Validation of the clearance of TSE agent by the heat and pressure process for manufacturing of gelatine and colloidal protein

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AUTHENTICATION

The undersigned Quality Assurance Officer of ID-Lelystad BV hereby declares that the work was performed under principles of good laboratory practice and that - to the best of his knowledge - this report reflects a true and faithful account of the results as they are collected and written down in the source data and records of the study.

Place: Beekbergen

Date : 15 – 12 – 02

Signature: .....

Name: Drs. ir. W.J.A.M. Meulepas

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SUMMARY REPORT.

The industrial manufacturing process for the production of gelatine and colloidal protein by the heat and pressure process was downscaled to an accurate laboratory scale model. Using this downscaled model process, gelatine was made from bones experimentally contaminated with mouse brain infected with the 301V strain of mouse-passaged Bovine Spongiform Encephalopathy (BSE) agent. Samples of input, intermediate, and output material were taken throughout the experimental process. To determine the capacity of the process to remove/inactivate 301V infectivity, a sample of the infectious brain and crude protein extract were assayed for the amount of infectivity present in each of these fractions. The infectivity present in these samples was determined by intracerebral inoculation in experimental mice. The measured infectivity of the infectious brain was $10^{8.7}$ ID_{50}/g, while no infectivity was detected in the extracted protein (≤ $10^{0.2}$ ID_{50}/g). The calculated clearance factor was: >=$10^{6.8}$ ID_{50}.

Introduction.

The Gelatin Manufacturers of Europe (GME) commissioned a validation study on the inactivation and removal effect of the gelatine manufacturing processes on Transmissible Spongiform Encephalopathies (TSE). The background, aims, approach and the 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 results of the inactivation and removal of TSE infectivity by the heat and pressure process for manufacture of gelatine and colloidal protein (usually called Heat and Pressure process or H+P process), a description of the experimental procedures, and all other data associated with this study.

Industrial manufacturing process.

The Heat and Pressure process is extensively described in Appendix 1 to this report. Here follows a brief description and a diagram.

The Heat and Pressure process consists of the following steps:

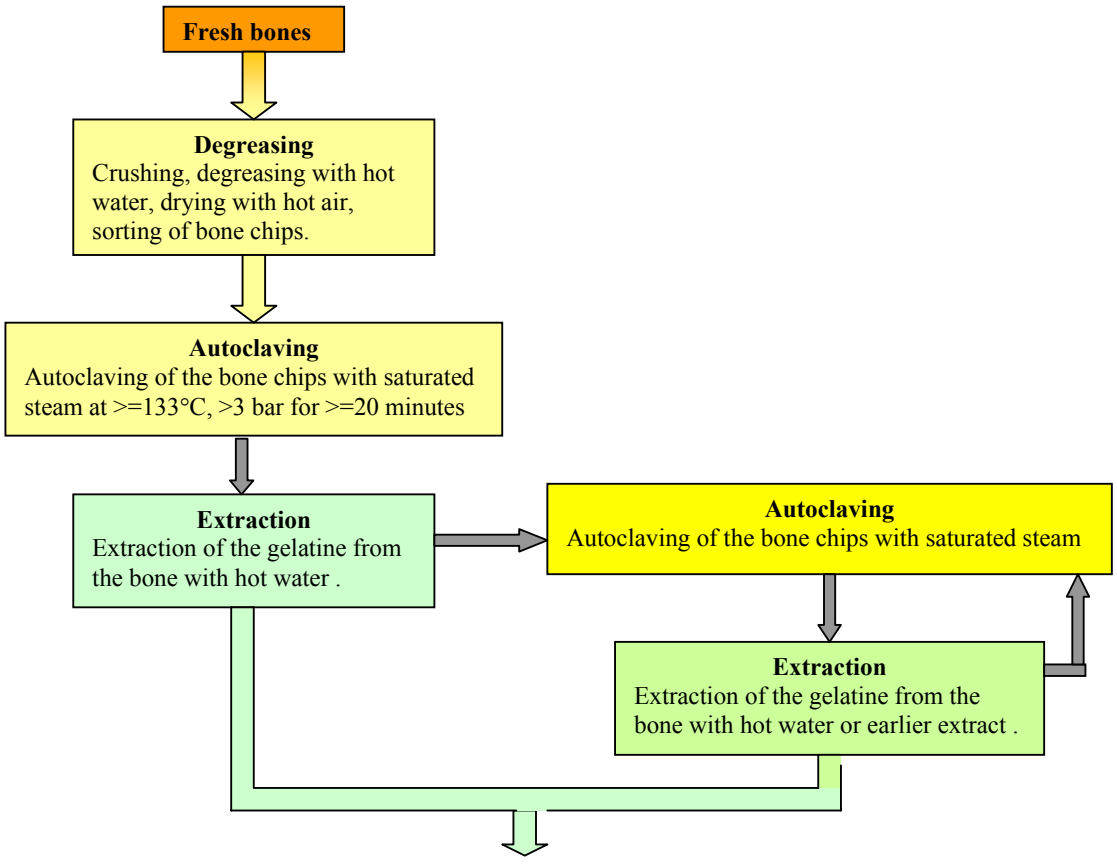
- Degreasing, in which the bones are crushed, degreased with hot water and dried in a stream of hot air.
- Autoclaving of the dried degreased crushed bone (bone chips) with saturated steam at a pressure greater than 3 bar and a minimum temperature of 133°C, for at least 20 minutes.
- Extraction of the protein with hot water.

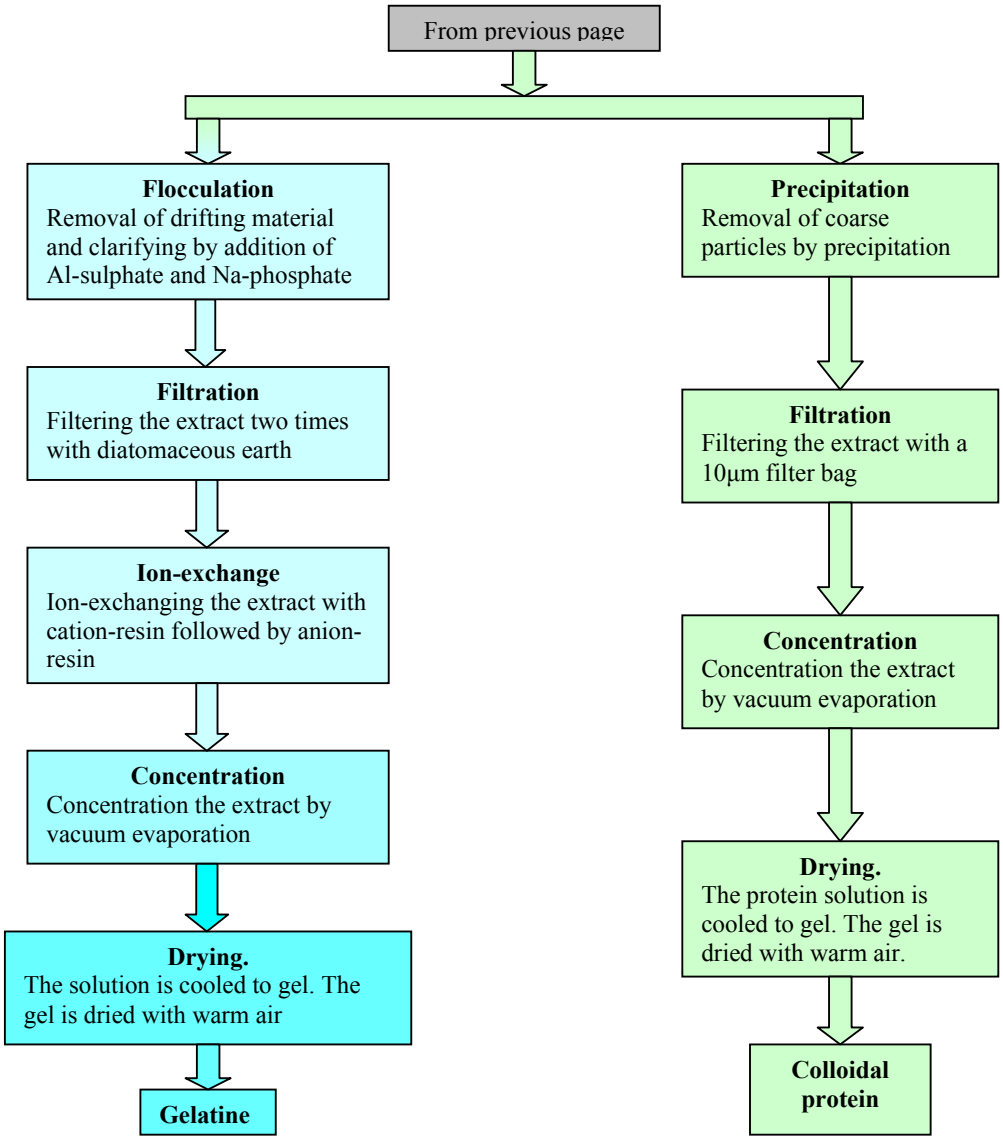
- Processing of the obtained protein extract.

After the first extraction the bone chips are autoclaved and extracted a further seven times, each consecutive step at a lower temperature for a shorter time. The final extractions are performed using earlier extracts to improve the concentration.

Depending on whether colloidal protein or gelatine is being manufactured, further processing will differ as follows:

- Colloidal protein:
  - Precipitation of coarse particles in a precipitation tank followed by filtration through a filter bag.
  - Concentration by vacuum-evaporation of most of the water content, after which the concentrated solution is cooled to gel. This gel is finely divided and dried in a stream of warm air.
- Gelatine:
  - Purification of the obtained extract, which is a dilute gelatine solution, consecutively by flocculation to remove drifting particles and to clarify the solution, by filtration to remove coarse particles and by ion-exchange to remove salts.
  - The purified gelatine solution is then concentrated by vacuum-evaporation of most of the water content, after which the concentrated solution is cooled to gel. This gelatine gel is finely divided and dried in a stream of warm air.





Limitations of the study.

Although the complete processes for manufacturing gelatine and colloidal protein are described above, this study was limited to degreasing, autoclaving and extraction, because no large contribution to inactivation was expected from the filter bag filtration, while diatomaceous earth filtration and ion-exchange were investigated in a separate experiment.

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. The downscaling procedure and the downscaled process are extensively described in Appendix 2 and Appendix 3. Here follow the main points of the downscaling.

Principles of the downscaling.

The process conditions of the individual process steps can vary slightly. 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 thus was representative for the industrial process used.

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 influenced inactivation.

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 the 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 strains.

TSE infected brain material was used as the infectious load. Specifically, the mouse-passaged BSE strain, 301V was used as this strain achieves high levels of infectivity in the brain of infected rodents, has a relatively short incubation time compared with other rodent-passaged TSE models, and is highly heat resistant. The 301V infected mouse brain material was obtained from 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.

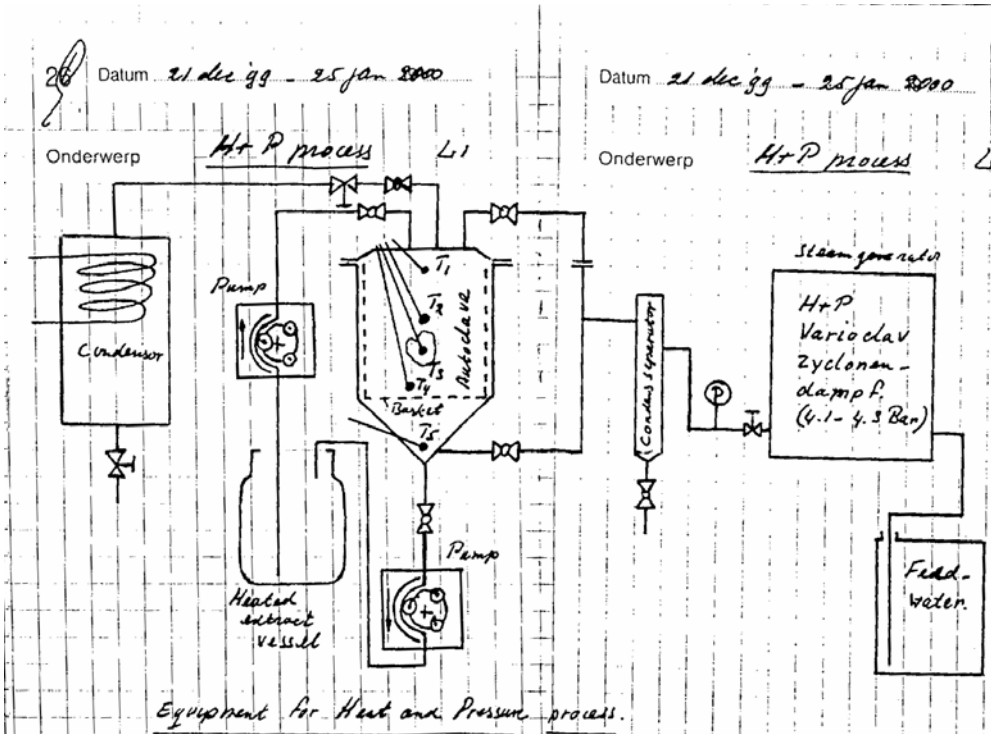
The starting materials for the experiment consisted of 0.75 kg of fresh crushed bone sampled from the industrial process and 0,25 kg of intact calf backbone. The bone material was spiked with approximately 4 g of infectious brain tissue. To imitate inclusion of BSE-infected CNS tissue, the spinal cord within the calf backbone was injected with 2 g of homogenised brain. To imitate cross contamination from infected CNS tissue, the remaining 2 g 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.

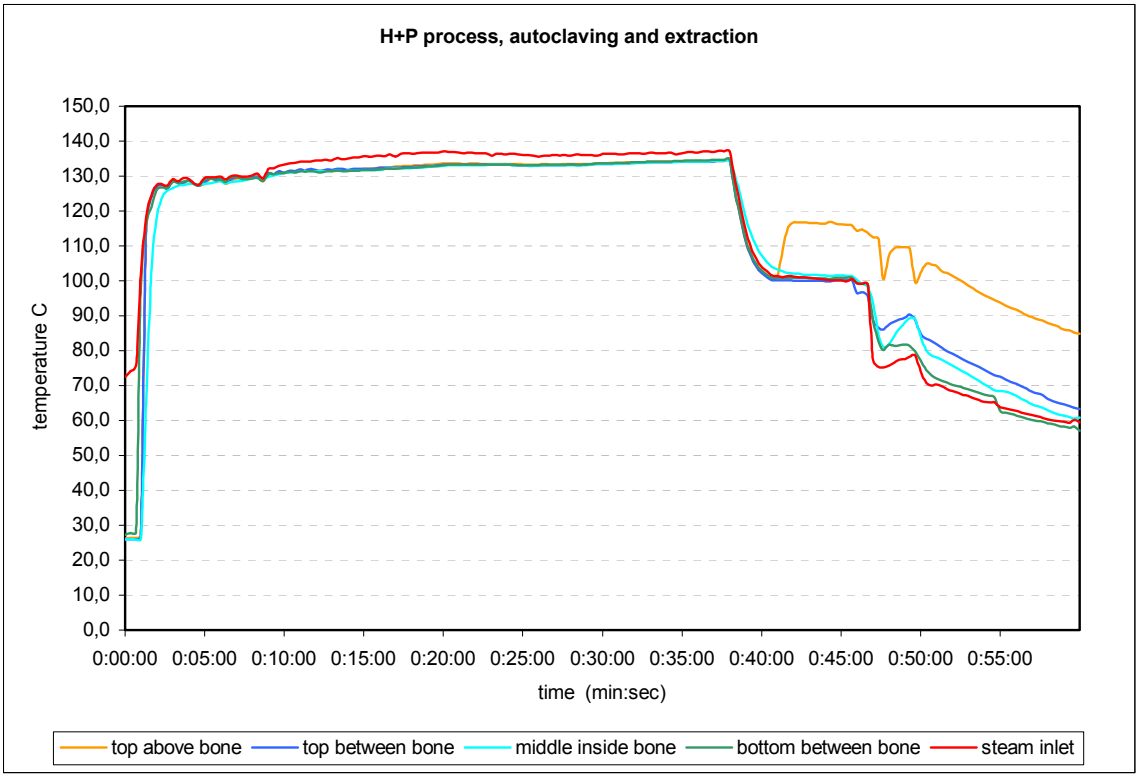
Experiments with the downscaled model processes of the Heat and Pressure process.

A complete and extensive description of the experiments is in Appendix 5 to this report.

Downscaled Heat and Pressure process with 301V spike.

The raw materials consisted of 1004 g of fresh crushed bone and backbone, which contained 3.92 g 301V infected mouse brain. The process was executed from degreasing up to extraction. The execution of the different experimental process steps is extensively described in appendix 5. The degreased bone chips obtained from the scaled down degreasing process were autoclaved. The autoclave was heated to autoclaving temperature in approximately 17 minutes after which the bone chips were autoclaved for 20 minutes at 132 to 134°C. After cooling to below 100°C, the autoclaved bone chips were extracted with water of 80°C for approximately 12 minutes. A total of 175 ml of crude gelatine was extracted. Samples of the crude gelatine extract were titrated by mouse bioassay to determine infectivity titres. Below is a diagram of the autoclaving/extraction equipment and a graph of the temperatures of the autoclaving and extraction process.


Datum 21 dec '99 - 25 jan 2000
Onderwerp
Ht P process
Condenser
Pump
Autoclave
Basket
Heated extract vessel
Pump
Equipment for Heat and Pressure process.
Datum 21 dec '99 - 25 jan 2000
Onderwerp
Ht P process
Steam generator
Ht P Varioclav Zyclonen - dampf. (4.1 - 4.3 Bar)
Feed-water.


H+P process, autoclaving and extraction
temperature C
150,0
140,0
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110,0
100,0
90,0
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10,0
0,0
0:00:00 0:05:00 0:10:00 0:15:00 0:20:00 0:25:00 0:30:00 0:35:00 0:40:00 0:45:00 0:50:00 0:55:00
time (min:sec)
top above bone
top between bone
middle inside bone
bottom between bone
steam inlet

Determination of the infectivity titres of the samples.

To determine the infectivity titres in the spike material and the output sample, 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 approximately 600 days post injection. The brains of all animals 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 the samples was calculated by the statistical method of Kärber. (1931) (*Archives of Experimental Pathology and Pharmacology* **162**, 480-483)

Results

Table 1 contains the infectivity titres measured for the 301V spike material and of the output gelatine produced

Table 1. Infective titres of 301V infected mouse brain and gelatine made by downscaled process.

Sample name	Study and sample number from protocol	Titre/result (ID_{50})	Observation time
Mouse brain titration		$10^{8.7}$	607 days
Heat and pressure process; extracted gelatine	Study 8 - Sample 13	No detectable infectivity	623 days

Infectivity clearance factors were calculated from the measured titre values using the calculation below. The calculations were corrected for samples taken and for any losses during processing.

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

The data for calculation of the clearance factors are in table 2. The calculated clearance factors are in table 3. No infectivity was detected from the extracted protein sample, therefore a theoretical maximum titre value was calculated using the Generalised Linear Model with C-loglog link and binomial distribution of the data. (Oberthür *et al*. *Die Risicoeinschätzung und –minimalisierung von BSE*. Prionen und Prionenkrankheiten. Edt. B. Hörnlimann et al. Walter de Gruyter. Berlin 2001), from which the minimum clearance value was calculated.

Table 2. Process data for calculation of clearance factor.

Sample name	Study and sample numbers from protocol	Amount of spike (g)	Titre of spike ID_{50}/g	Amount of gelatine (g)	Corr. factor	Titre of gelatine ID_{50}/g
H+P process - extracted gel.	Study 1 - sample 2	3.91	$10^{8.7}$	175 (+ <=121 absorbed by bone)	1.1	$<=10^{0.2}$

Table 3. Clearance factor.

Sample name	Total clearance factor ID_{50}
Heat and Pressure process - extracted gelatine	$>=10^{6.8}$

For the absorption of water by degreased bone different values have been found ranging from 25% to 58.5% of the weight of the dry bone. The latter value was found in a comparable experiment with autoclaved bone chips and is probably somewhat higher than the actual amount of extract absorbed by the bone chips in this experiment. However, being the worst case, this value was used here.

Discussion

The titration values recorded show a significant reduction of 301V infectivity of at least $10^{6.8}$ by the downscaled heat and pressure process following degreasing, autoclaving and extraction. Therefore, the majority of the inactivation/removal of infectivity occurs during this first part of the manufacturing process before the consecutive purification steps. The sample of the extract obtained failed to produce disease in any of the mice inoculated.

The results reported here provide a basis for a risk assessment of the industrial heat and pressure process for the manufacturing of colloidal protein and gelatine. In order to make a valid comparison, the model process must meet specific criteria for process validations as follows:

- The study has to represent reality, both concerning the process and the starting material.
- The level of infectivity must be as high as possible without influencing the composition of the starting material, and should be much higher than in reality.
- The detection method used must be sufficiently sensitive to detect very low levels of infectivity.

The downscaled model process used here was developed to maintain the same manufacturing conditions as those of the industrial process. The bone starting material was mainly industrial crushed bone, while the added backbone was treated such that it did not differ from industrial crushed bone.
A heavy load of infectious material was used, but introduced in a way that resembled incorporation of both directly infected and cross-contaminated raw material into the manufacturing process. Of the total raw material weight, 0.4% consisted of infectious mouse brain and approximately 0.5% calf spinal cord. This amount of cerebrospinal tissue is approximately 5 to 10 times greater than with the raw bones used for current European gelatine manufacture. However, before measures were taken to exclude CNS tissue from starting material, this figure could have been as high as 1.7%. Hence, 0.9% CNS tissue used in the experiment is not outwith the range encountered historically in the industrial process.
Nevertheless, based on current data regarding BSE infectivity in cows, the level of infectivity applied in this experiment is higher than should be encountered in current industrial practice, but is used to facilitate the measurement of definite clearance values.

301V infectivity titres were measured by experimental rodent bioassay, carried out by inoculating the samples by the intracerebral route. The transmission of disease in these models is most efficient by this route compared with peripheral challenge. The infectious material used, 301V, is a rodent adapted strain, which avoids the loss in sensitivity of detection of infective titre, observed when crossing between species (the species barrier). This is the most sensitive assay of infectivity that is currently available.

Conclusions

1. The downscaled model of the Heat and Pressure process for the manufacturing of colloidal protein and gelatine removed/inactivated 301V infectivity to below the level of detection.
2. A 301V infectivity reduction of at least $10^{6.8}$ ID_{50} was obtained by the downscaled model of the Heat and Pressure manufacturing process after extraction of the protein.
3. The Heat and Pressure process was successfully scaled down; colloidal protein/gelatine was prepared from industrial starting material.
4. The study complied with the requirements on a validation study.

In conclusion, these data provide actual measurement of clearance factors for the heat and pressure process that can be used to facilitate risk assessment of the safety of bovine bone gelatine with regard to BSE and human safety

Appendix 1. INDUSTRIAL MANUFACTURING PROCESS

Short description of the Heat and Pressure process

Fresh bones from healthy slaughtered animals, which have been officially declared fit for human consumption, are collected at slaughterhouses, meat processing plants and other places and are transported in special trucks to the degreasing plant. EU regulations or gelatine industry standards exclude the use of some bones and other tissues. In the degreasing plant the bones are crushed, degreased with hot water and dried with hot air. The degreased bone chips are autoclaved for at least 20 minutes with saturated steam at 133°C or more, and a pressure of over 3 bar to denature the protein of the bone. From the autoclaved bone, gelatine is extracted with hot water. The extract, which is a dilute gelatine solution, is purified either by precipitation and filtration with a 10µm filter bag to colloidal protein, or by flocculation, filtration and ion-exchange to gelatine. The purified solution is then concentrated by vacuum evaporation of most of the water content. The concentrated solution is cooled down to form a gel. The gel is finely divided and dried in a steam of warm air. The dried colloidal protein or gelatine is packed and stored until further use.

Description of the individual steps of the Heat and Pressure process for manufacturing of colloidal protein and gelatine

(The process will be described from degreasing up to extraction)

Degreasing

The fresh bones received at the degreasing plant still contain a large amount of meat and other soft tissue that together with the fat has to be removed. A typical example of the composition of a batch of fresh bones is:

Water content	46 %
Fat	15 %
Protein	19 %
Minerals	20 %

Degreasing is a continuous process that consists in general of the following steps:

a. Crushing of the bones.
b. Treatment of the bones with hot water. This process is done in a tank filled with hot water and bone. A stream of fresh crushed bones and a stream of clean hot water enter this tank at one end and a stream of treated bone chips, fat emulsion and small particles leaves the tank at the other end. The bone is transported over the bottom of the tank by a screw or by propellers which at the same time mix the bone and water.

The turbulent action of the hot water and the sliding and rubbing of the crushed bone, causes part of the meat and other soft tissue to loosen from the bone.

c. Fat emulsion and floating particles are decanted. The bone chips are immersed in a stream of hot water and the bone and soft tissue are separated by centrifuging. The bone chips are then removed from the water.
d. The wet bone chips are dried with hot air in a rotating drier. The rotation moves the chips through the drier and continuously mixes them causing remaining meat and other soft tissue to loosen from the bone.
e. Any smaller particles are separated from the dried degreased bone chips by sieving and the light particles are separated by gravity.

The conditions of the process are different for different installations in use and vary as follows:

A. The temperature of the hot water varies from 75 ^{o}C to 90 ^{o}C.
B. Degreasing time (step b) varies from 15 to 30 minutes, depending on the installation.
C. The input flow ratio of bone/water varies from 1/8 to 1/1.
D. The ratio of bone/water in the degreasing tank varies from 1/3 to 1/1.

The continuous mixing by the rotating drier results in each bone chip being exposed to the hot air for only a few seconds. This fact coupled with the cooling effect of the evaporation of the water ensures the temperature of the chips will not normally exceed 85°C, although the air entering the drier can be heated to over 400°C. The drying time varies from 20 to 60 minutes and the size of the equipment and the amount of bones that are processed per hour varies between the different installations.
Modern installations are made of stainless steel, whereas older installations are made of normal steel.
The amount of dried degreased bone chips obtained from 1 kg of crushed fresh bone is usually about 200g (20 %). The typical composition of these bone chips is:

Water content	approximately	8 %
Composition of the dry matter:		
Fat	less than	3 %
Ash	approximately	63 %

Autoclaving and extraction

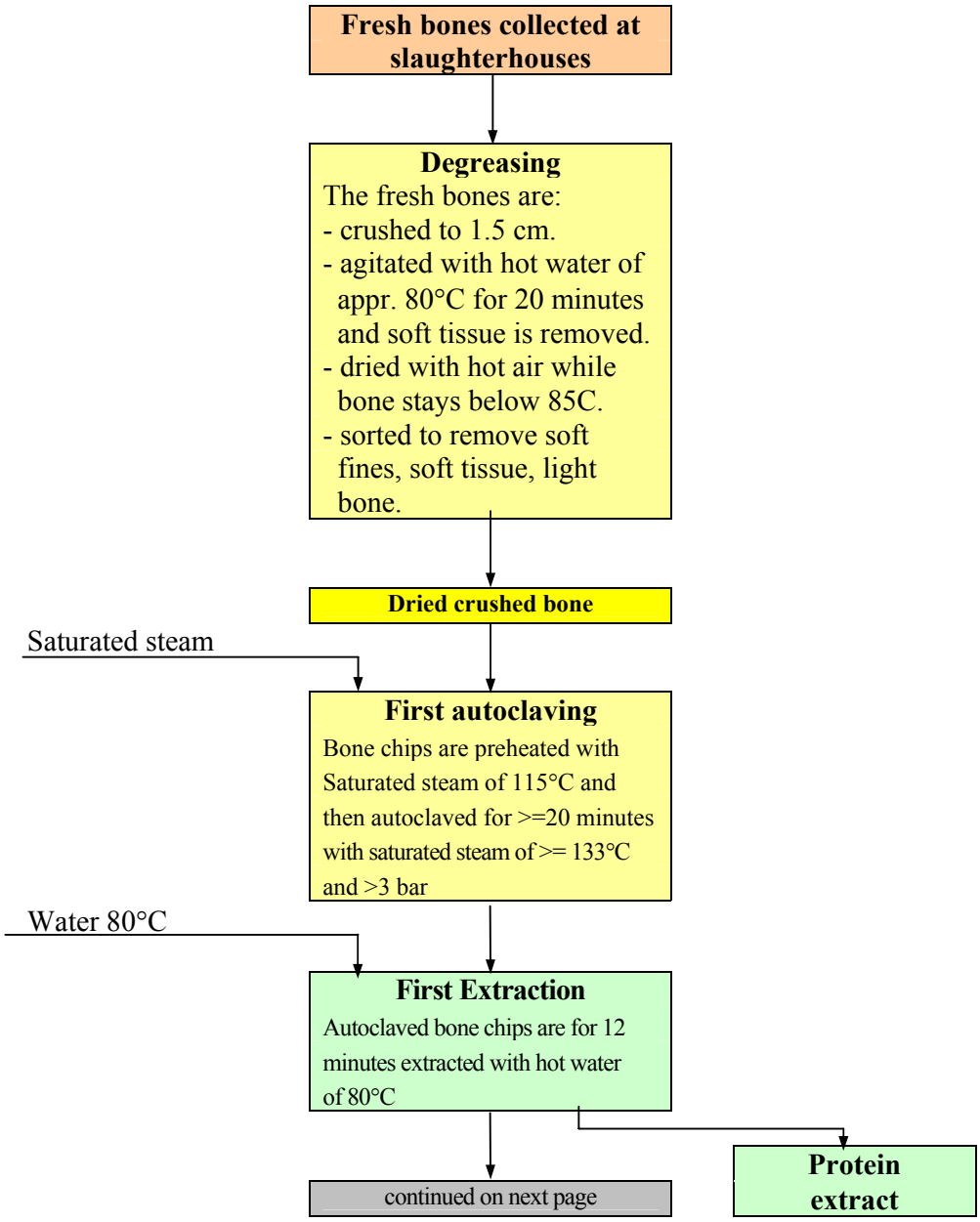
Bone chips are autoclaved and protein extracted in a series of eight similar cycles. There are some differences between consecutive cycles which are described below.
An autoclave with a volume of approximately 6,850 litres is filled with 2,300 kg of degreased bone chips. To start the process, the autoclave is pre-heated for 10 minutes by passing steam of 1.7 bar and 115 ^{o}C from the base of the chamber. After pre-heating, the exhaust is closed and the autoclave pressurised and heated with steam of more than 3 bar and 133 ^{o}C to 135 ^{o}C

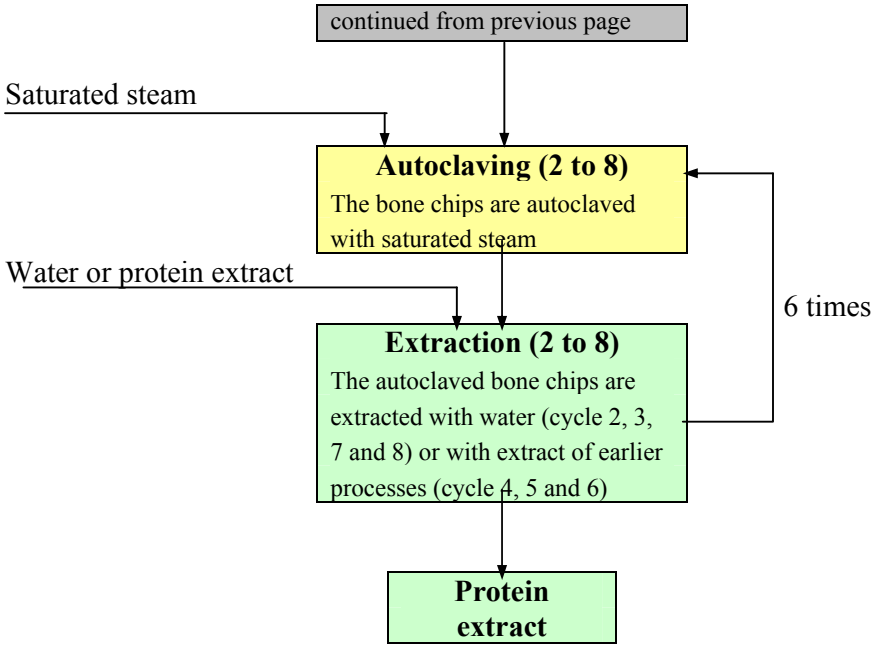
for a minimum time of 20 minutes. The chamber is then depressurised in 4 to 5 minutes before 1500 litres of water is sprayed over the bone chips to extract the gelatine. The resultant solution is pumped out during extraction and is continued for 12 minutes, after spraying has stopped. The second cycle then begins immediately as no preheating is required and is carried out in the same way as the first, except that from the second cycle on autoclaving times and temperatures are less than 20 minutes and below 133°C to prevent damage to the structure of the bone.
After depressurising in the third cycle the autoclave is filled with 1,500 litres of water of 10°C which is left to stand in the autoclave for 20 minutes. The extract from this and subsequent cycles is not pumped out, but extruded by steam pressure. In cycles 4 to 6 extraction is not carried out with water, but with extracts obtained from the fifth to eighth cycles of earlier processes. Hence part of the extracts from the fifth and sixth cycle are stored to be used in future extractions.
Extraction in the seventh and eighth cycle is done as in the third cycle and again the extracts are stored for future use.
After the eighth autoclaving and extraction the chips are left to drain for 20 minutes and the liquid extruded by steam.

Further processing.

After purification, concentration and drying the manufactured solid colloidal protein or gelatine is packed and stored under appropriate conditions of temperature and humidity. The batch is given a unique number which can be traced back to the original bones.
The equipment that comes into contact with the gelatine is made of stainless steel and at some points of synthetic material.

Flow sheet of the Heat and Pressure manufacturing process.




continued from previous page
Saturated steam
Autoclaving (2 to 8)
The bone chips are autoclaved with saturated steam
Water or protein extract
6 times
Extraction (2 to 8)
The autoclaved bone chips are extracted with water (cycle 2, 3, 7 and 8) or with extract of earlier processes (cycle 4, 5 and 6)
Protein extract

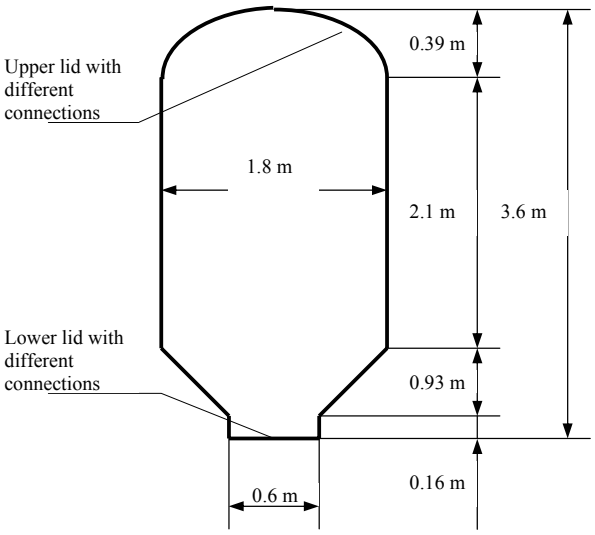
Summary of the conditions of the Heat and Pressure process.

Degreasing: Crushing of fresh bones to about 1.5 cm size.
Agitating the crushed bones with water of 80°C.
Fresh water continuously added and emulsion continuously removed.
Soft tissue and light material separated
Crushed degreased bone dried with hot air, bone stays below 85°C
Bone sorted. Fines, soft tissue and light material removed.

H+P:: Batch size: 2.3 tons bone chips.
Dimensions of reactor: height 3.6 m, diameter 1.8 m, volume 6850 litres (see draft below)

1e step	Duration of process: Preheating at 115°C	10 minutes
	Heating to autoclaving temperature	5 to 8 minutes
	Autoclaving at >=133°C	>=20 minutes
	Cooling to below 100°C	<=5 minutes
	Extraction	10 minutes
	Pumping	12 minutes

Temperature of extraction water 80°C
Volume of extraction water 1500 litres



Appendix 2. DOWNSCALING PROCEDURE.

Industrial gelatine is manufactured from a batch of between 11 tons and 12 tons of fresh bones. The scaled down process used approximately 1 kg of fresh crushed bone and backbone. This amount was sufficient to be representative of the large quantities of starting material and intermediate products of the industrial process, yet small enough to allow safe laboratory practice, much of which had to be carried out in a biological safety cabinet. Further, the design had to ensure the prevention of cross-contamination within and between process steps. The downscaling factor therefore was between 1:11,000 and 1:12,000.

Although the process is the same for each batch produced, the precise process conditions can differ somewhat between different batches.
This variation in conditions presented the problem to the design of the downscaled process of which process conditions to use. Therefore the minimum conditions for each step were identified and applied in the model process. This ensured a process, representative of the industrial process, compliant with minimum process conditions which inherently do not favour inactivation.

A second challenge, inherent in any up scaling and down scaling, was that it is not possible to keep all process conditions the same when the size of the equipment changes.

The classical example of this problem is the flow of a liquid through a tube. When this is downscaled with a certain factor one can observe several changes. When volume per unit of time and tube diameter are downscaled by the same factor, then the flow speed is this same factor higher, while the Reynolds number, which indicates the turbulence, remains the same. When one however wants to keep the flow speed the same, the scale factor for the diameter must be the square root of the scale factor for the volume, but now the Reynolds number will be different by this same square root of this factor and the turbulence is different.

However, not all process conditions have an equal influence on the gelatine process. Some conditions are essential for the process, others have no or very limited influence. It was therefore decided to apply the following rules for downscaling:

- Essential process conditions are precisely maintained.
- Other process conditions are maintained when possible, but when these cannot be maintained a deviation is allowed.
- In the case of a deviation from the minimum industrial process conditions, this will be devised such that it is ideally neutral, or does not favour inactivation.

The downscaled model process developed according to these rules was a precise imitation of an industrial process with a minimum capacity for TSE inactivation.

List of essential process conditions

Process step	Minimum process conditions of industrial process	Process conditions of downscaled process
Degreasing	20 minutes with emulsion of 80°C while part of it is replaced by fresh water, and some minutes with clean water. Mechanical separating bone and soft tissue. Drying with hot air, such that bone remains below 85°C, for 20 to 45 minutes. Sieving and mechanical sorting to remove unwanted material.	20 minutes with emulsion of 80-85°C, bone:emulsion=1:2, replacing 50% of emulsion with fresh water 3 minutes with clean water. Separating bone and soft tissue by hand. Drying with hot air such that bone remains below 85°C for 45 minutes. Sieving and sorting by hand.
Autoclaving	2.3 tons of bone chips Pre-heating to and at 115°C for 10 minutes. Heating to autoclaving temperature in 6 to 8 minutes. Air removed during pre-heating. Autoclaving at >=133°C for >=20 minutes. Pressure >3 bar. Cooling to below 100°C in less than 5 minutes.	200 to 250 g of bone chips Heating to >132°C in less than 20 minutes. Air removed by venting during heating up Autoclaving at > 132 for 20 to 22 minutes. Pressure of steam-generator between 4 and 4.5 bar. Cooling to below 100°C in less than 5 minutes.
Extraction	Spraying 1,500 litres water of 80°C on the autoclaved bone chips in 10 minutes, pumping out during spraying. Pumping for 12 minutes after spraying was completed.	Circulating 200 ml of water over the autoclaved bone chips for 10 minutes. Pumping out until none comes out for at least 1 minute.

Appendix 3. DOWNSCALED MODEL PROCESS.

Heat and Pressure process.

Degreasing

a. Amount of bones.

The starting material for the scaled down process consisted of 1,000 g of bone. From these 1,000 g approximately 200 to 250 g of dried bone chips will be obtained, depending on the quality of the crushed bone. This compared with a typical batch size of 2,300 kg used in the autoclaving/extraction on manufacturing scale

b. Crushing of the bones

The 1,000 g of bone consisted of approximately 750 g of industrial crushed bone and 250 g of intact spine. Spine material was added to model the inclusion of raw material from cows infected with BSE. (See appendix 4). The spine material was then sawn into pieces of 10 to 15 mm before mixing with the crushed bone pieces. Sawing of the spine was preferred to crushing in order to prevent shattering and spread of infected bone. This was advantageous in avoiding both contamination of surrounding equipment, and the exclusion of total infectious material from the process, and for the safety of the experimenters. The pieces obtained from the spine closely resembled the crushed bone pieces, and these two fractions were carefully mixed.

c. Degreasing the crushed bone.

The industrial degreasing process is a complicated continuous process. Making a scaled down version of this continuous process would have been extremely difficult and resulted in a very complicated and large installation, unsuitable for use in a research laboratory. Therefore, the scaled down degreasing was carried out as a batch process such that essential conditions representative of those in industrial scale manufacture were maintained but performed differently to give similar results.

The main steps during degreasing are:
a. Degreasing the bone chips with hot water.
b. Separating the bone chips from the emulsion of fat and drifting particles.
c. Separating the bone chips from the lighter soft material.
d. Pre-drying and drying of the bone chips.
e. Separating the small and light particles from the bone chips.

These steps had to be part of the scaled down process.

On scaling down the following had to be taken into account:

a. Degreasing is a continuous process carried out in fat/water emulsions to which a stream of clean hot water and fresh bone is added, while an equal amount of emulsion flows from the tank.
b. Bones are agitated to improve process efficiency.
c. Most of the water/fat emulsion is separated from the bone after agitation. Some of the emulsion remains with the bone.
d. Due to the continuous nature of the process, not all bone material will be treated for the same amount of time.
e. During degreasing and drying, soft tissue is removed from the bone due to the friction caused by contact between bone chips.
f. Soft tissue and small particles of bone are separated from the bone chips at two points in the process. The first of these involves immersion of the bone chips in clean hot water, where light density material is removed by gravity. Remaining soft tissue and light particles are removed by sieving after drying of the bone.
g. The temperature of the bone chips should not rise above 85°C during drying, done in a stream of hot air.

The model process was designed with respect to these observations. For example, it would be inappropriate to start the batch process with fresh bone in clean water as this state does not occur in reality and, although the end product would be similar, the process would be markedly different. Instead, batchwise degreasing was done in fat/water emulsions from an earlier fresh bone/clean water degreasing. In the continuous process, the fat/water emulsion will be in constant change which is not possible to imitate in a batch process. Therefore, as a compromise, the starting state was a degreasing emulsion that contained 60 % of the maximal amount of fat. This solution was replaced ¾ of the way through the process time with an amount of fresh water equal to the weight of the crushed bone being degreased. In the final quarter of the process no water was added and no emulsion taken out. In this way the process solutions will contain a higher amount of fat than the continuous process at one stage, and a lower amount at another, but the average process and end products are similar.
The possible difference in processing time of the individual chips was taken into account but this could only have been remedied by continual addition of the bone into the hot emulsion. This was not done due to the possible risk to the experimenters of splashing and aerosols. Instead all crushed bone was immersed in the emulsion liquid in a basket at the start of the process. The industrial process and the small scale model process differ here, in that the temperature of the industrial process is constant because of the relatively small volume of crushed bone added, compared with the total process volume. This is not the case in the small scale process, and the temperature of the emulsion will go down about 10 ^{o}C when the chips are first immersed in the emulsion. This will result in a slightly less efficient degreasing. At the end of the process, decanting of the bone from the emulsion would have been closest to reality but, again, this presented too great a risk of spilling and aerosols, so the emulsion was pumped out. To prevent, as far as possible, the freshly degreased bone chips becoming covered in the fat floating at the top of the emulsion, the pumping hose was kept at or near the emulsion surface to remove the floating matter.

To imitate the first separation of light particles and soft tissue (and some further fat), the emulsion was drained off and the bone was stirred with clean, hot water. At this point in the industrial process, the degreased bone is separated from the remainder by centrifugation, but in the model process was removed manually using forceps. Although very different from reality this has the same result and does not interfere with the different process steps.
In the industrial process the chips are dried in a rotating drier in a stream of air of over 400 ^{o}C. The chips do not overheat due to the large amount of chips from which water continuously evaporates, and the rotation which means individual chips are only briefly in direct contact with the hot air. Making a model of such a drier would be too difficult and therefore the chips were spread in a thin layer, held stationary and dried with air at a lower temperature.
The model dryer was made with converted hairdryer machinery, used for 20 minutes at full power with air of 105 to 115 ^{o}C, then 40 minutes at 85 ^{o}C minimum. Testing had shown that this regime resulted in bones with the same water content as industrial drying. During the first phase most water is evaporated and the chips stay below 85 ^{o}C due to the cooling effect of the evaporating water. During the second phase, the temperature is set at approximately 85°C but over a longer period so the remaining water evaporates. The mechanical abrasion of the different steps was imitated by mechanically tumbling the dried chips for 6 to 8 hours.

For safety reasons, it was required that the degreasing was carried out entirely in a biological safety cabinet which dictated the size of the equipment and amount of material used. The equipment used in industry is entirely made of (stainless) steel, while the equipment designed for use in the safety cabinet was made partly of glass and partly of stainless steel.

The degreasing step for the small scale process was carried out as follows:

2 litres of emulsion obtained from degreasing 600 g of uninfected bone chips were put in a beaker and heated to 85^{o}C.
1000 g of crushed bone chips were immersed in the emulsion and stirred for 20 minutes. During the first 15 minutes 1000 ml of the emulsion was pumped out at a continuous rate and, in portions, 1000ml of water was pumped in. After 20 minutes stirring was stopped. After waiting a few minutes, to allow fat to separate from the water, the supernatant liquid was pumped out such that the topmost part was removed. 2000 ml of water of about 85 °C was added to the chips and stirred for 3 minutes. The liquid was pumped out, the chips allowed to drain for about 5 minutes then the loose soft parts picked out with forceps. Chips were dried for 20 minutes with air of 105 ^{o}C then 40 minutes at 85 ^{o}C. The dried chips were tumbled overnight then sieved on a 4 mm sieve. Remaining soft parts were manually separated from the bone using forceps.

Autoclaving

The most important parameters of the industrial process are:
- The temperature profile of the process
- The use of saturated steam, thus correct balance between the steam pressure and the steam temperature, and condensed water and steam being in equilibrium.
- The absence of air in the autoclave chamber.

The downscaled model process was designed such that its temperature profile was as close as possible to that of the industrial process using the minimal autoclaving temperature. By using saturated steam the balance between pressure and temperature was maintained and water and steam were in equilibrium. The air was removed during pre-heating of the autoclave.

Equipment

Autoclaving (and extraction) was carried out in a down-scaled model of the industrial autoclave (see diagrams on page 11 and 48). The bone chips lie loose within the chamber of the industrial autoclave but in the downscaled one were contained in a basket made of stainless steel mesh. The basket was connected to the upper lid of the model autoclave. Further, the model autoclave had no lid at its base. The lid was equipped with 3 tubes with valves (a steam inlet, steam outlet and extraction water inlet) and 4 thermocouple ports. At the bottom, the model autoclave had 2 tubes with valves (steam inlet and extraction water outlet) and one thermocouple port. Saturated steam was obtained from a steam generator. The steam outlet was connected with a condenser + condensate vessel. The temperature was measured using five thermocouples, one of which was positioned at; the steam inlet, the top of the autoclave above the bone, the base of the bone chip basket, the top of the basket, and inside a bone chip (small hole drilled to accommodate the thermocouple) at the centre of the basket. During the entire process the temperature measured by each thermocouple was recorded at 20 second intervals.
A heating cord was wrapped around the outside of the autoclave and the final part of the steam tube and kept at approximately 120°C during autoclaving to minimise loss of heat through the walls of the tube and the autoclave which were also insulated with insulation material. The outside wall temperature of the autoclave and steam tube were measured continuously at three points using thermocouples.
The steam tube contained a condensate separator which was used to remove condensate and air from the steam before entering the autoclave.

Description of processing.

The dried bone chips obtained from the degreasing were put in the basket of the autoclave. The steam inlet valve was opened with the steam outlet of the autoclave fully open to expel all air from the chamber. The steam outlet valve was gradually closed such that the temperature in the autoclave increased continuously. After 7 to 10 minutes the valve was closed. If necessary, during the process any further air can be expelled by slight opening of

the outlet valve for 15 to 30 seconds, ensuring the temperature of the autoclave does not fall. Approximately 15 to 20 minutes was required all thermocouples to read more than 132°C. When the temperature rose above 132°C timing of the autoclaving began. After 20 minutes the steam inlet valve was closed and the steam outlet valve gradually opened up to its maximum over a period of less than 5 minutes. The temperature reading of the thermocouples should be approximately 100°C after this period.

Extraction

Extraction is the process of treating the autoclaved bone with hot water to dissolve the protein. In the industrial process the autoclaved bone chips are extracted with a certain amount of water for a fixed period of time. The same was done in the scaled down model process. Differences occurred between the processes due to the volumes involved. In the industrial process the water is sprayed over the bone chips and runs down over the chips from the top of the chamber to the bottom. The water is therefore in contact with a large amount of bone chips for a relatively long period. Due to downscaling in the model autoclave, the water would be in contact with a small amount of bone chips for a short period. This discrepancy was addressed in the model process by circulating water over the bone chips for the same period of time as the chips are sprayed in the industrial process. By this process the bone chips absorb more water, therefore the amount of water used in the down scaled model process was approximately 30% larger than in the industrial process.

Appendix 4. STARTING MATERIAL.

Bone gelatine is made from fresh bones collected at slaughterhouses or meat processing plants, from animals fit for human consumption. The first part of the gelatine manufacturing process requires the bones to be crushed into pieces of approximately 1.5 cm before further processing. Ideally, the scaled down process should use the same raw materials as the industrial process but the amount of bone required is very small. One specific cow bone would be sufficient but would not be representative of the heterogeneous mix of bones present in the industrial process. It was decided to use samples collected from the bone crusher of a European industrial gelatine plant. Eight 10 litre amounts were collected, thoroughly mixed then frozen in 1kg aliquots. The bone collected did not contain cattle heads, and because backbone would be added separately at spiking, any cattle backbone present was removed before crushing.

At the time these experiments were planned, European bone gelatine manufacturers excluded the use of cattle skulls but included backbone from which the spinal cord had been removed. Dorsal route ganglia, a tissue known to harbour BSE infectivity is attached to the backbone and could be included in gelatine manufacture, as could spinal cord tissue which is not always completely removed and can be spread over other tissue during slaughter. When the bones are crushed this potentially BSE infective tissue would become well mixed and smeared amongst the raw material.
To obtain a good imitation of the potential contamination of crushed bone starting material, two approaches were used in the model process.

- To imitate cross-contamination before and during crushing, approximately 2g of 301V infected mouse brain was smeared over 750g of crushed bone, after which this was left for at least one day in a refrigerator.
- To imitate contamination by CNS-tissue in the backbone, a further 2g of infected mouse brain was injected into the spinal cord of a 250g intact piece of calf backbone. This piece was kept overnight in a refrigerator then sawn into 1 to 1.5 cm pieces ensuring the spinal cord was cut and stayed connected to the bone. These pieces were then mixed with the spiked crushed bone and stored overnight in a refrigerator.

The amount of backbone used was relatively high compared to what might occur in reality. The bones of one whole cow, without the backbone, weighs approximately 20 to 29 kg, the backbone 5 to 6 kg; a ratio of 5:1. The ratio in the model process of crushed bone to sawn backbone was 3:1.

The amount of infectious brain material and spinal cord used in the model process, approximately 9 g on 1 kg of bone, is higher than the amount of CNS that could be present when the head and spinal cord are removed. Dorsal root ganglia weigh approximately 30 g, which coupled with an estimated cross-contamination by CNS tissue of 20 g, results in 50 g CNS on approximately 35 kg bone. This represents a ratio of 1:700 whereas the model

process used 1:110. However heads and spinal cords used to be included in the process, and in this case the ratio would be approximately 1:60. Therefore, the amount of CNS in the model process lies between the two values calculated for industry practices before and after SRM removal.

Industrial crushed bone contains approximately 42% water. The composition of the dry matter is in table 1.

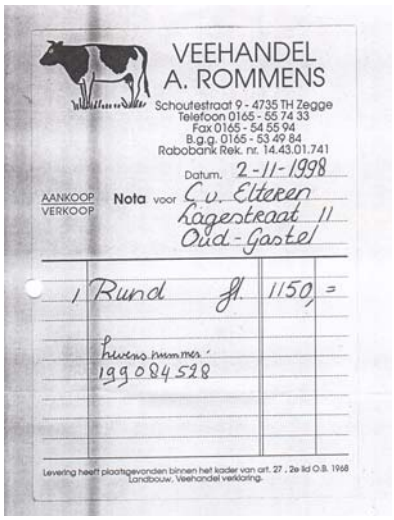
Table 1. Approximate composition of the fresh bone.

	Composition calculated on dry matter	**Composition calculated on dry matter excluding fat**
Fat	approx. 36 %	
Protein	approx. 32 %	approx. 50 %
Ash 550 ^{o}C	approx. 31 %	approx. 48 %
Ash 1100 ^{o}C	approx. 30.5 %	approx. 47.5 %

After degreasing the composition of the dried crushed bone is approximately:

Water	less than 12 %
Fat	less than 3 %
Ash	more than 58 %

The calf from which the backbone was obtained was bought and slaughtered specifically for use in these experiments. All the relevant associated papers are shown on the following pages together with the health certificate of the bone material used.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A. ROMMENS
Schoutestraat 9 - 4735 TH Zegge
Telefoon 0165 - 55 74 33
Fax 0165 - 54 55 94
B.g.g. 0165 - 53 49 84
Rabobank Rek. nr. 14.43.01.741

Datum 2-11-1998

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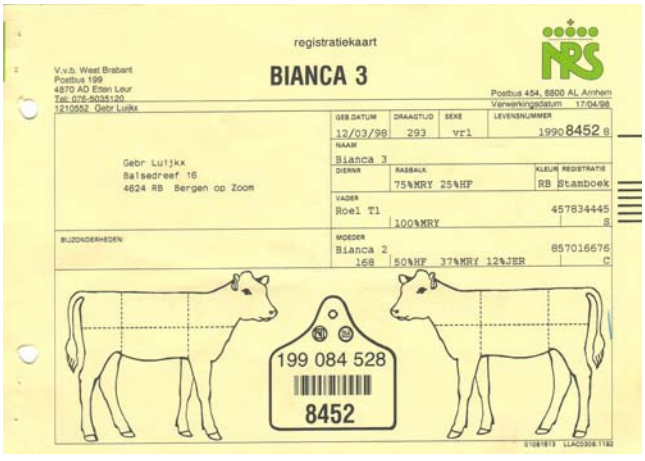
1 Rund fl. 1150,=

Levering heeft plaatsgevonden binnen het kader van art. 27, 2e lid O.B. 1968 Landbouw, Veehandel verkaring.



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Het genoemde rundveebeslag is evenals het/de betreffende rund(eren) ten tijde van afgifte van deze verklaring gekwalificeerd als:

Dierziekte	Kwalificatie
Brucellose	Officieel vrij
Tuberculose	Officieel vrij
Leucose	Officieel vrij
Leptospirose	Vrij
IBR	Niet vrij
Paratuberculose	
BVD	Onbekend
Salmonellose*	
BSE	Vrij

*eventueel aanvullende informatie veehouder/praktiserend dierenarts

Deze verklaring is **geldig tot maximaal zeven dagen na datum afgifte.**

Datum afgifte: 16-10-1998

de directeur.

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KONINKRIJK DER NEDERLANDEN

GEZONDHEIDSCERTIFICAAT

(Veterinair Certificaat)

Ministerie van Landbouw, Natuurbeheer en Visserij / Ministry of Agriculture, Nature Management and Fisheries / Ministerium für Landwirtschaft, Naturschutz und Fischerei / Ministère de l'Agriculture, de la Nature et de la Pêche / Ministerio de Agricultura, Naturaleza y Pesca

Rijksdienst voor de keuring van Vee en Vlees / National Inspection Service for Livestock and Meat / Staatlicher Dienst für Vieh und Fleischbeschau / Service National d'Inspection du Bétail et de la Viande / Servicio Nacional de Inspección de ganado y carne

Gezondheidscertificaat / Health Certificate / Gesundheitsbescheinigung / Certificat Sanitaire / Certificado Sanitario

I Certificering voor: / Certification for: / Bescheinigung für: / Certification pour: / Certificación para:

Naam produkt, diersoort / Name of the product, animal species / Name des Produktes, der Tierart / Nom du produit, espèce animale / Nombre del Producto, especie animal	BONE CHIPS (DRIED BONE PRODUCTS)
Identificering / Marks / Identifizierung / Identification / Marcas	BATCH NO. RESEARCH MR. A. GROBBEN
Aantal, gewicht / Number, weight / Zahl, Gewicht / Nombre, poids / Quantos, peso	CA. *5* KGS
Soort verpakking / Nature of packaging / Art der Verpackung / Sorte d'Emballage / Clase de embalaje	IN 1 CONTAINER
Naam en adres producent, herkomstbedrijf / Processing plant, Farm of origin / Erzeuger, Herkunftsbetrieb / Producteur, exploitation d'origine / Productor, establecimiento de origen	GELATINE DELFT BV ROTTERDAMSEWEG 268-270 2628 AT DELFT HOLLAND
Exporteur / Exporter / Absender / Expéditeur / Exportador	THE SAME APPROVAL NUMBER: NL7321 EEG
Naam en adres ontvanger / Name and address of consignee / Name und Anschrift des Empfängers / Nom et adresse du destinataire / Destinatario Land / Country / Land / Pays / País	CRODA COLLOIDS LIMITED (DMD) FOUNDRY LANE DITTON WIDNES CHESHIRE WA8 8UB UNITED KINGDOM
Middel van vervoer / Means of Transport / Transportmittel / Moyen de transport / Clase de transporte	BY DHL ON OR ABOUT 26-02-1999

II Ondergetekende verklaart / The undersigned certifies / Der Unterzeichnete / Le soussigné, certifie / El que suscribe, certifica

1. The raw material of which the degreased chipped bone is prepared are bovine and pig bones, obtained from healthy animals that were slaughtered in a slaughterhouse, were inspected prior and after slaughter by an offical veterinarian and were declared fit for human consumption.
2. The bovine bones are either obtained from animals from herds that never had a case of BSE reported or were imported from countries where no case of BSE has been officially declared. No bovine bones are obtained from the United Kingdom, Ireland, France, Oman, Switzerland or Portugal.
3. The product does not contain, and is not derived from, specified risk material as defined in Commission Decision 97/534/EC or mechanically recovered meal obtained from the vertebral coumn of bovine, ovine or caprine animals.
4. The crushed bones are defatted by treating them for 15 minutes with an aqueous solution of pH=5 at 90° C and are subsequently dried with air of 450°c.
5. The crushed bones are not intended to be diverted for direct use in human or animal food.
6. Traceable records of raw material for each batch of degreased chipped bone are maintained and are available for inspection by the authorities.

Gedaan te / Done at / Ausgefertigt in / Fait à / hecho en / Rotterdam op / on / am / le / el / 23-02 99

handtekening officiële functionaris / signature of the official functionary / Unterschrift des beamteten Funktionärs / Signature du fonctionnaire officiel / firma funcionario oficial.

drs H.T. v.d. Laan

naam in drukletters / name in capital letters / Name in Grossbuchstaben / nom en lettres capitales / nombre en letras capitales.

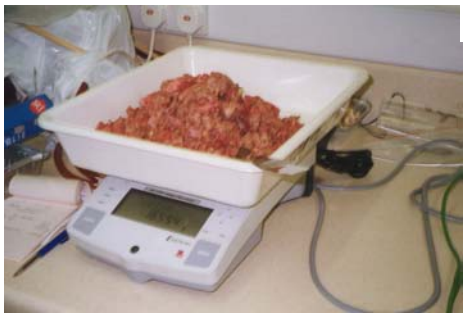
Rijksdienst voor de keuring van Vee en Vlees • Nederland •

Appendix 5. DETAILED DESCRIPTION OF EXPERIMENTAL PROCEDURES

Preparation of the starting material

Crushed bone starting material and calf backbone

A bag of crushed bone and a piece of intact backbone were taken from the freezer and were thawed in a refrigerator. The backbone was weighed on Ohaus Explorer scales (4100±0.1g cat no EOD120). The difference between the weight of the backbone and 1000 g was calculated and this amount of crushed bone was aliquoted into a tray.



Crushed bone

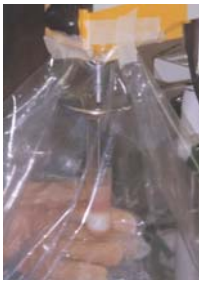
Homogenising equipment

Preparation of the 301V mouse brain homogenate for spiking

In the biological safety cabinet a Heidolph stirrer stand with a Heidolph RZR 2020 electronic stirrer (LS cat no 5141 12100) was set up. A hole was made in the top of a polythene glove bag, to fit over the stirrer controls, and was then fixed to the stirrer with adhesive tape. The stirrer was equipped with a 30ml Schütt homogeniser with a PTFE piston (LS cat no 1931 05143/55). Approximately 5.5 g of 301V infected mouse brain macerate was weighed from the stock jar into the homogeniser tube on a Scout SC 2020 scale (200±0.01g, cat no YSC2020). The tube was placed on the piston, the stirrer was switched on set at 1000 rpm, and the brain was homogenised by passing the piston three times along the tube wall. The homogenate was transferred to "jar-I" which was weighed. The jar, the homogeniser tubes and the spatulae were set aside. The glove bag was removed from the cabinet, together with the pistons of the homogenisers, and disposed of. The stirrer and stand were also removed from the cabinet and put in polythene bags until further use.

Jar with brain- macerate

Transfer to homogeniser tube



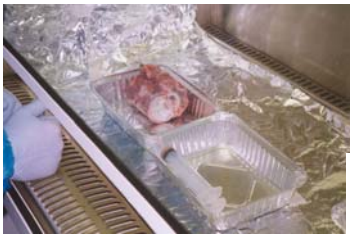
Homogenising

Injecting into the spinal cord of the backbone and spiking of the crushed bone.

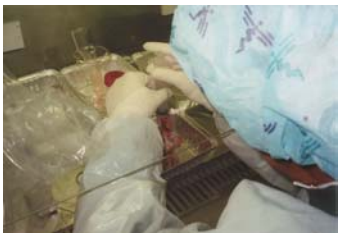
A Braun Omnifix 30 ml syringe (LS cat no 1942 13030) was filled with the brain homogenate from jar-I. A Beckton Dickinson 18G6 needle (cat no 408360) was fixed to it and the syringe weighed. The homogenate was injected into the spinal cord of the calf backbone after which the syringe was weighed again. The tray with the spiked backbone was put in a polythene bag and put in the refrigerator.

The tray with crushed bone was taken from the refrigerator and placed in the biological safety cabinet. The spatulae used previously with adherent brain were weighed before the brain tissue was smeared on the crushed bone. The spatulae were weighed again and disposed of. The brain tissue left in jar-I was also smeared on the crushed bone by wiping the inside of the jar with pieces of crushed bone. Jar-1 was weighed before and after the wiping. The brain tissue left in the homogeniser tube was transferred to the crushed bone in the same way. The homogeniser tube was also weighed before and after wiping. The tray with crushed bone was placed in a polythene bag and put in the refrigerator. The stock jar with brain macerate was put back in the laboratory freezer.

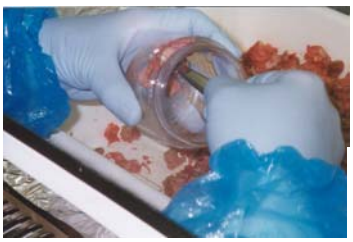
All materials and equipment remaining in the biological safety cabinet were disposed of and the cabinet decontaminated using a sodium hypo chlorite solution with 20,000 ppm free chlorine for an hour minimum.



Syringe with spike



Injecting in spinal cord



Smearing on crushed bone

Weight of crushed bone, backbone and brain tissue.

Weight of tray with crushed bone	1115 g
Weight of empty tray	365 g
Crushed bone	**750 g**
Tray and bag with backbone	287 g
Empty tray	15 g
Back bone	**254 g**
Total weight of starting material	**1004 g**

Stock jar before taking out			19.19 g	
Stock jar after taking out to homogeniser tube			13.59 g	
Taken from stock jar to homogeniser tube			5.60 g	
Homogeniser tube with mouse brain			170.74 g	
Homogeniser tube new			165.86 g	
Brain in tube			4.88 g	
Spatula with mouse brain			115.23 g	
Spatula new			114.77 g	
On spatula			0.46 g	
Brain taken from stockjar		5.60 g		
Brain in tube	4.88 g			
On spatula	0.46 g			
Total		5.34 g		
Difference		**0.26 g**		

This difference is probably caused by evaporation of water during transfer and weighing.

Syringe after filling with homogenate			22.97 g	
Syringe new			20.28 g	
Homogenate in syringe			2.69 g	
Syringe after filling with homogenate			22.97 g	
Syringe after injection			20.57 g	
Injected in backbone				2.40 g
Syringe after injection			20.57 g	
Syringe new			..20.28 g	
Left in syringe			0.29 g	
Weight of needle 2 after injection			0.82 g	
Weight of needle 2 new			0.76 g	
Left in needle			0.06 g	
Homogeniser tube before smearing on bone			166.65 g	

Homogeniser tube after smearing on bone		166.11 g	
Smeared on bone			0.54 g
Homogeniser tube after smearing on bone		166.11 g	
Homogeniser tube new		165.86 g	
Left in homogeniser tube		0.25 g	
Spatula 1 before smearing		115.32 g	
Spatula 1 after smearing		114.90 g	
Smeared on bone			0.33 g
Spatula 1 after smearing		114.90 g	
Spatula 1 new		114.77 g	
Left on spatula 1		0.13 g	
Spatula 2 before smearing		115.42 g	
Spatula 2 after smearing		114.96 g	
Smeared on bone			0.46 g
Spatula 2 after smearing		114.96 g	
Spatula 2 new		114.80 g	
Left on spatula 2		0.16 g	
Total spike			**3.73 g**

Losses:	
Left in homogeniser tube	0.25 g
Left in syringe	0.29 g
Left in needle 2	0.06 g
Left on spatula 1	0.13 g
Left on spatula 2	0.16 g
Total known amount left on equipment	0.89 g
Taken from stock jar	5.60 g
Total know left on equipment	0.89 g
	4.71 g
Total spike	3.73 g
Other losses	0.98 g
Other losses	0.98 g

Evaporation during transfer and weighting	0.26 g
Left on piston	0.72 g

Correction for known evaporation: 3.73/(1.68+3.73)*0.26=0.18 g

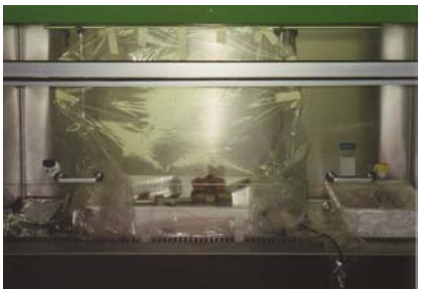
Total spike 3.73 + 0.18 = 3.91 g

Infective material was lost during spiking due to material left on surfaces of the used equipment. Part of the difference in weight is however also be caused by evaporation of water from the brain tissue. Part of the material left on the surfaces of equipment during smearing of brain tissue on the bone chips could be fat transferred from the bone chips to these surfaces. The amount of spike could therefore be in reality somewhat larger than the stated amount of 3.91 g.

Reference: Note book 9-9-99 to 14-12-99 page82 to 91. Date 22-11-99.

Sawing of the backbone

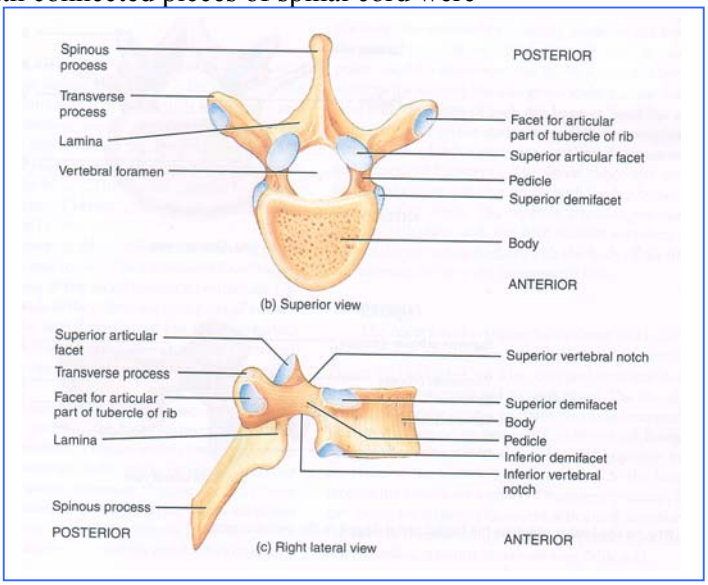
A glove bag was set up in the biological safety cabinet. One plastic and one aluminium tray, two pairs of tweezers (Bochem 18/8 steel LS cat no 3305 01000), two pairs of forceps (arterienklemme stainless steel LS cat no 3351 11038), two scalpels (scalpel handle LS cat no 3351 18061, blade LS cat no 3351 18064) and a vice were put in the bag. A hole was made in the side of the bag through which a Stryker TPS Micro Sagittal saw (ref 5100-34) with a Stryker Long Wide Aggressive Blade 34.5mm x 16.5mm (ref 2296-3-504) was put inside the glove bag. The saw handle and the cord of the saw were covered with a protective sleeve. The sleeve was taped to the hole to close this [hermetically]. The purpose of the glove bag was to protect against dispersal of sawdust created by sawing of the backbone. The tray with the backbone was taken from the refrigerator and put in the glove bag. The backbone was placed into the vice and sawn into 1.5 cm pieces as described below. The soft tissue on the backbone was cut away with scalpels and the backbone material was handled using the tweezers and forceps. Care was taken when separating the pieces that the spinal cord was cut to size and remained connected to the backbone. Care was also taken that the dorsal root ganglia remained between the vertebrae. The pieces of backbone were put into the tray which had originally contained the whole backbone.



Sawing the backbone

Description of sawing of the backbone:

The backbone was firmly clamped in the vice to facilitate easier and safer sawing. Before sawing, any muscle or fat surrounding the vertebrae was cut with a scalpel. The bone was sawn with short bursts of the saw, of approximately 10 seconds, to prevent overheating of the saw motor and blade. The backbone was dissected as follows. Firstly, the transverse processes were sawn in several directions to obtain pieces of approximately 1.5 cm. The spinous process was done in the same way. Thereafter the vertebral arch (lamina) was sawn at both sides from posterior to anterior cut precisely at both sides of the vertebral foramen. The lamina was also cut laterally such that 1.5 cm large pieces were obtained. The pieces were lifted from the vertebrae. The now visible spinal cord was cut into three with a scalpel, one connected to the body of the vertebrae, and one to each of the pedicles. The pedicles were removed by cutting the vertebral foramen at the base of the vertebral arch. The pedicles were also cut laterally to obtain pieces of 1.5 cm. The spinal cord connected to it was then also cut laterally with a scalpel. The pieces of the pedicles with connected pieces of spinal cord were removed with forceps. The left and right posterior parts below the pedicles were removed, after which the body was sawn from left to right such that the bone to which the remainder of the spinal cord was connected could be removed. Finally the body of the vertebrae was sawn to pieces of 1.5 cm. After sawing, the backbone pieces were placed in the original tray, covered with a polythene bag and transferred to the refrigerator.



The saw blade was removed from the handle and the handle was carefully drawn back in the sleeve, so it did not touch the sides of the sleeve. The sleeve was then cut so it covered the contaminated top of the saw. was The sleeve was closed with tape and the covered saw was put in a polythene bag and stored for further use. The scalpels, tweezers and forceps were removed from the glove bag, put in a small polythene bag and disposed of into a contaminated sharps bin. The opening in the glove bag was closed and the air let out through the air inlet/outlet. The glove bag and all remaining contents was removed from the

Sawing the backbone

biological safety cabinet and disposed of. The cabinet was decontaminated with sodium hypochlorite as before.

The trays with spiked crushed bone and the sawn backbone were taken from the refrigerator to the biological safety cabinet. The sawn backbone was carefully mixed with the bone using two spatulas to move the material around in the tray for approximately 15 minutes. The tray was then put in a polythene bag and returned to the refrigerator.
The materials left in the cabinet were put in polythene bags and disposed of. The cabinet was decontaminated as before.

Reference: Note book 9-9-99 to 14-12-99 page 89. Date 23-11-99.

Degreasing

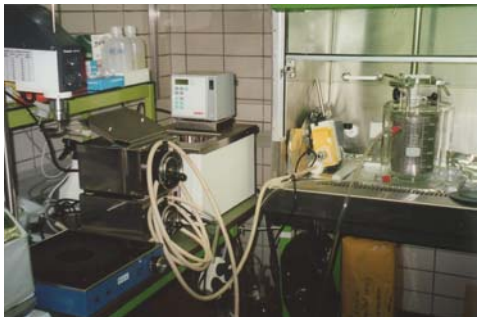
Preparation of the degreasing emulsions.

A bag of fresh crushed bone was taken from the refrigerator and after thawing, a portion of 600 g was put in aluminium foil trays. A 5000 ml beaker (Schott DURAN) with 2000 ml water and 1.4 ml 2M H_2SO_4, was heated on a hotplate (Schott-Geräte CERAN hotplate 1800W type 930 00) to 92°C. The 600 g portion of crushed bone was added to the water and stirred for 20 minutes at 80°C. (Heidolph electronic stirrer RZR 2020 with stainless steel 3 blade propeller stirrer PR33, diameter 66 mm, LS cat no 395093300000). The emulsion produced was then decanted and put in the tempering beaker of the degreasing equipment set up in the cabinet.

Preparing Degreasing emulsion

Degreasing of the crushed bone and sawn backbone spiked with 301V infected mouse brain.

The degreasing equipment was set up. (See pictures and drawing of degreasing equipment below). A Heidolph stirrer stand and Heidolph RZ2020 stirrer with a 3 blade stainless steel propeller of 66mm, was put in the cabinet. A 3 litre double walled tempering beaker was put in the cabinet and fixed to the stand with a chain clamp. Inside, this beaker was equipped with a stainless steel basket and topped with a PVC lid. The basket was hung in a ring clamped to the stirrer stand. The lid contained several holes for the stirrer, an inlet



Degreasing equipment

Beaker and basket

siphon, an outlet siphon and a thermometer. The double wall of the tempering beaker was connected to a Jubalo MW12 circulating thermostatic waterbath. Using a Verder Pericor peristaltic pump 9F-240, water heated on a hotplate could be pumped into the double walled beaker. A second identical peristaltic pump was used to pump the degreasing emulsions in and out of the double-walled beaker.

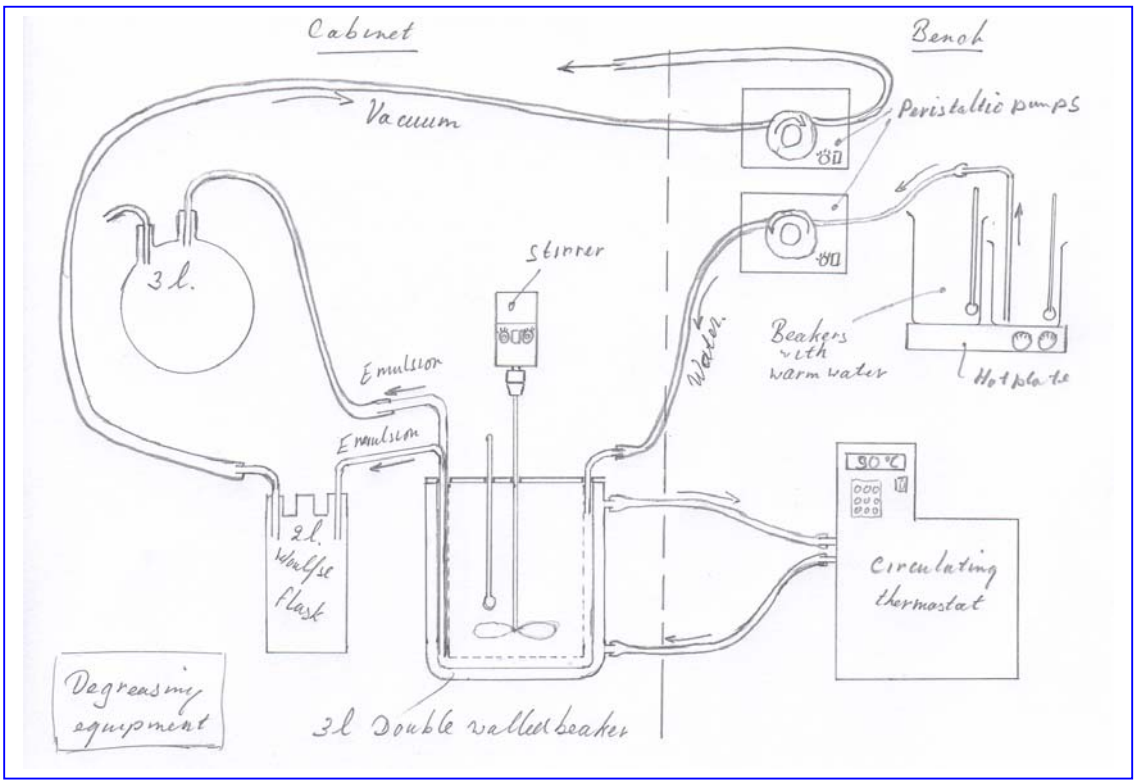
The basket was removed from the double walled beaker and the blade from the stirrer.

The 2 litre portion of degreasing emulsion was prepared and put into the double walled beaker and the circulating thermostat was switched on to warm the emulsion to 80°C. 1 litre and 2 litre beakers of water were warmed to 95°C on the hotplate. The spiked crushed bone and backbone mixture was taken from the refrigerator to the safety cabinet where it was transferred to the basket which was wrapped in a polythene bag to prevent spillage. The stirrer was placed in the basket and the lid fitted on top. The basket was fixed to the ring and the whole assembly was fitted on to the stand. The polythene bag was removed and the basket assembly lowered into the double-walled beaker, immersing it in the warm degreasing liquid. The propeller stirrer was fixed to the stirrer motor, the water inlet siphon connected and the emulsion outlet siphon put in the emulsion. The stirrer was started and the emulsion with bone was stirred vigorously for 20 minutes. During this period, at regular intervals, 4 x 250 ml of emulsion was pumped out of the double walled beaker into the Woulfse flask each time being replaced by the same volume of warm water. After 20 minutes 1 litre of emulsion was pumped out of the beaker and 1 litre of clean water was pumped in. After a further 20 minutes all degreasing liquid was pumped from the double walled beaker into a 3 litre round bottom flask. The pump and the stirrer were then switched off.

Filling the basket

Degreasing

Two litres of water heated to 80°C were pumped onto the bones, stirred for 3 minutes then the water pumped out into a second 3 litre flask. The temperature measured for this stage was 85-86°C. Samples were taken of the degreasing emulsion, the final washing liquid and the degreased wet bone.



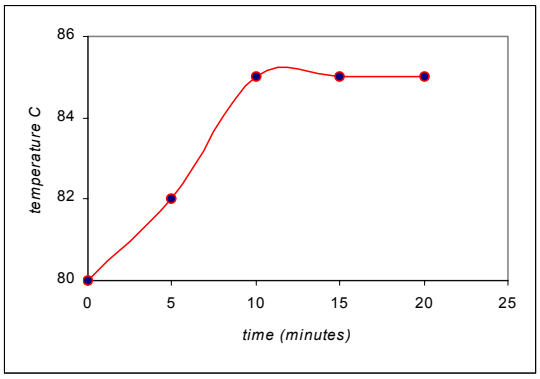
List of equipment:

tempering beaker 3 litre custom made
basket custom made
ring custom made
lid custom made
U-tube custom made
Large siphon custom made
Small siphon custom made
Small tube custom made
Thermometer –10…110° cat no 67370413
Woulfse flask 3 neck 2000 ml cat no 832502179
4 round bottom flask 3 litre cat no 832092583
2 cork rings cat no AS240020170
2 Verder Pericor peristaltic pump 9F-240
pumping hose Verderprene 8x1.6 cat no 58 84 81680
pumping hose Verderprene 9.6x3.2 cat no 58 84 83209
PVC tube 9x12 mm
Schott-Geräte CERAN hot plate type 930 00 1800W cat no 43 27 93000 (67740401
circulating thermostat bath Jubalo MW12
Heidolph electronic stirrer RZR 2020 cat no 5141 12100, with 3 blade propeller stirrer PR33, diam 66mm, cat no 395093300000
Beaker Schott DURAN 5000 ml cat no 11 21 10673
Beaker Schott DURAN 3000 ml cat no 11 21 10668)

The basket was lifted from the beaker. To avoid splashes and contamination, care was taken to wrap all equipment taken from the double walled beaker in polythene bags until further use. The basket was also immediately put in a polythene bag and the beaker covered with aluminium foil. The degreased bone was transferred from the basket into a plastic tray with stainless steel spatulae.

Temperature during degreasing.

Time (min)	**temperature degreasing emulsion**	**temperature fresh water**
initial	81°C	79°C
0	80°C	
5	82°C	
10	85°C	
15	85°C	
20	85°C	



Taking degreased bone and soft tissue from basket

Degreased bone

Tray with bag and degreased bone	924.6 g
Tray with bag	365.5 g
Bone and soft tissue after degreasing	550.1 g

A sample of 15.5 g was taken of the degreased bone and tissue.

The tray with degreased bone was put in a polythene bag and put in the refrigerator.

All equipment used in the cabinet except the stirrer stand, the clamps and the stirrer motor, was wrapped in polythene bags and disposed of. The stirrer stand, the clamps and the stirrer

were put in polythene bags and removed from the cabinet until further use. The cabinet was decontaminated as before.

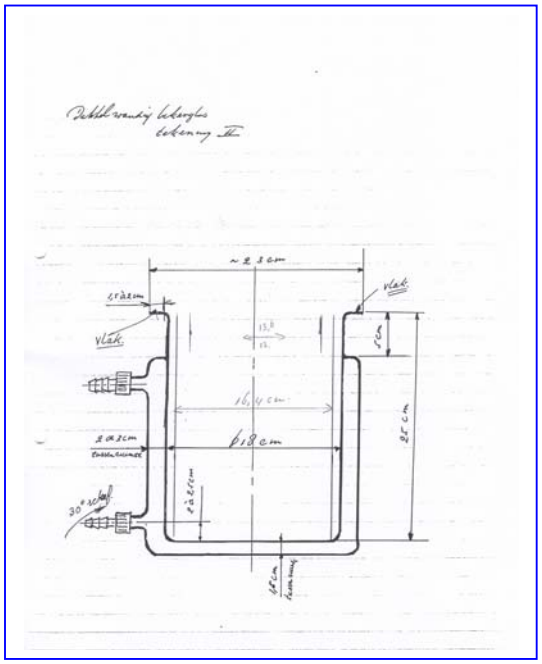
Separating bone and soft tissue

Separating degreased bone and

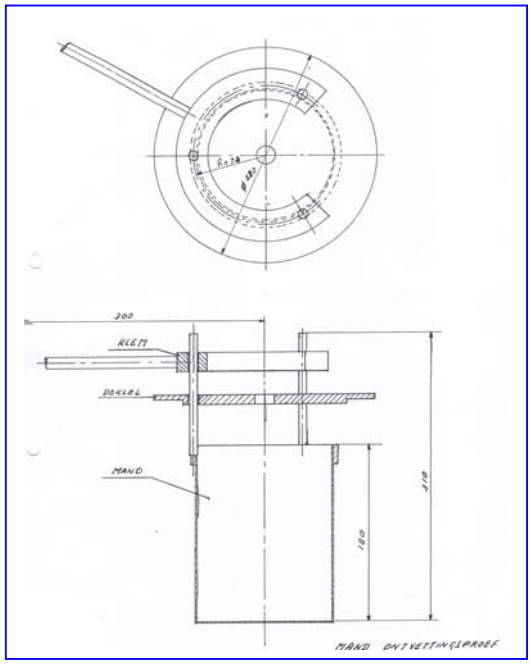
The degreased material was separated, using forceps, into bone and soft tissue fractions. Bone with a little soft tissue attached was placed with the bone fraction, otherwise it was placed with the soft tissue. Material of less than 4 mm was considered too fine and was not sorted. The soft tissue and fine material was disposed of. The tray with bone was put in a polythene bag and put in the refrigerator.
All used equipment was put in polythene bags and disposed of. The safety cabinet was decontaminated as before.

Tray and bag with sorted wet bone	330.7 g
Tray and bag	18.4 g
Sorted wet bone	312.3 g

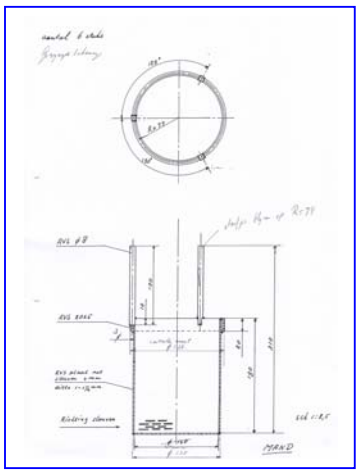
Custom made equipment



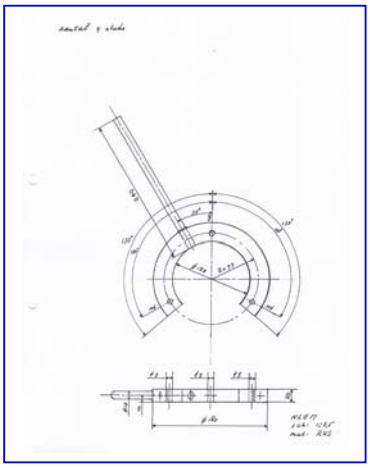
3 litre double walled beaker



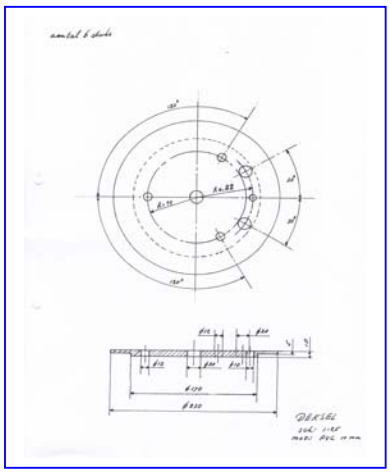
basket assembly



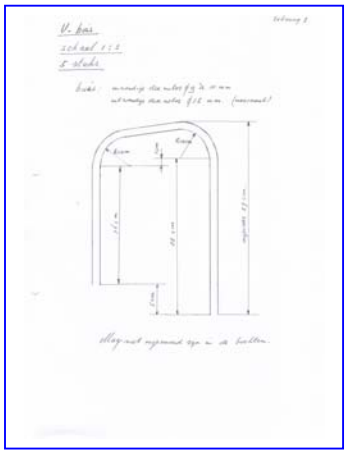
basket

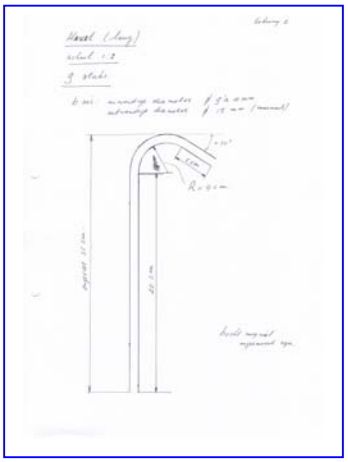


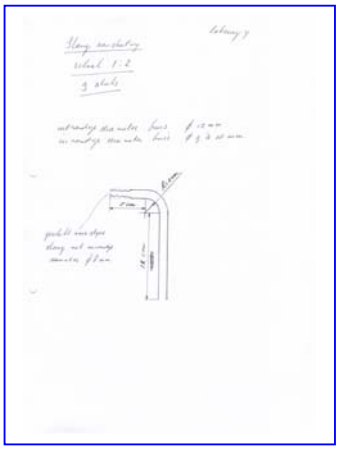
ring



lid







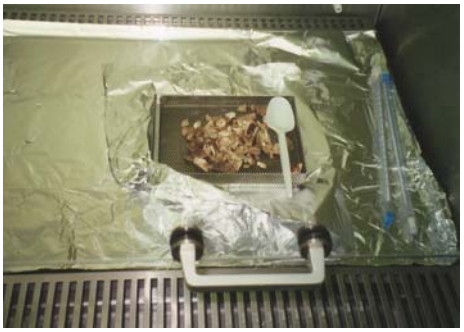
siphons

Drying of the bone

The custom-made bone drier was installed. The bone drier was a closed circuit drier using a system of 3 hairdryers, to heat and circulate air over stainless steel mesh trays containing the wet bone. Finally, two water-cooled heat exchangers cooled the air and condensed the water. A filter behind the heat exchangers prevented any drops of water entering the air flow.

Drier, view on inside with trays

The bone was taken from the refrigerator to the safety cabinet and transferred to the drier trays. A maximum thermometer (Amarell –10 … 150°C LS cat no 1610 15002) was put amongst the bone and another one in the air stream. The drier was closed and switched on for 10 minutes at 3,700W followed by 40 minutes at 2,100W. After cooling for 20 minutes the drier was opened and the dried bones on the trays were put in a 3.5 litre sealable plastic (tumbler) jar.
The maximum temperature of the air was 80°C and the maximum temperature between the bone was 69°C.

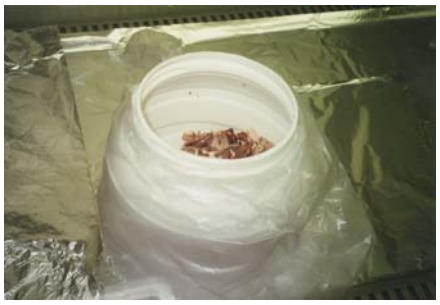


Tray with dried bone chips

All small equipment was wrapped in polythene bags and disposed of. The drier was closed and disposed of. The safety cabinet was decontaminated as before.

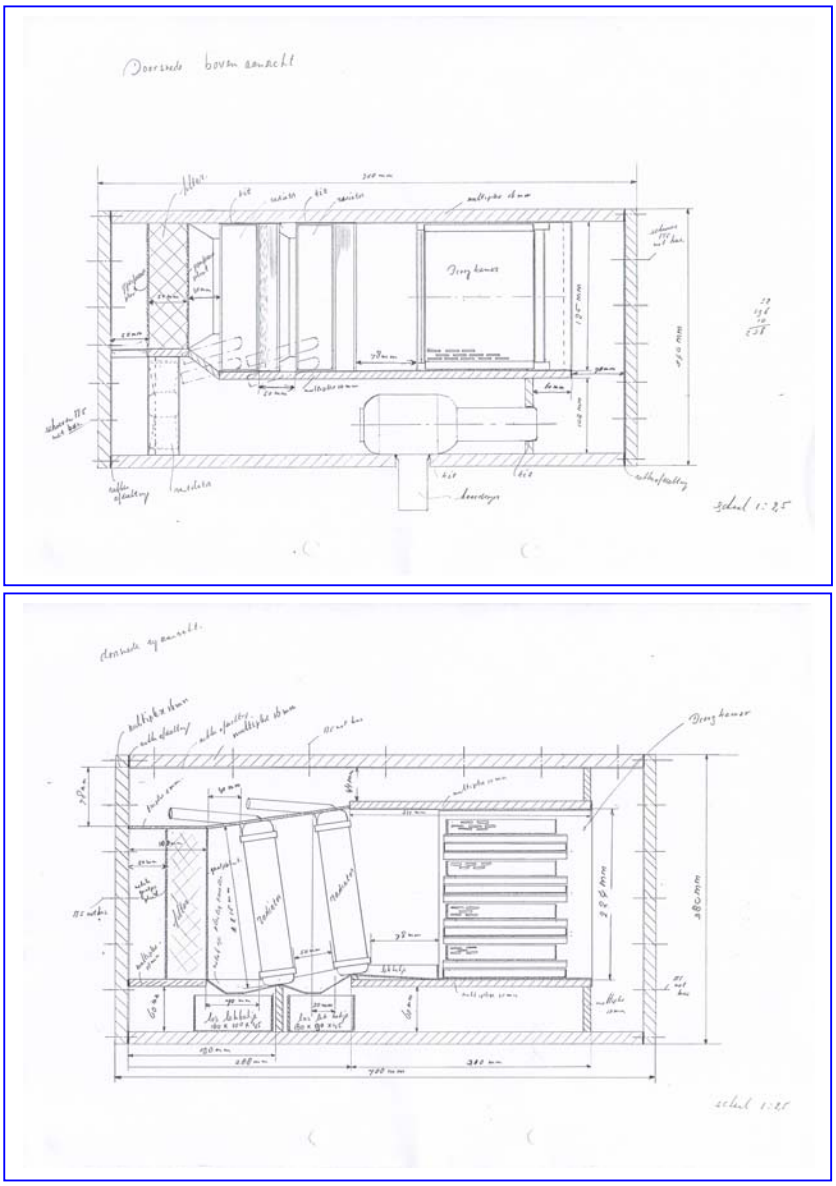
Tumbling, sieving and sorting of the dried bone.

The tumbler jar (Curtec Keg wide mouth HDPE 3.5 litre BDH cat no 215/0380/12), was closed and sealed with a piece of steel wire. The jar was put on a roller bank and rotated for 4 hours.



Tumbler jar with dried bone chips

Tumbler jar on roller bank



Drier, side view and top view

Jar with dried bone	600.0 g
Empty jar	360.1 g
Dried bone chips	239.9 g

The contents of the jar were then transferred, as demonstrated in the six pictures below, to a 4 mm stainless steel sieve with a sieve bottom and lid. The jar was disposed of.

1

2

3

4

5

6

The sieve was taped shut and the bone chips were sieved for 10 minutes. (Sieve 4.00 mm, 200x50 mm DIN ISO 3310/1 C rvs LS cat no 01310310066, Sieve lid 200mm rvs LS cat no 01051070252, Sieve bottom 200x50 mm rvs LS cat no 0105010022) The bone was then transferred to a tray and the sieve assembly disposed of.

Sieve with dried tumbled bone

Sieving the bone

Sorting of the bone chips.

The sieved bone chips were sorted by hand with forceps to remove loose soft tissue and highly porous material. From the remaining material, bone chips of sufficient size, 5 mm and larger, were transferred to an aluminium foil tray, remaining fine material was sorted again for pieces which had a sufficient size and density to be included for further processing to gelatine. Anything remaining at this point was disposed of. The bone chips to be processed further still contained bone with soft tissue connected to it.

Jar and bone chips	589.4 g
Empty jar	369.5 g
Sorted degreased dried bone chips	**219.9 g**

A sample of 14.9 g was taken of the bone chips.

Sorted degreased dried bone chips	219.9 g
Sample of bone chips	13.3 g
Bone chips for autoclaving	**206.6 g**

The bone chips were put in a jar and, stored at -20°C until further use.

All equipment used was put in polythene bags and disposed of. The safety cabinet decontaminated as before.

Reference: Note book 9-9-99 to 14-12-99 page 90 to 97. Date 25 to 30-11-99.

Autoclaving

Introduction

In situ testing of the autoclaving equipment found that the desired autoclaving temperature was not reached. Temperature in the equipment did not rise above 125 to 128°C. A number of changes were then made to the equipment:

- Insulation of pipes and autoclave was improved.
- The steam pipe between the steam generator and the autoclave was shortened.
- A electrical heating wire was wound around the autoclave and the end of the steam pipe.
- A condense-trap/air-trap was installed just before the connection to the autoclave.
- The adjustment of the steam generator was changed, increasing the steam pressure of the generator from 3.5 bar to 4.2 bar.

Because of these changes the temperature of the autoclave was increased to 132 to 134°C. After making these changes 7 test runs were made using industrial bone chips.

Setting up equipment

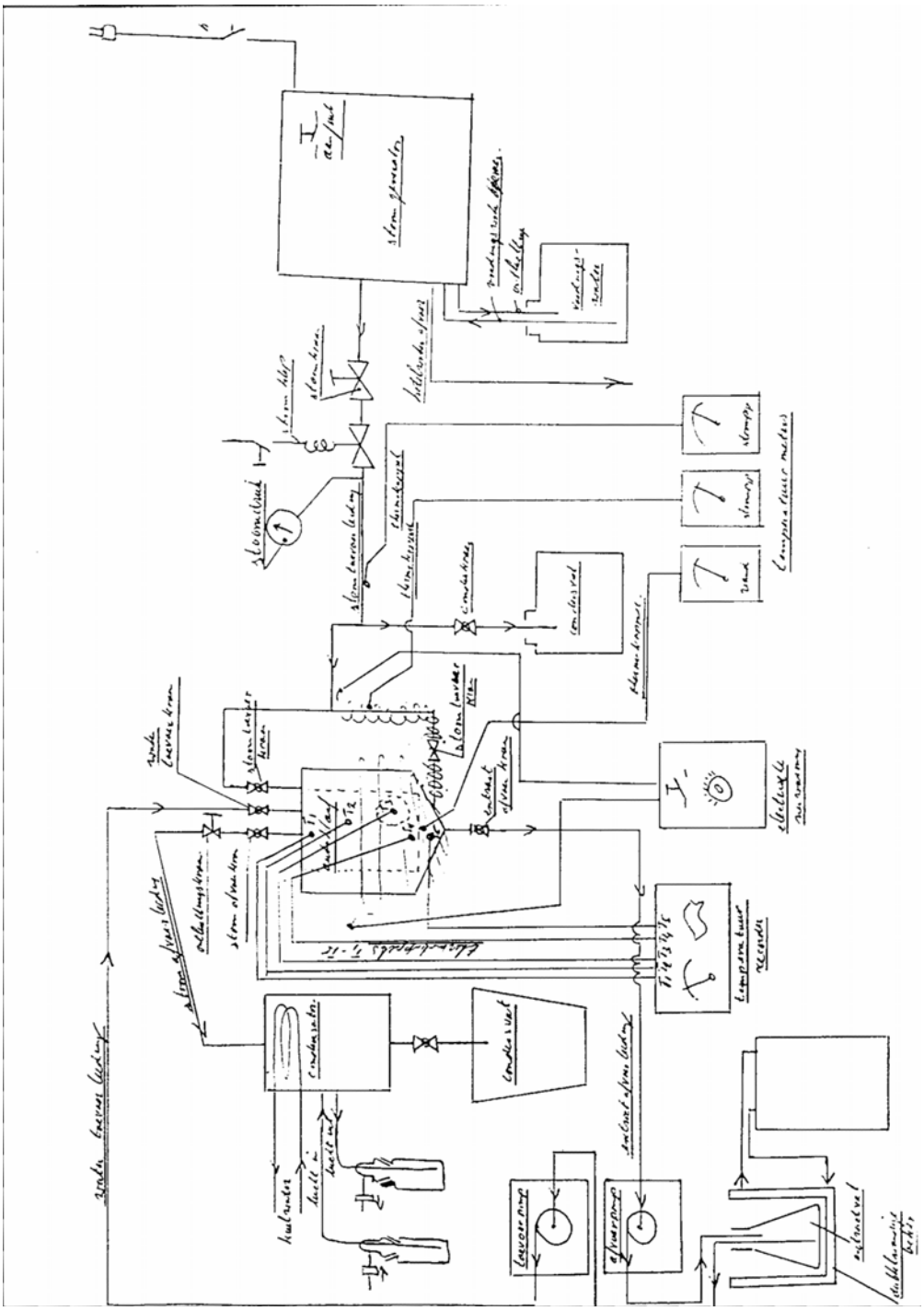
The autoclave was placed in the cabinet. The condenser/condense vessel was connected to the steam outlet pipe on the lid. The condense-trap/air-trap was connected to the steam inlet pipe. The calibrated thermocouples (100 cm x 1 mm type K) (see calibration) were inserted in the ports and their length adjusted. The steam generator (H+P Varioclav Zyclonendampf) was connected to the trap.
Next the steam generator was switched on and all connections and valves were carefully checked for leaking of steam and/or water.
The heating wire was wrapped around the autoclave and the end of the steam pipe and connected to its control panel. Three thermocouples (type K) to measure the outsidetemperature of steam pipes and autoclave were installed and connected to the meters (Voltcraft M3860M). The autoclave and steam pipes were insulated.

Autoclave and condenser

Washing bottles with sodium hydroxide were connected to the air inlet and air outlet of the condenser/condense vessel. The thermocouples were connected to the recorder. (Kane May 1242)
The temperature meters, recorder and steam generator were switched on. When temperatures were constant the heating wire was switched on and the control adjusted such that the temperature measured by the thermocouples inside the autoclave remained between 133 and 135°C. The outside temperature of the autoclave was approximately 130°C.

Autoclaving equipment

After testing and adjusting the steam valve was closed and the steam generator switched off. Once cooled, the other valves were opened and the temperature meters and recorder were switched off.



Autoclaving

The jar with bone chips was taken from the freezer and left to thaw. The insulation was removed from the autoclave, the lid was taken off and the basket disconnected from the lid. A piece of bone with a hole drilled in it, was attached to a thermocouple which would be placed in the middle of the basket with bone. The bone chips were carefully transferred from the jar into the basket.

Jar with bone chips	589.5 g
Jar empty	369.3 g
Bone chips in autoclave	220.2 g

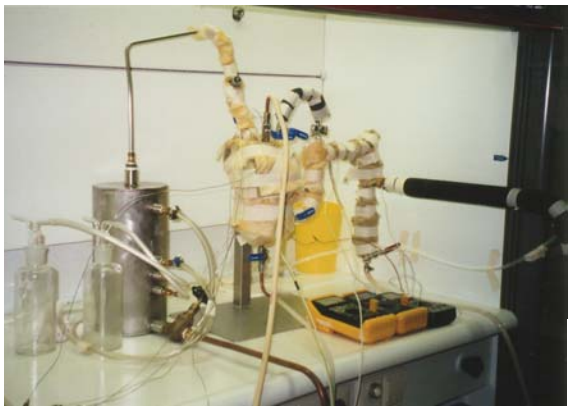
Basket

The basket was carefully connected to the bottom of the lid. Three thermocouples were carefully positioned one between the bone chips in the basket, one above the bone chips and the other at the steam inlet. The autoclave lid was closed and all necessary steam pipe and thermocouple connections made. The insulation of the autoclave was replaced. The temperature meters were switched on and the temperature recorder was switched to standby. The steam inlet valve, the water inlet valve and the water outlet valve were closed. The steam outlet valve opened and the needle valve opened 1 ½ turn, the drain valve of the trap was also opened, the condenser cooling water put on and the steam generator switched on with the main steam valve closed. When the steam generator was at full pressure the main steam valve was opened. When no more water came from the condense trap the valve of this trap was gradually closed until it only bled a little steam, thus any air would be removed from the inlet steam. The steam pressure was measured at 4.1 to 4.2 bar. After 10 minutes the temperature recorder was switched on, registering the temperatures inside the autoclave every 20 seconds, after two of which intervals the lower steam valve was opened, the valve of the trap closed, the heating wire controls witched on and the chronometer started. When the steam pressure reached 3.1 to 3.3 bar the valve of the trap was opened again such that some steam and any air would be very slowly released. After 4 minutes the needle valve to the condenser was closed to ¾ turn, at 6 minutes to ½ turn, at 7 minutes to ¼ turn and from there gradually further until it was fully closed after 8 ½ minutes from the start of heating.

Placing lid with basket on autoclave

When all thermocouples inside the autoclave registered more then 132°C and the average of the 4 topmost ones was more than 132.5°C, autoclaving started and the time on the chronometer and the temperature recorder were registered. Autoclaving was continued for

20 minutes. At this point, the time was noted. The condense needle valve was opened one turn, the lower steam inlet valve was closed and the upper steam inlet valve opened for 5 seconds, to expel any water from the condense pipe, and then closed. The heating wire control was switched off and the steam valve of the steam generator closed to ½ turn. When all thermocouples within the bone chips and the one at the chamber base registered less than 105°C, the needle valve was completely opened. When the same thermocouples registered approximately 100°C and less than 100°C respectively, cooling down was completed and the time was recorded.



Autoclaving extraction setup.

	Chronometer (min:sec)	**Temperature recorder** (hrs:min:sec)	**Duration of process step** (min:sec)
Preheating started	0:00	13:31:18	17:32
Preheating finished/Autoclaving started	17:32	13:48:18	20:11
Autoclaving finished/Cooling started	37:43	14:08:38	
Cooling finished	44:30	14:15:38	6:47

Reference: Note book 20-1-2000 to 21-1-2000 page 41 to 97. Date 25 to 30-11-99.

Temperature data autoclaving and extraction

T1 Top autoclave above bone chips
T2 Top of basket in between bone chips
T3 Middle of basket inside bone chip
T4 Bottom of basket in between bone chips
T5 Bottom of autoclave steam inlet temperature

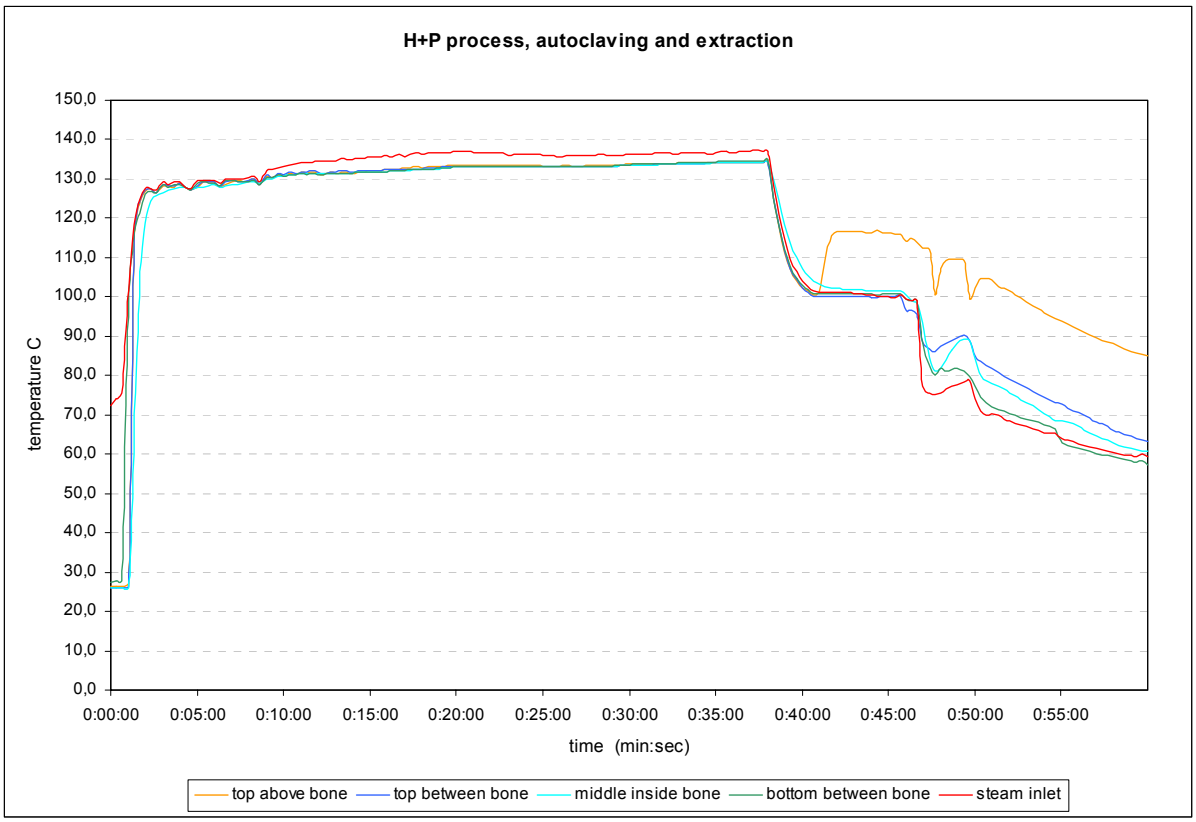
Time (h:m:s)	**Time** (m:s)	**T1** top autoclave above bone ©	**T2** top basket between bone ©	**T3** middle basket inside bone ©	**T4** bottom basket between bone ©	**T5** steam inlet temperature ©	observations
13:30:38	0:00:00	26,2	26,0	25,9	27,3	72,5	
13:30:58	0:00:20	26,4	26,1	26,0	27,7	74,2	
13:31:18	0:00:40	26,3	26,1	25,9	27,9	75,7	steam on
13:31:38	0:01:00	27,0	26,5	26,1	98,0	101,4	
13:31:58	0:01:20	115,7	116,4	65,3	116,3	118,4	
13:32:18	0:01:40	124,1	124,2	103,6	121,2	124,6	
13:32:38	0:02:00	126,9	127,1	118,8	126,2	127,6	
13:32:58	0:02:20	127,5	127,4	124,2	126,8	127,6	
13:33:18	0:02:40	126,4	126,3	125,8	126,5	127,2	
13:33:38	0:03:00	128,2	128,5	126,5	128,4	129,2	
13:33:58	0:03:20	128,3	128,2	127,3	128,0	128,5	
13:34:18	0:03:40	127,7	128,1	127,5	128,5	129,4	
13:34:38	0:04:00	129,0	129,0	127,8	128,6	129,2	
13:34:58	0:04:20	127,8	127,8	127,8	127,7	128,0	
13:35:18	0:04:40	127,3	127,2	127,6	127,2	127,6	
13:35:38	0:05:00	127,7	128,3	127,7	128,9	129,5	
13:35:58	0:05:20	129,1	129,3	127,9	129,3	129,6	
13:36:18	0:05:40	129,2	129,1	128,2	128,8	129,6	
13:36:38	0:06:00	129,3	129,2	128,5	129,0	129,8	
13:36:58	0:06:20	127,8	128,1	128,0	128,2	128,9	
13:37:18	0:06:40	128,7	129,1	128,3	129,6	130,0	
13:37:38	0:07:00	129,3	129,7	128,4	129,8	130,1	
13:37:58	0:07:20	129,5	129,4	128,7	129,1	129,9	
13:38:18	0:07:40	129,2	129,2	128,9	129,1	129,9	
13:38:38	0:08:00	129,6	129,5	129,3	129,3	130,2	
13:38:58	0:08:20	129,9	129,9	129,4	129,5	130,7	
13:39:18	0:08:40	128,5	128,6	129,0	128,7	129,3	
13:39:38	0:09:00	130,8	130,9	129,9	130,5	132,1	
13:39:58	0:09:20	130,4	130,5	130,1	130,5	132,3	
13:40:18	0:09:40	131,2	131,3	130,6	130,9	132,9	
13:40:38	0:10:00	130,8	131,0	130,8	130,8	133,2	
13:40:58	0:10:20	131,4	131,6	131,1	131,2	133,6	

13:41:18	0:10:40	131,0	131,3	131,1	131,1	133,8	
13:41:38	0:11:00	131,6	131,9	131,4	131,4	134,1	
13:41:58	0:11:20	131,3	131,6	131,4	131,2	134,2	
13:42:18	0:11:40	131,6	132,0	131,4	131,4	134,2	
13:42:38	0:12:00	131,4	131,8	131,4	131,2	134,4	
13:42:58	0:12:20	131,2	131,5	131,4	131,1	134,5	
13:43:18	0:12:40	131,6	131,9	131,4	131,4	134,7	
13:43:38	0:13:00	131,4	131,8	131,4	131,3	134,5	
13:43:58	0:13:20	131,6	132,0	131,5	131,5	135,2	
13:44:18	0:13:40	131,7	132,0	131,4	131,4	134,9	
13:44:38	0:14:00	131,5	131,8	131,5	131,4	135,0	
13:44:58	0:14:20	131,8	132,1	131,6	131,6	135,4	
13:45:18	0:14:40	131,8	132,1	131,6	131,6	135,3	
13:45:38	0:15:00	132,0	132,1	131,7	131,8	135,7	
13:45:58	0:15:20	132,1	132,1	131,7	131,7	135,5	
13:46:18	0:15:40	132,2	132,0	131,8	131,8	135,7	
13:46:38	0:16:00	132,5	132,4	131,8	131,9	135,8	
13:46:58	0:16:20	132,4	132,4	132,0	132,0	135,7	
13:47:18	0:16:40	132,5	132,4	132,1	132,1	136,2	
13:47:38	0:17:00	132,7	132,4	132,2	132,1	135,6	
13:47:58	0:17:20	132,8	132,5	132,2	132,3	136,4	
13:48:18	0:17:40	133,0	132,6	132,3	132,4	136,5	begin autoclaving
13:48:38	0:18:00	132,9	132,6	132,3	132,4	136,4	
13:48:58	0:18:20	133,1	132,7	132,4	132,4	136,6	
13:49:18	0:18:40	133,1	132,8	132,4	132,6	136,8	
13:49:38	0:19:00	133,2	132,9	132,6	132,7	136,7	
13:49:58	0:19:20	133,3	133,0	132,7	132,8	136,8	
13:50:18	0:19:40	133,4	133,1	132,8	132,9	136,7	
13:50:38	0:20:00	133,6	133,2	133,0	133,1	137,0	
13:50:58	0:20:20	133,6	133,3	133,1	133,2	136,9	
13:51:18	0:20:40	133,6	133,3	133,1	133,2	136,9	
13:51:38	0:21:00	133,6	133,3	133,1	133,2	136,7	
13:51:58	0:21:20	133,5	133,3	133,1	133,2	136,6	
13:52:18	0:21:40	133,6	133,3	133,1	133,2	136,8	
13:52:38	0:22:00	133,6	133,3	133,1	133,3	136,5	
13:52:58	0:22:20	133,5	133,3	133,1	133,3	136,5	
13:53:18	0:22:40	133,5	133,2	133,1	133,2	136,5	
13:53:38	0:23:00	133,5	133,3	133,2	133,3	135,9	
13:53:58	0:23:20	133,5	133,2	133,2	133,3	136,3	
13:54:18	0:23:40	133,5	133,2	133,2	133,3	136,4	
13:54:38	0:24:00	133,5	133,2	133,2	133,3	136,2	
13:54:58	0:24:20	133,5	133,1	133,1	133,2	136,4	
13:55:18	0:24:40	133,4	133,1	133,1	133,1	136,2	
13:55:38	0:25:00	133,3	133,1	133,1	133,1	136,0	
13:55:58	0:25:20	133,2	133,0	133,0	133,1	136,1	
13:56:18	0:25:40	133,2	133,0	133,0	133,1	135,9	

13:56:38	0:26:00	133,2	133,1	133,0	133,1	135,6	
13:56:58	0:26:20	133,4	133,1	133,1	133,2	135,8	
13:57:18	0:26:40	133,3	133,1	133,1	133,2	135,9	
13:57:38	0:27:00	133,3	133,1	133,1	133,2	136,1	
13:57:58	0:27:20	133,3	133,1	133,1	133,3	135,9	
13:58:18	0:27:40	133,4	133,1	133,1	133,3	136,1	
13:58:38	0:28:00	133,4	133,2	133,1	133,3	135,8	
13:58:58	0:28:20	133,4	133,1	133,1	133,2	136,2	
13:59:18	0:28:40	133,4	133,1	133,1	133,3	136,1	
13:59:38	0:29:00	133,4	133,2	133,1	133,3	136,1	
13:59:58	0:29:20	133,5	133,2	133,2	133,4	136,1	
14:00:18	0:29:40	133,6	133,4	133,4	133,6	135,8	
14:00:38	0:30:00	133,7	133,5	133,5	133,6	136,3	
14:00:58	0:30:20	133,7	133,5	133,5	133,7	136,4	
14:01:18	0:30:40	133,8	133,5	133,5	133,7	136,3	
14:01:38	0:31:00	133,8	133,6	133,5	133,8	136,2	
14:01:58	0:31:20	133,8	133,6	133,6	133,8	136,2	
14:02:18	0:31:40	133,9	133,7	133,6	133,8	136,5	
14:02:38	0:32:00	133,9	133,8	133,7	133,9	136,5	
14:02:58	0:32:20	133,9	133,8	133,8	133,9	136,5	
14:03:18	0:32:40	134,0	133,8	133,8	134,0	136,4	
14:03:38	0:33:00	134,1	133,9	133,8	134,1	136,8	
14:03:58	0:33:20	134,1	133,9	133,9	134,1	136,5	
14:04:18	0:33:40	134,1	133,9	133,9	134,1	136,6	
14:04:38	0:34:00	134,1	134,0	134,0	134,2	136,6	
14:04:58	0:34:20	134,1	134,0	134,0	134,2	136,7	
14:05:18	0:34:40	134,1	134,1	134,0	134,3	136,2	
14:05:38	0:35:00	134,1	134,1	134,1	134,3	136,6	
14:05:58	0:35:20	134,1	134,1	134,1	134,4	136,9	
14:06:18	0:35:40	134,2	134,1	134,1	134,5	136,9	
14:06:38	0:36:00	134,3	134,1	134,1	134,5	136,7	
14:06:58	0:36:20	134,2	134,1	134,1	134,5	137,0	
14:07:18	0:36:40	134,3	134,2	134,2	134,5	137,1	
14:07:38	0:37:00	134,3	134,2	134,3	134,6	136,9	
14:07:58	0:37:20	134,3	134,3	134,3	134,6	137,2	
14:08:18	0:37:40	134,3	134,3	134,3	134,7	137,0	
14:08:38	0:38:00	134,4	134,3	134,3	134,8	137,0	autoclaving stopped
14:08:58	0:38:20	125,0	125,2	130,0	125,2	128,5	
14:09:18	0:38:40	117,7	118,0	124,2	118,2	121,0	
14:09:38	0:39:00	111,5	111,8	118,5	112,2	114,8	
14:09:58	0:39:20	107,2	107,3	113,7	107,9	109,6	
14:10:18	0:39:40	104,1	104,2	110,0	104,8	106,3	
14:10:38	0:40:00	102,3	102,2	107,3	103,0	104,0	
14:10:58	0:40:20	101,4	101,1	105,4	101,8	102,7	
14:11:18	0:40:40	100,6	100,2	104,0	100,9	101,6	
14:11:38	0:41:00	101,1	100,1	103,2	100,8	101,3	

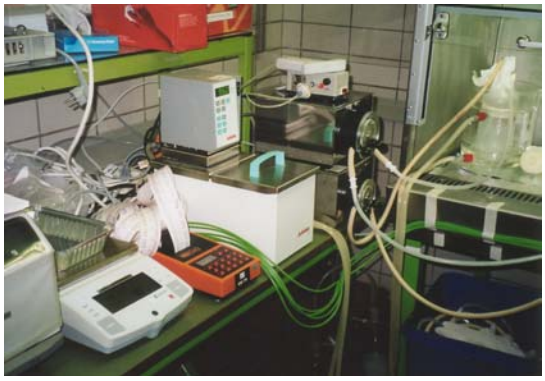
14:11:58	0:41:20	108,9	100,1	102,7	100,8	101,1	
14:12:18	0:41:40	115,4	100,1	102,3	100,8	101,3	
14:12:38	0:42:00	116,7	100,0	102,1	100,8	101,2	
14:12:58	0:42:20	116,8	100,0	102,0	100,8	101,1	
14:13:18	0:42:40	116,8	100,0	101,8	100,8	101,0	
14:13:38	0:43:00	116,6	100,0	101,7	100,7	100,8	
14:13:58	0:43:20	116,5	100,0	101,7	100,7	100,7	
14:14:18	0:43:40	116,4	100,0	101,6	100,7	100,5	
14:14:38	0:44:00	116,4	99,9	101,5	100,7	100,4	
14:14:58	0:44:20	116,9	99,9	101,4	100,6	100,2	
14:15:18	0:44:40	116,3	100,1	101,6	100,8	100,2	
14:15:38	0:45:00	116,2	100,1	101,5	100,8	100,1	begin extraction
14:15:58	0:45:20	116,0	100,1	101,4	100,8	99,9	
14:16:18	0:45:40	115,9	100,1	101,4	100,8	100,6	
14:16:38	0:46:00	114,3	96,5	100,3	99,3	99,3	
14:16:58	0:46:20	114,7	96,7	99,1	99,1	99,1	
14:17:18	0:46:40	113,8	95,7	98,3	99,0	99,2	
14:17:38	0:47:00	112,4	88,6	93,6	88,7	77,3	
14:17:58	0:47:20	112,1	86,6	84,5	82,6	75,4	
14:18:18	0:47:40	100,5	86,1	81,1	80,2	75,1	
14:18:38	0:48:00	107,1	87,4	81,8	81,8	75,7	
14:18:58	0:48:20	109,3	88,3	84,1	81,3	76,5	
14:19:18	0:48:40	109,6	88,9	86,4	81,6	77,4	
14:19:38	0:49:00	109,7	89,5	88,1	81,8	77,6	
14:19:58	0:49:20	109,3	90,3	89,3	81,3	78,3	
14:20:18	0:49:40	99,6	88,8	88,9	79,9	78,6	
14:20:38	0:50:00	102,2	84,9	84,2	77,2	74,2	
14:20:58	0:50:20	104,8	83,5	80,2	74,5	70,9	
14:21:18	0:50:40	104,7	82,7	78,7	72,9	70,0	
14:21:38	0:51:00	104,3	81,9	78,1	72,0	70,4	
14:21:58	0:51:20	102,7	80,9	77,4	71,4	69,9	
14:22:18	0:51:40	102,2	79,9	76,5	70,8	69,0	
14:22:38	0:52:00	101,4	79,1	75,7	70,2	68,4	
14:22:58	0:52:20	100,6	78,3	74,8	69,8	67,9	
14:23:18	0:52:40	99,6	77,5	73,9	69,3	67,3	
14:23:38	0:53:00	98,7	76,8	73,2	69,0	67,1	
14:23:58	0:53:20	97,8	76,0	72,3	68,5	66,3	
14:24:18	0:53:40	96,8	75,3	71,4	68,0	65,9	
14:24:38	0:54:00	95,9	74,5	70,3	67,5	65,3	
14:24:58	0:54:20	95,2	73,6	69,4	67,0	65,2	
14:25:18	0:54:40	94,4	72,9	68,6	66,5	65,2	
14:25:38	0:55:00	93,8	72,6	68,4	62,8	63,8	
14:25:58	0:55:20	93,0	71,7	68,2	62,3	63,5	
14:26:18	0:55:40	92,3	71,1	67,7	61,9	63,1	
14:26:38	0:56:00	91,8	70,5	67,0	61,4	62,7	
14:26:58	0:56:20	91,0	69,8	66,2	61,0	62,2	

14:27:18	0:56:40	90,2	69,1	65,5	60,6	61,9	
14:27:38	0:57:00	89,6	68,3	64,7	60,2	61,5	
14:27:58	0:57:20	89,0	67,8	64,1	59,8	61,2	
14:28:18	0:57:40	88,6	67,2	63,6	59,6	60,7	
14:28:38	0:58:00	88,0	66,2	62,9	59,2	60,3	
14:28:58	0:58:20	87,4	65,6	62,2	58,9	60,0	
14:29:18	0:58:40	86,9	65,0	61,7	58,5	59,8	
14:29:38	0:59:00	86,0	64,7	61,3	58,2	59,6	extraction stopped
14:29:58	0:59:20	85,8	64,1	61,0	57,9	59,3	
14:30:18	0:59:40	85,2	63,7	60,6	58,2	60,2	
14:30:38	1:00:00	84,9	63,2	60,9	57,1	59,5	



Extraction.

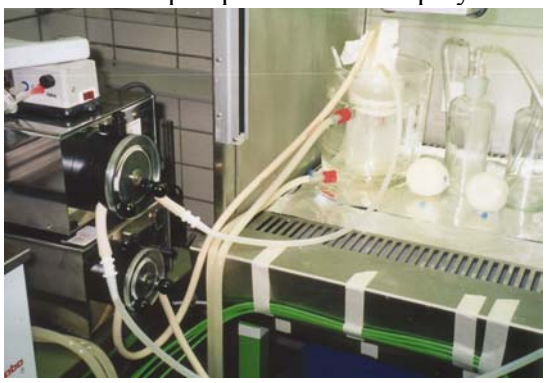
At the set-up of the equipment a double walled beaker was put in the biological safety cabinet. The beaker was connected to a pumping thermostat on the bench and hot water of 85°C was pumped through the double wall of the beaker. The beaker was partly filled with water to serve as water bath. A wide neck polythene bottle was weighed, filled with 200 ml of water and was placed in the double walled beaker, and allowed to heat up to 80°C for use in extraction. Two peristaltic pumps (Verder Pericor peristaltic pump 9F-240, with pumping hose Verderprene 8x1.6 cat no 58 84 81680/pumping hose Verderprene 9.6x3.2 cat no 58 84 83209) were set-up at the bench. Each of the pumping hoses was equipped with a siphon, and put into the polythene bottle.



Extraction equipment

After autoclaving the other ends of the pumping hoses were connected to the water inlet and outlet of the autoclave.

After the autoclave had cooled to less than 100°C, both pumping hoses were connected. The steam outlet valve was left open to serve as an air inlet/outlet. The water outlet valve of the autoclave was opened and all condensate in the autoclave was pumped out into the polythene bottle. The outlet valve was closed and the pump switched off. The water inlet valve of the autoclave was opened, the inlet pump switched on and the hot water in the polythene bottle was pumped into the autoclave. The pump was switched off and the water was left in the autoclave for 1 minute, then the outlet valve was opened, both pumps were switched on and the water was circulated through the autoclave for 10 minutes. The siphon of the inlet pump was then lifted out of the extracted liquid and the system drained into the bottle. After the hose was empty, the pump was switched off, the inlet valve closed and the siphon wrapped in a plastic bag. After 3 minutes the outlet pump was switched off and both the inlet and outlet valves of the autoclave were closed. The siphon of the outlet hose was taken from the extract and also wrapped in a plastic bag. The lid was screwed on the polythene bottle. The hoses were carefully removed from the autoclave and the ends wrapped in plastic bags. Capped pieces of tubing were placed on the autoclave water inlet and outlet to protect against possible contamination.



Extraction

The bottle was taken from the double walled beaker, dried with tissue, put in a plastic bag and weighed. The bottle was put back in the cabinet and the index of refraction of the extract was determined, after which the bottle was again put in a plastic bag again and stored frozen. Samples of the extract were inoculated into mice for bioassay titration.

Bottle with extract	279,9 g
Empty bottle with plastic bag	105,1 g
Extract	174,8 g
Concentration	4.6 Brix

Reference: Note book 20-1-2000 to 21-2000 page 46 to 65. Date 21-1-2000.

Extract

Continuation of test.

The experiment was continued by twice repeating autoclaving/extraction procedure. Because this part of the test is not relevant for the validation, this part will not be reported.

After testing was finished the equipment was broken down. All equipment which gad been used in the cabinet was put in plastic bags and disposed of. This included the autoclave which was left closed and was disposed of with the extracted bone chips in it. The cabinet was decontaminated with hypo chlorite solution as described before.

Calibration.

Balances

Balances used were: Scout SC 2020 and Ohaus Explorer no EOD120. Both had an internal calibration procedure.
Apart from the calibration procedure the scales were tested before use with a 200 g weight. No differences outside the precision of the scales were observed.

Thermocouples.

Two thermocouples were calibrated against a calibrated PT-100. The PT 100 had, at 121°C, an upward deviation of 0.20°C, thus 121°C on the PT-100 is in reality 120.80°C, and at 137°C an upward deviation of 0.21°C, thus 137°C on the PT-100 is in reality 136.79°C. Calibration involved placing both thermocouples and the PT-100 in the same stabilised calibration oil bath. When the PT-100 indicated that the temperature of the bath was stable, it was left for further 30 minutes, after which the thermocouples were calibrated. Calibration was done at approximately 121°C and 137°C. The thermocouples were connected to the first two channels of a Kane May KM1242 temperature recorder.

PT-100 (°C)	KM 1242 Channel 1 (°C)	KM 1242 Channel 2 (°C)
121.56	121.6	121.4
121.58	121.5	121.4
121.57	121.6	121.5
121.56	121.4	121.3
121.56	121.5	121.4
121.566	121.52	121.4

PT-100 (°C)	KM 1242 Channel 1 (°C)	KM 1242 Channel 2 (°C)
137.58	137.8	137.6
137.61	137.9	137.7
137.59	137.7	137.6
137.58	137.7	137.6
137.58	137.9	137.8
137.58	137.8	137.7
137.58	137.9	137.8
137.586	137.94	137.69

Results: 121°C: Channel 1: 0.15°C too high
Channel 2: 0.03°C too high

137°C: Channel 1: 0.56°C too high
Channel 2: 0.31°C too high

The deviation of the other 3 thermocouples was determined by comparing the readings of these on channel 3, 4 and 5 of the Kane May KM 1242 temperature recorder with those on channel 1 and 2. All thermocouples were in the empty autoclave, which was heated with saturated steam.

Ch1	Ch2	Ch3	Ch4	Ch5
123.2	123.0	123.1	123.1	122.9
124.2	124.2	124.3	123.8	124.1
123.2	123.1	123.2	123.2	122.8
123.8	123.6	123.7	123.6	123.2
119.8	120.5	120.9	120.3	120.6
123.3	123.2	123.3	123.5	123.2
123.7	123.6	123.8	123.9	123.7
124.0	123.9	124.0	124.2	124.0
124.0	123.9	124.0	124.2	123.8
123.3	123.2	123.3	123.6	123.1
122.7	122.6	122.7	123.1	122.6
123.20	123.16	123.30	123.32	123.09

Ch1	Ch2	Ch3	Ch4	Ch5
126.2	126.0	126.2	126.1	126.1
125.8	125.8	126.0	125.7	125.6
125.4	125.3	125.5	125.5	125.7

Ch1	Ch2	Ch3	Ch4	Ch5
116.8	116.7	116.8	116.7	116.5
115.8	115.7	116.0	116.0	116.1
116.0	116.1	116.4	116.4	116.7
115.8	116.0	116.3	116.3	116.7
116.6	116.7	116.9	116.9	117.1
117.0	117.1	117.2	117.2	117.3
116.33	116.38	116.60	116.58	117.73

Ch1	Ch2	Ch3	Ch4	Ch5
125.9	125.8	125.9	125.7	125.8
125.0	124.9	125.1	125.1	125.2
125.5	125.4	125.5	125.6	125.7
125.7	125.5	125.8	125.8	125.9
125.9	125.6	125.9	125.9	125.8
124.6	124.4	124.6	124.7	125.0
125.4	125.2	125.5	125.5	125.7
125.8	125.7	125.9	125.9	125.9

124.3	124.6	124.9	125.3	125.4
125.43	125.59	125.65	125.65	125.70

Ch1	Ch2	Ch3	Ch4	Ch5
125.3	125.2	125.4	125.5	125.5
124.9	125.0	125.3	124.8	125.2
125.9	125.9	126.1	125.8	125.9
125.3	125.4	125.7	125.5	125.4
124.6	124.4	124.6	124.9	124.9
124.8	124.6	124.9	125.0	125.0
125.1	124.9	125.2	125.2	125.2
125.13	125.06	125.31	125.24	125.30

Ch1	Ch2	Ch3	Ch4	Ch5
134.6	134.5	134.5	134.7	134.8
134.8	134.8	135.0	135.0	134.9
135.1	135.0	135.5	135.2	135.2
135.2	135.1	135.2	135.3	135.4
135.3	135.2	135.3	135.4	135.3
135.2	135.0	135.3	135.3	135.2
134.9	134.8	135.2	135.3	135.2
135.0	135.0	135.3	135.2	135.2
135.2	135.1	135.3	135.3	135.3
135.2	135.1	135.3	135.4	135.3
135.2	135.1	135.4	135.4	135.3
135.06	134.97	135.21	135.23	135.19

Ch1	Ch2	Ch3	Ch4	Ch5
133.3	133.2	133.5	133.4	133.2
133.5	133.5	133.6	133.5	133.5
133.4	133.4	133.5	133.5	133.5
133.2	133.1	133.4	133.4	133.2
133.2	133.1	133.4	133.4	132.9
133.3	133.1	133.4	133.4	133.2
133.1	132.9	133.3	133.3	133.0
133.0	133.0	133.2	133.2	133.1
133.1	133.1	133.4	133.4	133.2
133.1	133.1	133.3	133.3	133.1
133.1	133.0	133.3	133.3	133.2
133.21	133.14	133.39	133.37	133.19

125.48	125.31	125.53	125.53	125.63

Ch1	Ch2	Ch3	Ch4	Ch5
125.0	124.9	125.2	125.2	125.1
125.2	124.9	125.1	125.2	125.1
124.9	124.8	124.9	124.9	124.9
124.7	124.6	124.7	124.7	124.7
124.6	124.4	124.6	124.6	124.6
124.3	124.3	124.3	124.3	124.4
124.3	124.2	124.4	124.4	124.3
124.4	124.2	124.5	124.5	124.5
125.1	124.9	125.2	125.2	125.2

125.5	125.3	125.5	125.5	125.5
125.5	125.3	125.5	125.5	125.5
124.6	124.4	124.6	124.6	124.6
124.84	124.68	124.88	124.88	124.87

Ch1	Ch2	Ch3	Ch4	Ch5
133.1	133.1	133.1	133.1	133.0
133.2	133.2	133.3	133.4	133.2
133.5	133.5	133.6	133.6	133.5
133.5	133.4	133.5	133.6	133.4
133.1	133.0	133.1	133.4	133.4
132.2	132.1	132.3	132.3	132.2
132.5	132.4	132.6	132.7	132.5
132.9	132.8	133.1	133.1	132.9
133.0	132.9	133.1	133.1	133.0
133.3	133.2	133.5	133.4	133.3
133.03	132.96	133.12	133.17	133.04

Ch1	Ch2	Ch3	Ch4	Ch5
133.1	133.0	133.2	133.2	133.1
133.1	133.1	133.3	133.3	133.1
133.1	132.9	133.2	133.2	133.1
132.1	132.1	132.5	132.5	132.1
132.85	132.78	133.05	133.05	132.85

The deviations of the thermocouples on channels 3, 4 and 5 were calculated from the average of the readings of those on channel 1 and 2. It is assumed that the deviation of the thermocouples on channel 1 and 2 is linear between 121°C and 137°C.

The temperatures and the deviations in the table are in °C, "+" means that the temperature reading is to high and "–" that it is to low.

Table of calculated deviations of the thermocouples.

Ch 1		Ch 2		Ch 3		Ch 4		Ch 5	
Av.Temp.	Dev.	Av.Temp.	Dev.	Av.Temp.	Dev.	Av.Temp.	Dev.	Av.Temp.	Dev.
116.33	+0.03	116.38	-0.05	116.60	+0.23	116.58	+0.21	117.73	+1.36
123.20	+0.21	123.16	+0.07	123.30	+0.26	123.32	+0.28	123.09	+0.05
124.84	+0.25	124.68	+0.09	124.88	+0.29	124.88	+0.29	124.87	+0.28
125.13	+0.26	125.06	+0.10	125.31	+0.40	125.24	+0.33	125.30	+0.39
125.48	+0.26	125.31	+0.11	125.53	+0.33	125.53	+0.33	125.63	+0.43
125.43	+0.26	125.59	+0.07	125.65	+0.31	125.65	+0.31	125.70	+0.36
132.85	+0.45	132.78	+0.24	133.05	+0.58	133.05	+0.58	132.85	+0.38
133.03	+0.46	132.96	+0.24	133.12	+0.47	133.17	+0.52	133.04	+0.39
133.21	+0.47	133.14	+0.24	133.39	+0.57	133.37	+0.55	133.19	+0.37
135.06	+0.51	134.97	+0.24	135.21	+0.57	135.23	+0.59	135.19	+0.55

Near the autoclaving temperature the thermocouple readings are between 0.2 and 0.6°C to high, meaning that the real autoclaving temperature is fractionally lower than indicated in the table and the graph.

Reference: Note book 14-12-99 to 17-1-2000 page 30 to 44. Date 11-1-2000.

General observation.

The water used in all experiments was laboratory quality demineralised water, unless otherwise indicated. The cooling water for the condenser was tap water.

Appendix 6: CORRECTION FACTORS.

Because samples were taken at most of the different manufacturing steps, and because in some of the steps material was lost in the equipment, the obtained amount of gelatine was reduced.
This is compensated for by a calculated correction factor.

Amount of starting material.

Total amount of 301V infected mouse brain on the bone starting material	3.91 g
	=====
Crushed bone	750 g
Backbone	254 g
301V infective mouse brain	3.91 g
Total amount of spiked bone starting material	1008 g
	=======

Correction factor for extracted gelatine

Amount of degreased wet bone and soft tissue	550.1 g
Sample taken	15.5 g
Remaining degreased wet bone and soft tissue	534.6 g
Amount of sorted dried bone chips	219.9 g
Sample taken of sorted dried bone before weighting	13.3 g
Remaining amount of sorted dried bone	206.6 g

Calculated correction factor = (550.1/534.6) x (219.9/206.6) = 1.095

Correction factor for extracted gelatine 1.1

Obtained extract 175 ml

Bone at atmospheric pressure and approximately 20°C absorbs approximately 25 % of water.

At earlier Heat and Pressure experiments the autoclaved extracted bone material absorbed up to 58.6% of water.

Summary of important data for the calculation of the clearance factors

A. Total amount of 301V infected mouse brain on the bone starting material 3.91 g

B. Extracted gelatine

Total volume of extracted gelatine	**175 ml**

Gelatine extract absorbed by autoclaved extracted bone chips: 52 to 121 ml.

Maximum volume of extracted gelatine	**296 ml**
Correction factor for extracted gelatine	**1.1**

Appendix 7: PREVENTION OF CROSS-CONTAMINATION.

The whole series of experiments was done in a special laboratory room, which is expected to be free off any background contamination. During the entire series of experiments special care was given to prevent background contamination. Handling of infectivity was exclusively done in a biological safety cabinet, which was decontaminated on a regular basis. Experiments were exclusively done in this cabinet or in completely closed and well sealed equipment, which was also covered. Transport of infective materials was exclusively done in closed and wrapped or covered containers. Gloves used in manipulations were regularly refreshed and disposed off during manipulations and were always immediately disposed off after manipulations.

Special attention was given to the prevention of cross-contamination.

Cross-contamination could occur between the different steps of one experiment and within one step of an experiment.

Only new and clean equipment was used for each experiment. This equipment was used exclusively for one experiment and then disposed of. Some pieces of large or expensive equipment were used in more than one experiment, such as the biological safety cabinet, an electric stirrer and a balance. This equipment was protected from any direct contact with any potentially infectious material and were possible, decontaminated after every use with sodium hypo chlorite solution (20,000 ppm for 1 hour min.).

Cross-contamination between steps of the experimental process was prevented by carrying out one step at any point in time. This was made easier by the consecutive nature of each step within the process. Between each step, the safety cabinet was decontaminated with sodium hypo chlorite as before. New equipment was used for each step and disposed of immediately after use.

Cross-contamination within one process step was prevented by the use of new equipment for each manipulation and disposing of all equipment immediately after use. The risk of cross contamination in this way was small as in most cases starting material and end product did not co-exist. In the cases where both were present, these fractions were kept physically separate such that no cross-contamination could occur.

During all manipulations, very good care was taken that no equipment was contaminated by spilling, contaminated equipment and tools or contaminated gloves. Gloves were changed regularly and always on suspicion or any doubt of contamination. Tools were disposed of or cleaned with sodium hypo chlorite when there had been any risk of contamination.

Appendix 8: BIOASSAY PROCEDURES AT ID-Lelystad.

All samples for bioassay were produced in a Category 3 containment laboratory within the Institute of Animal health, Edinburgh or at ID-Lelystad. These samples were then taken to the experimental animal unit also within ID-Lelystad.
All samples were administered by the intracerebral inoculation of 20 µl into weanling mice of the VM strain. To measure the infectivity of each sample, these were serially diluted and injected ("titration").

Samples for bioassay in this study were:

Untreated 301V- infected mouse brain (pool 2). Undiluted macerated tissue (10^0 log dilution) was serially diluted with saline to produce a series of log 10 dilutions for assessing initial infectivity titre. Each mouse was injected with 20µl i.c. of the appropriate dilution (see table).

Heat and Pressure process - treated concentrated gelatine. Undiluted heat-treated concentrated gelatine (10^0 log dilution) was serially diluted with saline to produce a series of log 10 dilutions. Each mouse was injected with 20µl i.c. and in addition 10µl intraperitoneally (i.p.) of the appropriate dilution (see table).

Prepared as follows:

	301V –infected mouse brain	H+P process - crude gelatine extract
10^0	-	18
10^{-1}	-	18
10^{-2}	-	18
10^{-3}	-	18
10^{-4}	-	18
10^{-5}	12	
10^{-6}	12	
10^{-7}	12	
10^{-8}	12	
10^{-9}	12	

Bioassay procedure

All tissues prepared for bioassay are done so in a biological safety cabinet. This protects the operator from potentially infectious materials and avoids airborne contamination of the sample. To prevent cross-contamination between samples, only one tissue is processed at one time with sterile unused equipment discarded after use, and the cabinet decontaminated with sodium hypochlorite solution between tissues of different TSE agent strain. General equipment, not in direct contact with any tissue (e.g. syringe rests) are not disposed of, but are covered for each use in aluminium foil, and autoclaved after use.

Titration method
The biological safety cabinet was switched on and the working surface covered with an absorbent sheet. Syringe rests (one for test samples, one for diluent i.e. 0.85% saline) and test tube racks covered with foil were placed in the cabinet. One sterile test tube and one sterile 1 ml syringe per dilution group were put into the cabinet and labelled with the appropriate dilution group number. One sterile syringe was placed in the cabinet for use with sterile saline only. A 10-fold dilution series was then made up for each sample as follows.

Untreated 301V- infected mouse brain (pool 2 for titration).
Inoculation date 7/3/00.

A 10% brain homogenate was prepared from the same undiluted macerated 301V-infected brain tissue as prepared by the IAH-E, used to spike the model gelatine process. The homogenate was prepared with an Ultra-Turrax T25 homogeniser. 500 mg of macerate was weighed using a electronic balance, then transferred to a new, sterile, glass, tissue homogenising tube. Ten ml of sterile 0.85% saline was then added to the tube and the tissue further homogenised. The homogenate produced was then transferred to a glass test tube labelled as 10^{-2}. Using the syringe labelled 10^{-2}, 0.1 ml of homogenate was removed from the tube labelled 10^{-2} and deposited in the tube labelled 10^{-3}. Using the syringe labelled for saline, 0.9 ml of 0.85% saline was added to the tube labelled 10^{-3}. The resultant solution in the 10^{-3} tube was mixed by drawing up and down in the syringe labelled 10^{-3}. Using the same syringe, 0.1 ml of the 10^{-2} solution was removed to the tube labelled 10^{-4}. This process was continued up to and including the production of a 10^{-9} dilution.
Only the dilutions 10^{-5} to 10^{-9} were subsequently inoculated in groups of 12 mice each. For results see table (Annex 9).

Crude gelatine extract. Inoculation date 28/2/00 (<u>Pilot group of 10 VM mice</u>).

The undiluted gelatine extract was warmed in a water bath at 50°C to liquefy it. The warmed undiluted (10^{0} group) gelatine extract was inoculated into 2x5 VM mice, 5 by i.c and i.p. route, and 5 by i.c. route only. This pilot served to assess the tolerance of the mice for the gelatine extract. Initially complications occurred and it was decided to heat the inoculum for 10 minutes at 100^{0}C. As this bacteriological sterile material was tolerated without further complications, the main experiment could go ahead.

Heat & Pressure process - treated concentrated gelatine.
Inoculation date: 23/3/00.

The spiked undiluted concentrated gelatine extract was warmed in a water bath at 50°C to liquify it. Dilutions were then made of this solution as for the 301V-infected mouse brain, up to and including 10^{-4}. All dilutions (10^{-0} to 10^{-4}) were subsequently inoculated in groups of 18 VM mice each. For results see table (Annex 9).

Inoculation of experimental animals

Prior to inoculation, the experimental protocol for the bioassay was completed and the requisite number of cages of mice allocated to the relevant groups. Group details and a unique experimental number were written on to a data card attached to each cage.

The safety cabinet was then set up for inoculation of each group of mice. Test tubes containing the sample dilution series for inoculation were put into the cabinet, along with a labelled syringe for each tube placed in dilution order in needle stands.

Starting with the most dilute group, the syringe was filled with inoculum and placed on the syringe rest. One cage of mice was anaesthetised and then one mouse at a time, placed into the cabinet. Each mouse was then inoculated intracerebrally with 20µl of the inoculum (the HP treated group received in addition 10µl intraperitoneally). After inoculation, each mouse was removed to a clean cage to recover. The next cage of mice was then anaesthetised and the process repeated. On completion of a group, the tube of inoculum was sealed and the used needle sheathed and discarded before commencing inoculation of the next experimental group.

When all groups were completed, the cages were moved to an experimental animal room where they were routinely observed up to approximately 600 days for the onset of TSE-associated clinical symptoms.

Pathological examination: From all mice surviving the first ten days the brains are examined histologically, after euthanasia and after having shown clinical signs consistently or at the end of the observation period. These are immersion-fixed in 10% formol-saline and histological sections, prepared from five different coronal sections, are stained with haematoxylin and eosin and examined microscopically for the presence of spongiform lesions. All brains are also examined by immunohistochemistry (Van Keulen *et al*. 1995), consisting of PrP^{Sc} staining with a peptide-based antibody, R505, made in-house and directed against murine PrP^{Sc}. Positive results are based on both these techniques in which the presence of PrP^{Sc} is considered decisive.

References

Keulen, LJM van, Schreuder BEC, Meloen RH *et al*. Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie. Vet Pathol 1995; 32:299-308

Appendix 9: TABLES.

Titration data of brain pools (spiking material).

301V mouse brain pool 2 at ID-Lelystad
Titration GME 298.47047.16 (exper. group No. 38)
Final 607 days post injection.

-log dilution	Number of mice**	Mice positive on 301V	Average incubation period (days)
5	12	12	-*
6	12	12	-*
7	11	4	228
8	12	2	236
9	11	0	-

*As soon as the first animal from these groups had succumbed and was confirmed as suffering from TSE, the remaining mice from these two groups were removed for storage in -70^0 freezer, for further propagation of the strain of TSE.
Dates of removal: appr. 5 months post inoculation (150-160 days).
**These are actually the numbers of mice surviving the minimum incubation period and that were suitable for pathological diagnosis.

Titration data of HP-treated gelatine samples at ID-Lelystad.

Sample of extracted gelatine
GME 298.47047.16 (exper. group No. 37)
Final 623 days post injection.

-log dilution	Number of mice**	Mice positive on 301V	Average incubation period (days)
0	16	0	-
1	17	0	-
2	15	0	-
3	15	0	-
4	17	0	-

**These are actually the numbers of mice surviving the minimum incubation period and that were suitable for pathological diagnosis.