectivity by this process. Published data suggest treatments of 1-2M NaOH for 1-2 hours but these concentrations would be likely to influence the quality of the gelatine. However, further experiments reported in scientific literature have shown that treatment with concentrations of 0.1M and 0.3M sodium hydroxide can have the

same effect on TSE infectivity, and as these were less likely to have a deleterious effect on gelatine quality, a treatment of 0.3M NaOH for 2 hours (at pH 13 minimum) was used.

Despite using a less aggressive NaOH treatment, it was recognised that this step may demonstrate improved clearance of infectivity but be of limited practical application in industry because of the possible influence of gelatine quality and dependent on the quality of raw material.

### **Manufacturing process steps.**

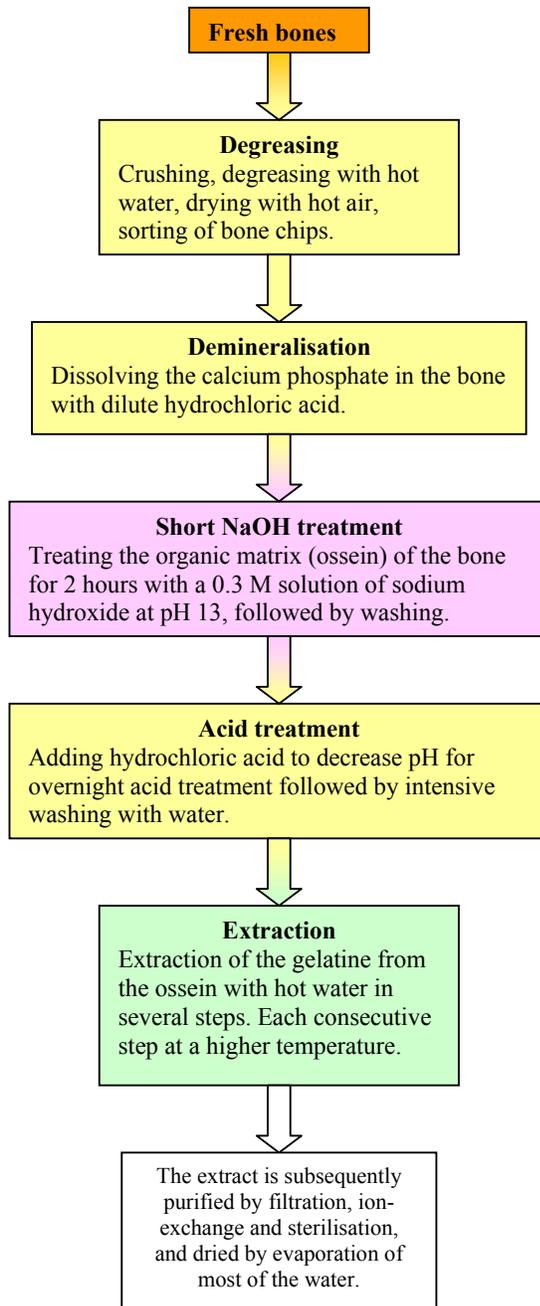
The complete acid bone gelatine manufacturing process is extensively described in the report *Validation of the clearance of TSE agent by the acid gelatine manufacturing process* of 20 August 2002. Here follows a brief description and diagram of the modified initial steps of this process:

- Degreasing, in which the bones are crushed, degreased with hot water and dried in a stream of hot air.
- Demineralising, the treatment of dried degreased bone chips with dilute hydrochloric acid to dissolve the calcium phosphate content. The resulting organic matrix, called ossein, is washed with water.
- Short NaOH treatment, in which the washed ossein is treated for 2 hours with 0.3M NaOH. Initially small volumes of 1M NaOH added to maintain a pH of at least 13 to counteract neutralisation by HCl present with the ossein after washing.
- Neutralisation, in which dilute hydrochloric acid is added to the washed NaOH treated ossein to decrease the pH prior to acid pre-treatment. Washed several times with water to remove excess acid.
- Stepwise extraction of the gelatine with hot water of increasing temperature.

In the complete industrial process the obtained extract would then be purified by filtration, ion-exchange and sterilisation. Solid gelatine is consequently obtained by evaporation of water.

The diagram of the modified process is on page 7.

**Diagram of the acid bone with additional short NaOH treatment:**



## **Development of the study.**

### *Demands on the study*

In order to make a valid comparison between the industrial and model process, the study had to meet specific criteria for process validation as follows:

- The study had to represent reality, both concerning the process and the starting material.
- To allow demonstration of accurate clearance values, the starting level of infectivity should be as high as possible without influencing the composition of the starting material.
- The detection method used had to be sufficiently sensitive to detect very low levels of infectivity.

These demands were met by:

- Using an accurately downscaled model of the industrial process, developed to maintain the same manufacturing conditions as those of the industrial process.
- Using industrial crushed bone as starting material. Backbone, which was added to facilitate spiking with infectious material, was treated such that it did not differ from industrial crushed bone.
- Adding infectious material to represent both direct infection and cross contamination of bone material.
- The use of 301V infected mouse brain, mouse passaged BSE which achieves high titres of infectivity, and is known to be relatively resistant to inactivation.
- Detection of 301V infectivity by mouse bioassay.

### *Downscaled model process*

The study was performed using an accurately downscaled model of the industrial process using the same conditions as in the industrial process. Here follow the main points of the downscaling.

### *Principles of the downscaling*

All acid bone gelatine manufacturers of the GME use the same manufacturing process steps. However, the process conditions of individual steps can vary slightly between manufacturers (Appendix 1). The mildest industrial conditions identified for each process step were applied in the downscaled model process, hence reflecting real conditions but not favouring inactivation. The process was downscaled such that all steps of the industrial process were performed in the downscaled model process, using small amounts of material in laboratory-scale equipment. The laboratory process was thus representative for the industrial process used by GME members. The downscaling followed these principles:

1. The essential conditions of every process step were established and were maintained in the downscaled model process.
2. When possible, non-essential conditions were also maintained in the downscaled model process.
3. Non-essential conditions, which could not be kept the same in the scaled down process, were adjusted such that these did not affect the process, nor influence inactivation.

Parameters for which no mutual minimal conditions could be established were tested individually. (This principle was not applicable here, there were no such conditions in this part of the process.)

The initial part of the acid bone manufacturing process was downscaled to laboratory size according to the above rules, with the addition of a short, novel NaOH treatment.

#### *Validation of the downscaled model process.*

The properties of all intermediate products and final gelatine produced by the industrial process were defined. The corresponding intermediates and final gelatine from the model process had to have these same properties. When developing the model process, conditions were recorded and the different intermediates and final gelatine were analysed. The results were compared with process conditions of the study protocol and the demands on intermediates and final product in part IV.3 of the study protocol. When each step was finalised, three validation tests were carried out for the entire process, the results of which will be reported separately.

## **Materials and methods.**

### *Agent strain.*

TSE infected brain material was used as the infectious load. Specifically, the 301V strain of mouse passaged BSE, which achieves high levels of infectivity in the brain of infected rodents and is known to be relatively resistant to inactivation. The 301V infected mouse brain material was prepared by IAH-E. To determine the amount of infectivity present, the brain material was titrated by intracerebral inoculation into mice.

### *Spiking (addition of infectious TSE brain) of the starting material*

The starting materials for the experiment consisted of 1575 g of fresh crushed bone sampled from the industrial process and 430 g of intact calf backbone. The bone material was spiked with approximately 10 g of infectious brain tissue. To imitate inclusion of BSE-infected CNS tissue, the spinal cord within the calf backbone was injected with 5 g of homogenised brain.

To imitate cross contamination from infected CNS tissue, the remaining 5g of brain was smeared over the crushed bones and stored chilled for two days prior to further processing. The backbone, with spinal cord intact, was then cut into pieces similar to the crushed bone. The backbone pieces with spinal cord were mixed with the crushed bone. The total amount of spiked bone was 2025 g, containing a total amount of 10.32 g 301V infected mouse brain.

*Experiments with the downscaled model process of the acid bone process with short NaOH treatment.*

### *Degreasing*

The mixture of crushed bone and backbone was added in portions of approximately 1000 g to 2 litres of degreasing solution held at 85 °C and stirred for 20 minutes at a temperature of approximately 80 °C. The degreasing solution was prepared by stirring 600 g of unspiked crushed bone from the same batch with 2200 ml of water of 80 °C. During the degreasing of the spiked test material 1 litre of degreasing solution was pumped out and 1 litre of water pumped in, both in 4 approximately equal portions, every 5 minutes. After 20 minutes all degreasing solution was removed and 2000 ml of clean water at 80 °C was added to the bone and stirred for 3 minutes. The degreased mixture of bone and soft tissue was then removed from the vessel. The temperatures during the degreasing were in all cases maintained between 78 and 84 °C; during the 3 minute rinse the temperature was between 81 and 83 °C.

After the degreasing step the loose soft tissue was manually removed from the degreased bone with forceps and the remaining bone and residues of connected soft tissue were dried in a stream of warm air. The temperature of the air was 119 °C which was equivalent to a temperature of 69°C measured at the surface of the bone. After drying the bone chips were agitated for 6 hours to loosen the connected soft tissue and then sieved on a 4 mm sieve, after which loose soft tissue and extremely porous material was manually removed.

### *Demineralisation*

The dried degreased bone chips were transferred to the demineralisation reactor through which an acid solution was pumped at a speed of 138 ml/hour (0.35 to 0.40 ml/hr.gram bone chips). For the first 24 hours a solution, containing 94.3 ml 36 % HCl and 65 g bone ash per litre, was pumped through, followed for 29 hours of a solution of 94.3 ml 36 % HCl and 28.5 g bone ash per litre, and finally 48 hours of a solution of 94.3 ml 36 % HCl per litre. These solutions contained 0.5 %, 2.5 % and 4 % free hydrochloric acid.

The acid solution was drained and replaced with 400 ml of water and held for one hour. This rinsing step was repeated one further time, before transferring the ossein to a beaker for a further 10 minute wash in 800 ml of water.

### *Short NaOH treatment.*

400 ml 0.3 M NaOH was added to the demineralised and washed ossein and stirred for 2 hours. The pH was measured continuously and maintained at 13.0. Initially the pH dropped to 12.5 and was adjusted to 13.0 by addition of some 1 M NaOH. The solution then stayed at pH 13 for the remainder of the treatment. After 2 hours the NaOH was drained and the ossein stirred twice for 15 minutes with 400 ml of water and once for 10 minutes with 800 ml water, after which 400 ml water was added to the ossein and 7.5 ml 2M hydrochloric acid were added to the ossein to decrease the pH. The pH measured 1.2 after addition of the 2M HCl increased to 8.4 overnight. Addition of 1 to 3 ml amounts of hydrochloric acid was continued until the pH remained 2.0 for several hours. The ossein was then washed 5 times with 400 ml water. The pH of the final washing was 2.7.

### *Extraction of the gelatine.*

The ossein was transferred to the extraction vessel and stirred with warm water. pH and temperature were measured continuously and gelatine concentration at intervals. The first extraction was done at 60 °C with 800 ml of water. When the gelatine concentration measured 6.5 %, the liquid was pumped out and replaced by 700 ml fresh water. The extraction temperature was increased to 70 °C. After the second extraction no ossein remained. Both extracts were collected and a sample taken for bioassay. The total volume of the extracts was 825 ml. The difference between the added water and volume of the extracts was caused by evaporation during extraction.

### *Determination of infectivity titres.*

To determine the infectivity titres of the spike material and the output samples, a series of tenfold dilutions were prepared of each sample and injected intracerebrally into groups of mice (20µl/mouse). The animals were scored according to standard protocols to detect clinical signs of neurological disease. The animals were culled when they developed unequivocal symptoms of neurological disease. Animals which did not develop clinical disease were culled 420 and 358 days (spike material) and 666 days (gelatine extract) post-injection. Animals injected with output samples were kept for more than 600 days because treatment of the agent potentially modifies the dose-response curve. The brains of all animals injected were removed and fixed in formol-saline. Sections were subsequently cut and stained with haematoxylin and eosin. These were examined microscopically for the spongiform lesions that are pathognomonic for 301V infection in mice. Using the ratios of positive and negative animals in each dilution group, the titre of infectivity in each sample was calculated by the statistical method of Kärber. (1931) (*Archives of Experimental Pathology and Pharmacology* **162**, 480-483) and by the Generalised Linear Model with C-loglog link and binomial distribution of the data. (Oberthür and others, *Die Risicoeinschätzung und –minimalisierung von BSE. In Prionen und Prionenkrankheiten.* Eds. B. Hörnlimann, D. Riesner and H. Kretzschmar. Berlin, De Gruyter. pp 456-469).

## Results

Table 1 contains the results of two bioassay titrations of the 301V spike material and of the gelatine extract, table 2 contains the infective titres calculated from these titrations.

**Table 1: Titration data.**

a. 301V mouse brain pool 1, 420 dpi

-log dilution	Number of mice	Mice positive for 301V	Average incubation period (days)
-	-	-	-
-	-	-	-
-	-	-	-
4	12	12	149
5	12	11	164
6	16	9	215
7	18	0	-
8	17	1	196
9	18	0	-

b. 301V mouse brain pool 1, 358 dpi

-log dilution	Number of mice	Mice positive for 301V	Average incubation period (days)
1	2	2	112
2	2	2	112
3	2	2	112
4	6	6	140
5	6	6	164
6	6	2	196
7	11	1	203
8	12	0	-
9	6	0	-

c. Sample of extracted gelatine, 666 dpi

-log dilution	Number of mice	Mice positive for 301V
0	16	0
1	17	0
2	15	0
3	16	0
4	18	0
5	18	0

**Table 2. Infective titres of 301V infected mouse brain and gelatine extract.**

Sample name	Titre/result (ID <sub>50</sub> /g)
301V strain infected mouse brain pool 1 – titration 1	10 <sup>7.7</sup>
301V strain infected mouse brain pool 1 – titration 2	10 <sup>7.6</sup>
Extracted gelatine	No detectable infectivity: ≤10 <sup>0.34</sup>

After 666 days post injection none of the mice inoculated intracerebrally with the obtained gelatine extract showed any clinical or pathological signs of 301V infection. This period is well beyond the maximally reported incubation period for 301V in VM mice. The infective

titre of the obtained extract is therefore expressed as less or equal to the detection level of the bioassay.

Infectivity clearance factors were calculated from the calculated titre values, the amount of 301V infected mouse brain used to spike the bone starting material and the obtained volume of gelatine extract the using the calculation below. The calculation was corrected for samples taken and for any losses during processing. This correction factor was 1.1. Because the detection limit of the bioassay was used as infective titre of the obtained extract, the calculated clearance factor must be expressed as a lower limit.

$$\text{clearance factor} = (\text{gram spike} \times 10^{\log \text{ titre spike}}) / (\text{ml gelatine} \times \text{corr.fact.} \times 10^{\log \text{ titre gelatine}}) \text{ ID}_{50}$$

The calculated clearance factor for the process was  $\geq 10^{5.4} \text{ ID}_{50}$ .

## Discussion

Gelatine was produced by the acid bone gelatine process with the inclusion of a novel NaOH washing step from materials with an infectivity titre of  $10^{8.4} \text{ ID}_{50}/\text{kg}$ . The gelatine extract failed to produce TSE disease when injected intracerebrally into mice. The animals were kept 1.5 times longer than the longest observed 301V incubation period and were scored both clinically and pathologically for disease markers. The clearance factor calculated from these data was  $\geq 10^{5.4} \text{ ID}_{50}$ . The clearance factor for acid bone gelatine extract produced without this additional short NaOH treatment reported in *Validation of the clearance of TSE agent by the acid gelatine manufacturing process* of 20 August 2002 was  $10^{2.6} \text{ ID}_{50}$ . Thus the additional clearance factor by the 2 hour treatment with 0.3M NaOH was  $\geq 10^{2.8} \text{ ID}_{50}$ .

The clearance of  $10^{5.4} \text{ ID}_{50}$  obtained in this experiment is greater than the  $10^{3.7} \text{ ID}_{50}$  observed in equivalent process steps in the alkaline manufacturing process (reported in *Validation of the clearance of TSE agent by the alkaline gelatine manufacturing process* of 20 August 2002). A short treatment with sodium hydroxide appears to have a higher capacity for inactivation than the long treatment with calcium hydroxide performed in the alkaline process. This difference may be explained by the studies of Brown and others, (*Journal of Infectious Diseases* **153**, 1145-1148.) which showed that alkaline inactivation with a sufficient hydroxyl ion ( $\text{OH}^-$ ) concentration takes place in the first 15 minutes of the process. With lower concentrations this can take up to several hours. Exposure of CJD infected Guinea pig brain and 263K infected hamster brain to an  $\text{OH}^-$  concentration of 0.01M for one hour resulted in only 1 log of inactivation, while 0.1M  $\text{OH}^-$  for 15 minutes gave a 4 to 5 log reduction. This suggests there is a threshold to inactivation dependent on hydroxyl ion concentration. The  $\text{OH}^-$  concentration of saturated  $\text{Ca}(\text{OH})_2$  is approximately 0.03M, and is between 0.1M and 0.3M for a 0.3M NaOH solution of  $\text{pH} \geq 13$ . The  $\text{OH}^-$  concentration of the 0.3M NaOH solution used in the process is therefore higher than this threshold but for calcium hydroxide is apparently approximately at or below this point.

## **Conclusions**

1. Inclusion of a short NaOH treatment after the demineralisation step in the manufacturing process of bone gelatine resulted in significant inactivation/removal of TSE infectivity to below the detection level of bioassay, currently the most sensitive method of detection available.
2. Inclusion of this treatment step considerably enhances the TSE clearance capacity of the acid bone manufacturing process..
3. The gelatine manufacturing process was successfully scaled down; gelatine was prepared from industrial starting material.
4. The study complies with the requirements of a validation study.