Validation of the clearance of TSE agent by the acid bone gelatine manufacturing process

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AUTHENTICATION

I, the undersigned, hereby declare that this work was performed under my direction using the principles of good laboratory practice, and that this report represents a true and accurate record of the results obtained.

Edinburgh, 22 August 2002

(from 1/1/00)
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SUMMARY REPORT.

The industrial manufacturing process for the production of acid bone gelatine 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, crude gelatine extract, and sterilised concentrated gelatine 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 $10^{7.8}$ ID_{50}/g, the crude extract $10^{2.8}$ ID_{50}/g, while no infectivity was detected in the sterilised gelatine (< $10^{1.3}$ ID_{50}/g). Calculated clearance factors were: $10^{2.6}$ ID_{50} for the process steps up to extraction, and >=$10^{4.8}$ ID_{50} for the complete process following concentration and sterilisation.

Introduction.

The Gelatin Manufacturers of Europe (GME) commissioned a validation study on the inactivation and removal effect of the gelatine manufacturing process on Transmissible Spongiform Encephalopathies (TSE). The background, aims, approach and 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.

This report contains the results of the inactivation and removal of TSE infectivity by the acid bone gelatine manufacturing process (usually called acid bone process), a description of the experiment done, and all other data associated with this study.

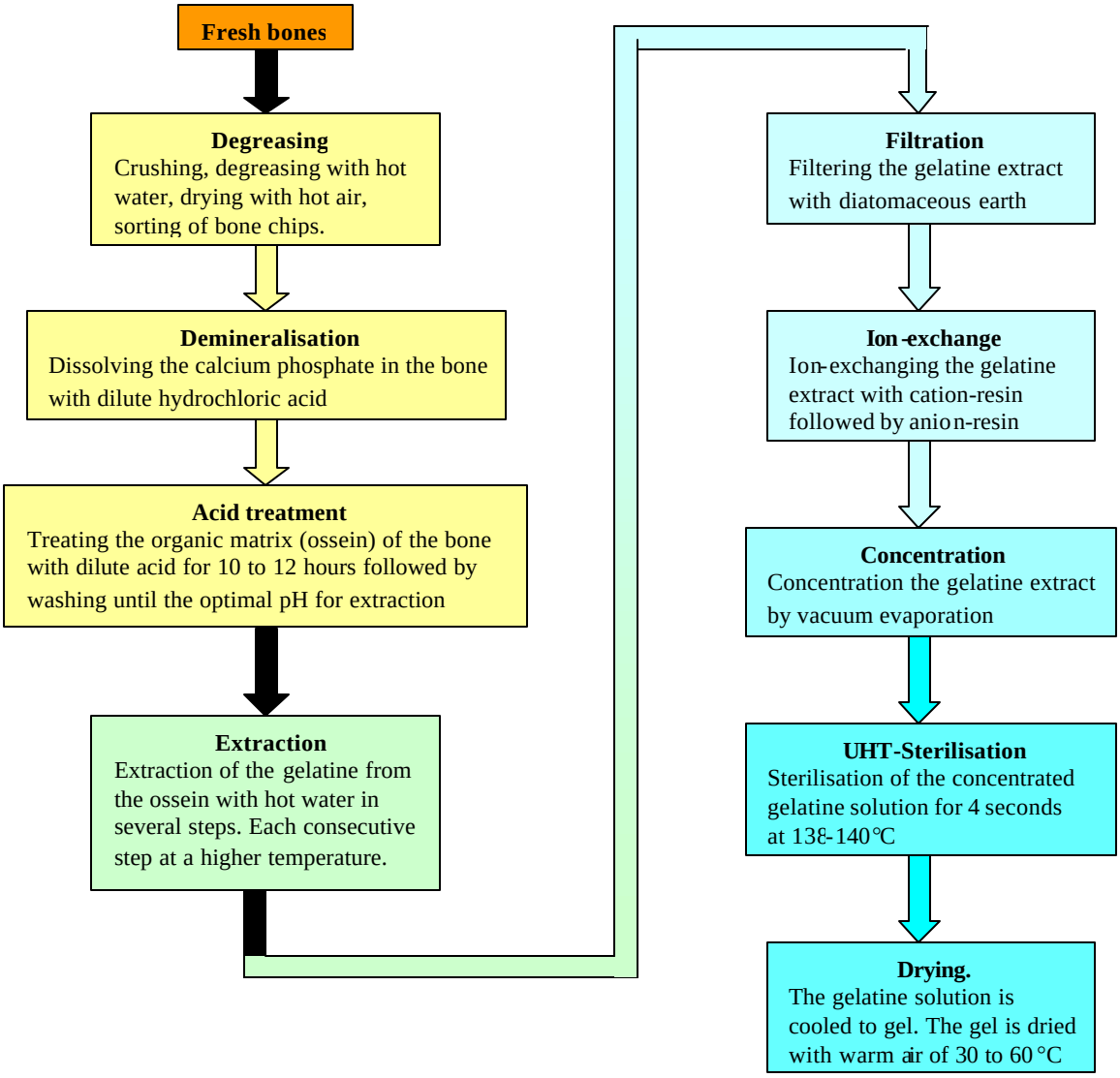
Industrial manufacturing process.

The acid bone manufacturing process is extensively described in Appendix 1 to this report. Here follows a brief description and a diagram.

The Acid bone 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.
- Demineralising with dilute hydrochloric acid to dissolve the calcium phosphate of the dried degreased bone chips, after which the remaining organic matrix, called ossein, is washed with water.

- Acid treatment. The washed ossein is kept for 10 to 12 hours in dilute acid and is then washed several times with water to remove excess acid and obtain the appropriate pH for extraction.
- Stepwise extraction of the gelatine with hot water of increasing temperature.
- Purification of the obtained extract, which is a dilute gelatine 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, after which the concentrated solution is UHT-sterilised and cooled to gel. This gelatine gel is finely divided and dried in a stream of warm air.



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

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

The downscaling followed these principles:

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

Parameters for which no mutual minimal conditions could be established were tested individually. Specifically, this was done for the different filter materials and ion-exchanger resins used by the producers.

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 prepared by IAH-E. To determine the amount of infectivity present, the brain material was titrated by intracerebral inoculation into mice.

Spiking (addition of infectious TSE brain) of the starting material.

The starting materials for the experiment consisted of 1.5 kg of fresh crushed bone sampled from the industrial process and 0,5 kg of intact calf backbone. The bone material was spiked with approximately 10 g of infectious brain tissue. To imitate inclusion of BSE-infected CNS tissue, the spinal cord within the calf backbone was injected with 5 g of homogenised brain. To imitate cross contamination from infected CNS tissue, the remaining 5g of brain was smeared over the crushed bones and stored chilled for two days prior to further processing. The backbone, with spinal cord intact, was then cut into pieces similar to the crushed bone. The backbone pieces with spinal cord were mixed with the crushed bone.

Experiments with the downscaled model processes of the acid bone process.

The complete and extensive description of the experiments are in Appendix 5 to this report.

Downscaled acid bone manufacturing process with 301V spike.

The raw materials consisted of 2001 g of fresh crushed bone and backbone, which contained 8.94 g 301V infected mouse brain. The entire process was executed from degreasing up to UHT-sterilisation. From the ossein produced by demineralisation a total of 1850 ml of crude gelatine was extracted, purified by filtration and ion-exchange then concentrated to 170 ml. Approximately 2 ml of this concentrated solution was UHT-sterilised. Samples of the crude gelatine extract and the UHT-sterilised sample were titrated by mouse bioassay to determine infectivity titres.

Determination of the infectivity titres of the samples.

To determine the infectivity titres in the spike material and the output samples, a series of tenfold dilutions were prepared of each sample and injected intracerebrally into groups of mice (20µl/mouse). The animals were scored according to standard protocols to detect clinical signs of neurological disease. The animals were culled either when they developed

unequivocal symptoms of neurological disease. Animals which did not develop clinical disease were culled 385 days (spike material) or approximately 600 days (output samples) 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		$10^{7.79}$	385 days
Acid bone process; extracted gelatine	Study 3 - Sample 3	$10^{2.82}$	658 days
Acid bone process; sterilised gelatine	Study 3 - Sample 3a	No detectable infectivity	604 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 sterilised gelatine 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 factors.

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
Acid bone process - extracted gel.	Study 1 - sample 1	8.94	$10^{7.8}$	1850	1.1	$10^{2.8}$
Acid bone process - sterilised gel.	Study 1 - sample 2	8.94	$10^{7.8}$	190	2.6	$<=10^{1.3}$

Table 3. Clearance factors.

Sample name	Total clearance factor ID_{50}
Acid bone process - extracted gelatine	$10^{2.6}$
Acid bone process - sterilised gelatine	$>=10^{4.8}$

Discussion

The titration values recorded show a significant reduction of 301V infectivity of at least $10^{4.8}$ by the downscaled alkaline manufacturing process. A titre reduction of $10^{2.6}$ was observed following degreasing, demineralisation and extraction. Filtration, ion-exchange and UHT-sterilisation added a further reduction of $>=10^{2.2}$. These data suggest that the majority of the inactivation/removal of infectivity occurs during degreasing where fat and soft tissue is removed from the bone. The final gelatine sample obtained following filtration and sterilisation failed to produce disease in any of the mice inoculated.

The results reported here provide a basis for a risk assessment of the industrial gelatine manufacturing process. In order to make a valid comparison, the model process must meets specific criteria for process validations.

Requirements for validation studies are:

- 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, 0.5% 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 in 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, 1% 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 acid bone gelatine manufacturing process removed/inactivated 301V infectivity to below the level of detection.
2. A 301V infectivity reduction of at least $10^{4.8}$ ID_{50} was obtained by the downscaled model of the acid bone gelatine manufacturing process.
3. The 301V infectivity was decreased substantially, with a factor $10^{2.6}$, by the pre-treatment steps of the process; degreasing and demineralisation followed by final washing. Most infectivity was removed/inactivated by these process steps.
4. Purification of the gelatine extract, filtration, ion-exchange and UHT-sterilisation, contributed to the removal/inactivation of infectivity. This contribution was $>=10^{2.2}$ ID_{50}.
5. The gelatine manufacturing process was successfully scaled down; gelatine was prepared from industrial starting material.
6. The study complied with the requirements on a validation study.

In conclusion, these data provide actual measurement of clearance factors for the acid bone gelatine manufacturing 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 acid bone processes

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 bone chips are treated for several days with dilute hydrochloric acid to remove the phosphate content. The obtained de-mineralised bone chips, the so called ossein, are washed with water and then treated for half a day up to 2 days with dilute acid, usually sulphuric acid, after which the ossein is washed several times to remove excess acid and to obtain the optimal pH for extraction. From the acid treated ossein, gelatine is extracted with warm water. The extract, which is a dilute gelatine solution, is purified by filtration and ion-exchange. The purified solution is then concentrated by vacuum evaporation of most of the water. The concentrated solution is sterilised and then cooled down to form a gel. The gel is extruded through a perforated metal sheet to form spaghetti like noodles, spread on a conveyor belt and dried in a steam of warm air. The dried gelatine is packed and stored until further use.

Description of the individual steps of the limed bone gelatine manufacturing process

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 %

The degreasing process 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 in some installations to 90 ^{o}C in other installations.
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 tact cou[led 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 composition of these bone chips is:

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

Demineralisation (also called acidulation)

In the demineralisation process the degreased dried bone chips are treated with hydrochloric acid to remove the inorganic component of the bone, the apatite, which consists mainly of calcium phosphate and partly of calcium carbonate. By reaction with the hydrochloric acid the insoluble calcium phosphate is converted into mono-calcium phosphate and calcium chloride,

which are both soluble. The reaction with hydrochloric acid converts calcium carbonate into soluble calcium chloride and carbon dioxide gas.

Reaction	$Ca_3(PO_4)_2 + 4\ HCl \rightarrow 2\ CaCl_2 + Ca(H_2PO_4)_2$			
Mol. weight	310.3	36.5	111	234.2
Equivalent weight	310.3	146		

Reaction	$CaCO_3 + 2\ HCl \rightarrow CaCl_2 + H_2O + CO_2$				
Mol. weight	100	36.5	111	18	44
Equivalent weight	100	73			

For the demineralisation of one ton of bone chips, containing 8% water and 63% hydroxy-apatite on dry matter one needs

0.90 x 0.63 x 1000 x (0.89 / 310.3 x 146 + 0.11 x 100 x 73) = 283 kg HCl

Which means that approximately 7,000 litres 4% hydrochloric acid is needed for complete conversion of one ton of bones.

For optimal demineralisation of the bone the concentration of hydrochloric acid must be more than 2% and less than 7%. At concentrations below 2%, hardly any reaction will occur, while at concentrations of 7% and more, protein will be broken down. To prevent breakdown of protein at concentrations less than 7%, the temperature of the acid must be kept below 18°C. Demineralisation is carried out batch wise in a counter current cascade process. In this cascade process up to six reactors are placed in series; see figure 1. Each reactor contains a batch of bone chips. Fresh 4% hydrochloric acid enters the first reactor. The acid runs through all reactors of the cascade and reacts with the apatite of the bone as described above. The spent acid which leaves the last reactor has an acid concentration of 0.5% or less. The bone chips within the different reactors of the cascade are each at a different stage of processing. The bone chips in the first reactor have been processed for the longest time and contain only a minimal amount of phosphate whereas those in the last reactor have been processed the shortest time and still contain most or all phosphate. When the acid leaving the first reactor is at the same concentration as the fresh acid for a period of 48 hours, the reactor is closed off from the cascade, the second reactor becoming the first. The ossein (demineralised bone) in the finished reactor is washed with water then removed from the reactor for further processing. The empty reactor is then filled with fresh bone chips and is connected to the end of the cascade. The reactor with the fresh bone is now the last reactor of the cascade. Effectively all batches have shifted one place forward, the first one is taken out and a new batch is put at the end.

The concentration of acid run into the last reactor with fresh bones is initially approximately 0.5%, and no or a negligible reaction will take place. This acid concentration gradually increases due to the progress of the process in the other reactors of the cascade and the acid will start reacting with the apatite of the bone. The reaction speed is determined by the acid concentration, which is the limiting factor, and increases due to the continuous increase of the concentration of the acid that runs into the reactor. The concentration of the effluent acid from the new reactor during this stage stays at approximately 0.5%. When the majority of the phosphate is removed from the bone the reaction speed is determined by the phosphate concentration. Because the acid concentration of the acid running into the reactor continues to increase, the acid running from the reactor is now more than 0.5%. The reacting batch has also shifted forward 1 or 2 places. Finally the acid that runs into the reactor is 4%, and after a while, when the apatite is completely dissolved, the acid running from the reactor is also 4%.

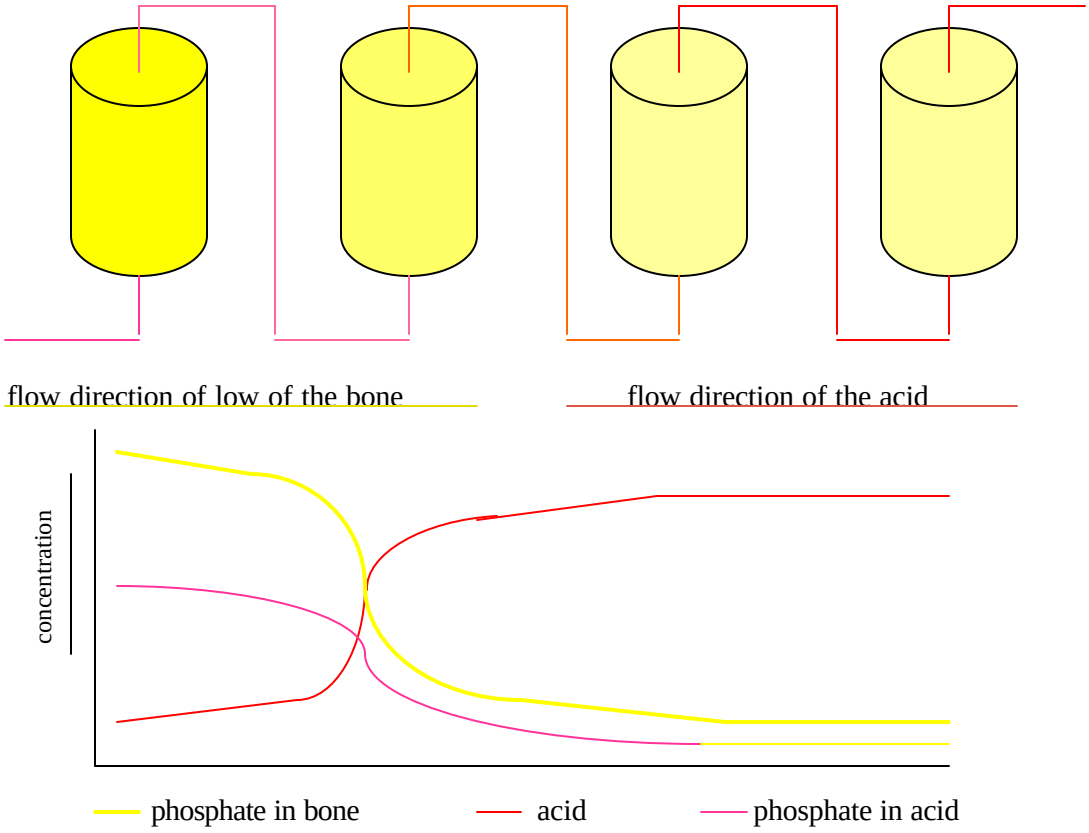


fig. 1 cascade process

A reactor tank in the process may typically contain a batch of about 20 to 50 tons of bone chips. The size of such a reactor tank is for instance 7.5 metres high with a diameter of 3.75 metres. The reactors are usually made from plastic coated steel.

The cascade processes can be arranged such that it has 4 reactor vessels, of which every day one is emptied and filled again. In 4 days 4 batches of 40,000 to 50,000 kg are treated, which is in total approximately 200,000 kg of degreased bone chips every four days.
Approximately, to demineralise these one needs 1,400,000 litres of 4% HCl in 4 days, or in industrial practice 1,700,000 litres in 4 days or 17,800 litres per hour.
The used volume of the reactor is 63,000 litres. The volume of the acid in the reactor is 36,000 litres, therefore the volume of bone is 27,000 litres. From these data it can be calculated that with a flow of 17,800 litres per hour the acid remains in a reactor for approximately two hours, indicating a flow rate between 1 and 5 meters per hour.
After the demineralisation of a batch is finalised the acid is pumped from the reactor in approximately two hours and the ossein is washed twice with approximately 50 tons of water to wash away the remaining acid. Filling the reactor with water takes approximately an hour. The ossein is left in the water for an hour and draining takes also an hour. Hereafter the ossein is left in the reactor for the acid treatment or pumped in a flow of water to the installation where this will be done.
Although basically the same, details of the installations and conditions will differ with the different manufacturers. Differences can be:

- There can be for instance 6 reactors in the cascade. With one set of bone chips a day this will result in a longer treatment of each batch. With more than one batch a day, the time of the treatment can be equivalent, but the output will be larger.
- The number of sets per day can vary. When more than one a day, the flow has to be larger or the concentration of the acid more than 4 %, when less than one set a day the flow will be lower.
- The acid concentration is at least 4 % but also higher concentrations, i.e. 5 or 6 % are used.
- Usually batch sizes are 20 to 50 tons but smaller batches may be used.
- The temperature of the acid can be approximately room temperature or below. At the end of the cascade it can be ambient temperature.

Acid treatment

When left in the reactor tank this is filled again with the same amount of water as the original weight of the bone chips, and is left to stand in it for about half a day to one day. The ossein does still contain sufficient acid to keep the pH below 2. After the vessel is emptied the ossein is washed again one or more times to obtain a pH of about 2.5 or higher and it is transferred to the extractors in a stream of water.

Extraction.

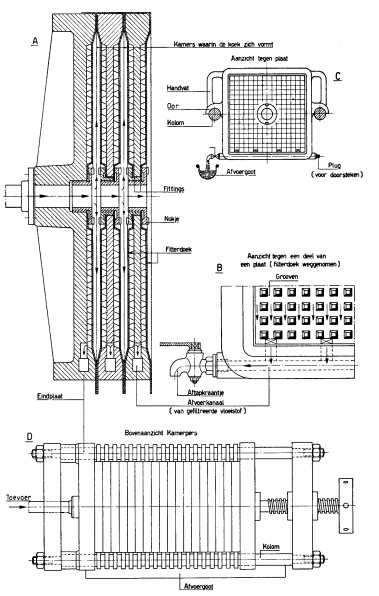
Gelatine is extracted from the denatured collagen in the ossein with warm water and is done in a number of steps, each being an individual extraction. The water temperature is increased with each consecutive extraction. The first is carried out usually at 50 to 60 ^{o}C, the last one at

100 °C. Individual consecutive extractions can however be done at the same temperature. The gelatine concentration of the extract is normally between 3 and 8 %.
By the extraction process, H-bridge bonds and VanderWaals forces maintaining the collagen helix are broken, allowing individual collagen molecules to dissolve. Breakage of peptide bonds is promoted by an acid environment and elevated temperature. The first extracted gelatine therefore has the highest gel strength. Later extracts at higher temperature have lower gel strengths because of more thermal breakdown
Industrially, extraction is executed as follows. In the extraction vessels, pre-heated water is added to the ossein, or cold water is added, which is then heated in the extractors. The extractors are either stirred or the water is circulated over the ossein bed. This is continued until a gelatine concentration of about 5 % is reached. The extract is then drained, the extractor is filled again with water and extraction continued, usually at higher temperature. The drained extract usually passes a sieve or a mesh to trap large particles. If during an extraction step the concentration does not increase sufficiently, the temperature is increased. Extraction is stopped when the concentration does not reach 3 % at the final extraction temperature, or when insufficient ossein is left. Temperature is generally not increased by more than 10°C, both at consecutive steps and during an extraction step.
The gelatine yield of the limed bone process is on average 20%, based on dried degreased bone chips: 50 tons of bone chips gives 10,000 kg of gelatine. The amount which is extracted in an extraction step depends on the amount of water used per step and the final gelatine concentration of the extract of that step. Normal amounts are between 1,500 and 4,000 kg per extract in a process which consists of 3 to 5 consecutive steps.
The essentials of the extraction equipment are the same for all manufacturers. All equipment consists of vessels containing a means to heat the water and to maintain movement between water and ossein. Individual differences include the size and number of individual extractors and the method of agitation.. The installations are made of stainless or coated steel.

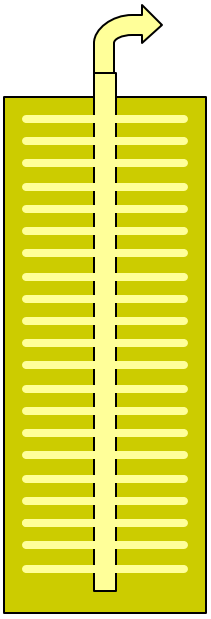
Filtration

Following extraction, crude gelatine extract is filtered with a diatomaceous earth or cellulose filter, to remove insoluble particles and any suspended drops of fat. Diatomaceous earth filtration is presently the common process in the gelatine industry, although some producers still use cellulose filtration.
Before the actual filter process the extract is first passed through commercial available cloth filter bags to remove large coarse particles. During filtration the temperature of the extract is kept at 55 to 60 °C to aid flow. Filtration installations generally consist of two or more parallel filters. When the one in use becomes saturated the flow is switched to the fresh parallel filter.
Cellulose filtration is done with a filter press containing cellulose pulp with a thickness of approximately five centimetres. The pads are used until the pressure over the filter press

becomes too high, at which point the flow is switched to the parallel filter. The cellulose from the spent filter pads is regenerated to make new ones. The diatomaceous earth filter layer is made freshly before use, by suspending the filter material in water and running this suspension through the filter installation. The equipment contains a mesh on which the filter material settles, forming a filter layer several millimetres thick. More earth is added to the gelatine solution as filter aid to prevent blocking of the filter layer. The pressure must not become to high to prevent collapse of the filter layer. When the maximum pressure is reached, flow is switched to the parallel filter and the filter meshes of the first filter are automatically cleaned and covered with fresh diatomaceous earth.

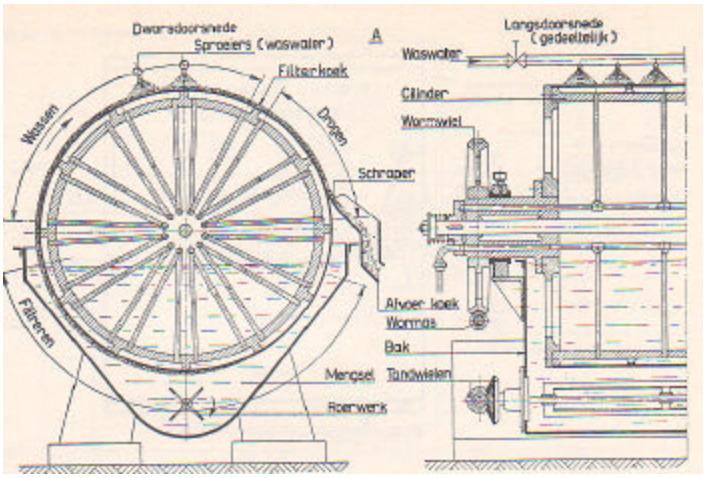


filter press



disk filter

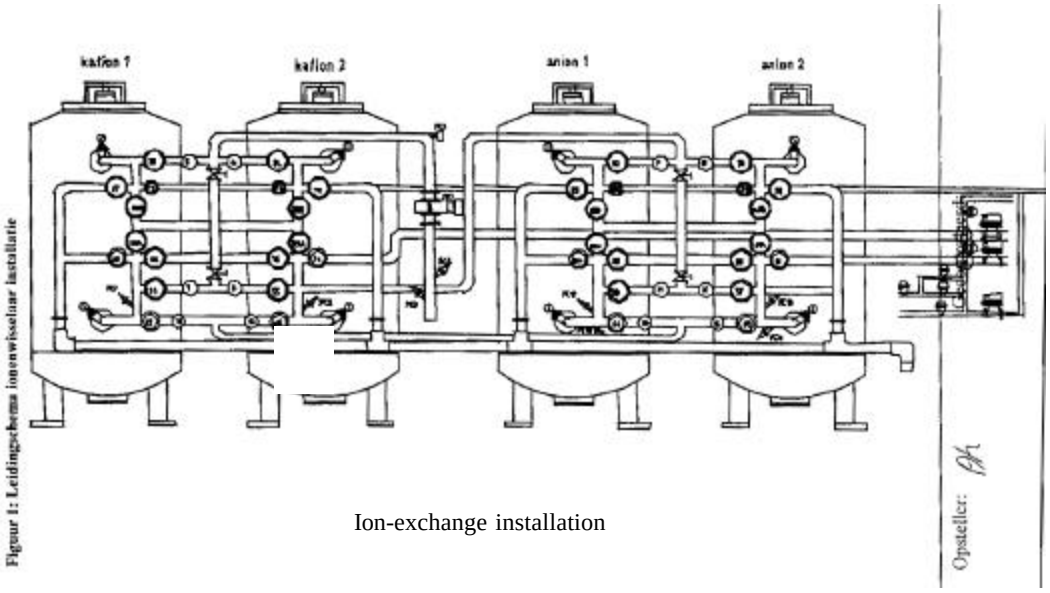
Diatomaceous earth filtration can also be performed with a rotating vacuum filter, where the filter layer is on the outside of a rotating drum. The drum is partially immersed in gelatine extract in which filter aid is suspended. The pressure inside the drum is be low atmospheric pressure.



Rotating vacuum filter

Ion-exchange.

The filtered gelatine solution is ion-exchanged over cation and anion resins. Limed bone gelatine normally passes the cation column first and then the anion column. Most installations consist of two cation columns and two anion columns of which one of each is in use, while the other columns are regenerating or standby. Modern installations have automated pH and conductivity control.



Ion-exchange installation

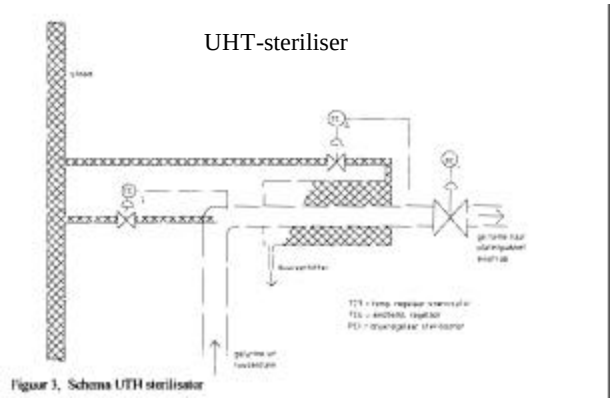
When pH and conductivity get out of the pre-adjusted range, the gelatine flow is automatically switched to a parallel column and the first column is regenerated. Cation resin is regenerated with 5 % hydrochloric acid, the anion resin with 5 % sodium hydroxide. After regeneration the columns are rinsed with de-ionised water or condensate until a pre-adjusted conductivity is reached.
After ion-exchange the pH of the solution is equal to the iso-ionic point of the gelatine, which is usually more than 7 for acid bone gelatine.
The industrial size columns have a bed size of 157 cm diameter by 175 cm high. The flow through such a column is about 7000 litres per hour or slightly more than two times the bed volume per hour. Flow rates of two to six times the bed volume are recommended by the resin manufacturers.
During ion-exchange the temperature of the solution is generally kept at 55 to 60 ^{o}C.
The ion-exchange installation is made of synthetic material or metal with plastic coating.

Concentration.

After ion-exchange, the gelatine solution is concentrated, by evaporation of water, from between 3 and 10 % to between 20 and 25 % gelatine.
Different kinds of equipment can be used but most common is evaporation in several steps in one or a series of vacuum evaporators. In each step the solution is heated in a heat exchanger and then injected into the vacuum evaporator where part of the water evaporates.

Sterilisation.

The concentrated gelatine solution is sterilised by direct injection of steam into a turbulent flow of the gelatine solution, by which the temperature is quickly raised to 138 -140 °C and maintained for at least 4 seconds. The solution is then quickly cooled by expansion. At a temperature of 138 to 140 °C, a pressure of more then 4 bar is maintained. The temperature of the gelatine is measured and monitored continuously.



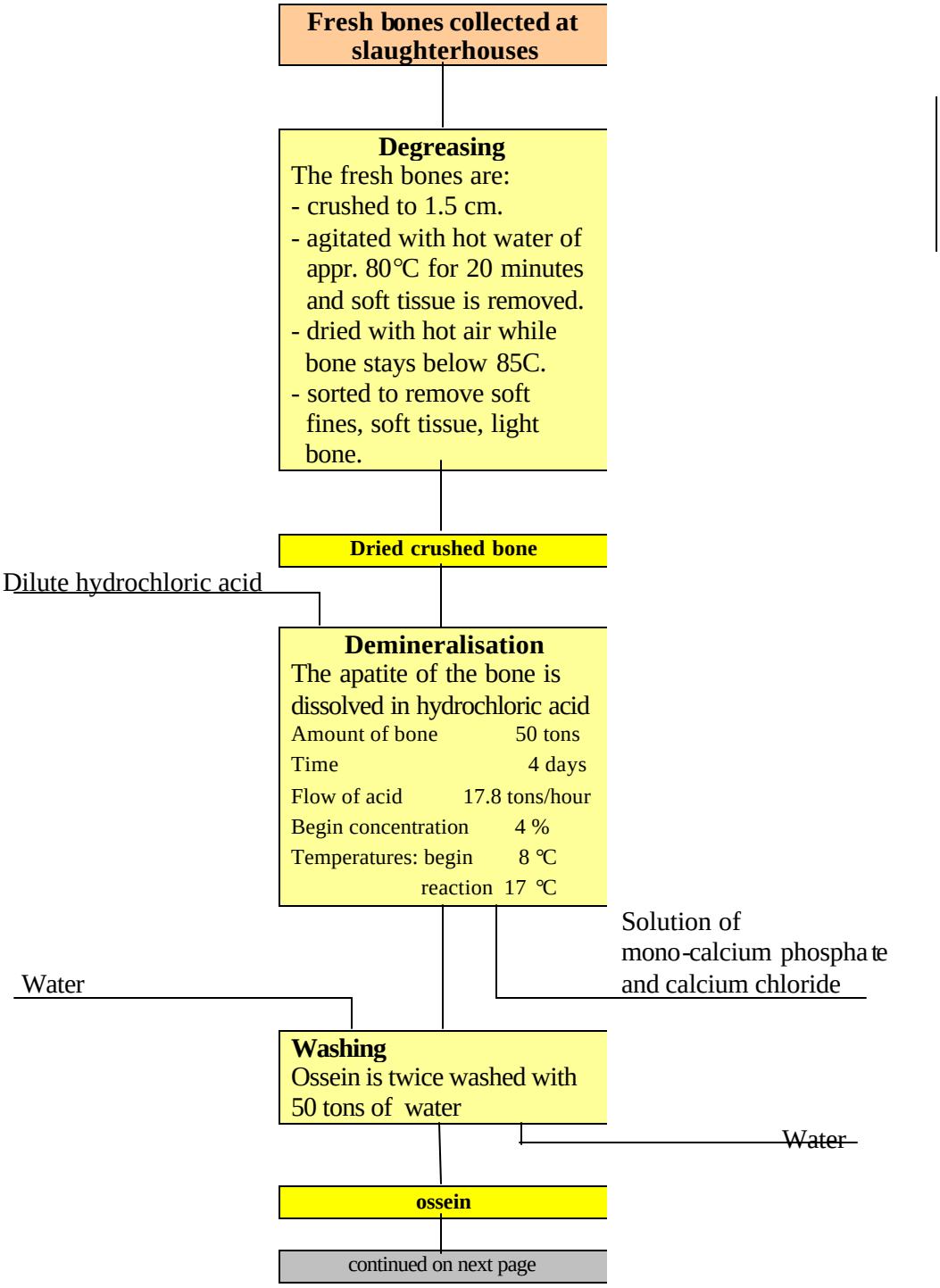
Figuur 3, Schema UTH sterilisator

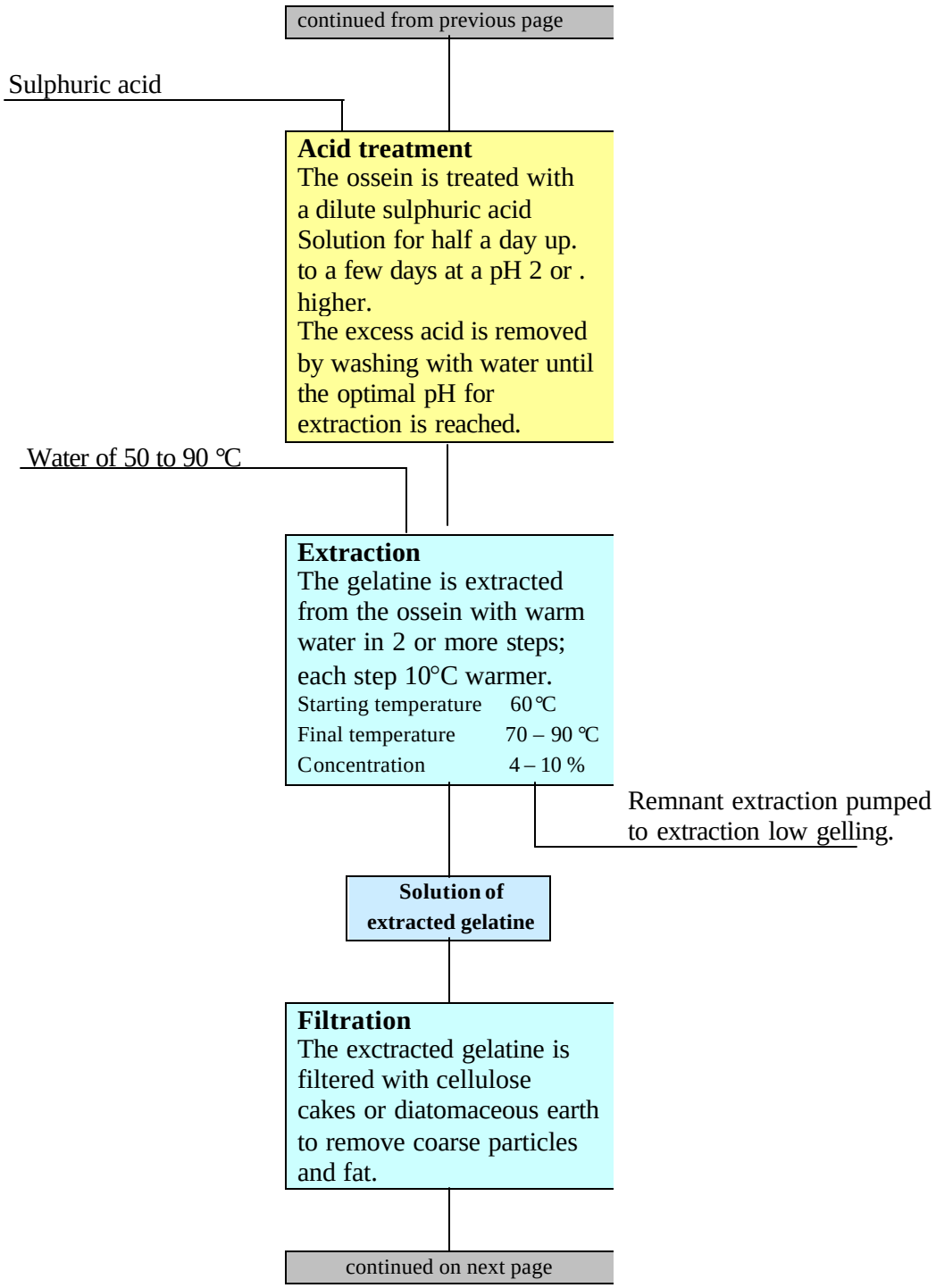
The sterilising equipment is made of stainless steel.

Drying, cutting, blending and packing of the gelatine.

The sterilised concentrated gelatine solution is pumped through a heat exchanger and cooled to less than 30 °C to form a gel. The gel is extruded through a perforated sheet to form thin threads. These threads are put on a large metal wire conveyor belt which runs through a segmented tunnel dryer where the gel is dried with clean, purified, pre-dried warm air. Each segment of this tunnel has a different temperature, the lowest (25 to 30 °C;) in the first segment increasing in each next segment up to 60 °C. The drying can take up to 6 hours. Gel entering the drying tunnel contains about 80 % water, the dried gelatine contains between 9 % and 15 % water; usually approximately 11 %.
At the end of the drier the solid gelatine is removed from the conveyor band and transported to a cutting mill where the threads of solid gelatine are cut to pieces of about 10 mm. The pieces are transported by a stream of clean air to a blender and blended to a uniform batch, which is packed and stored under the right conditions of temperature and humidity. The batch is given an 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 acid bone gelatine manufacturing process.

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Sulphuric acid
Acid treatment
The ossein is treated with a dilute sulphuric acid Solution for half a day up. to a few days at a pH 2 or . higher.
The excess acid is removed by washing with water until the optimal pH for extraction is reached.
Water of 50 to 90 °C
Extraction
The gelatine is extracted from the ossein with warm water in 2 or more steps; each step 10°C warmer.
Starting temperature 60 °C
Final temperature 70 – 90 °C
Concentration 4 – 10 %
Remnant extraction pumped to extraction low gelling.
Solution of extracted gelatine
Filtration
The exctracted gelatine is filtered with cellulose cakes or diatomaceous earth to remove coarse particles and fat.
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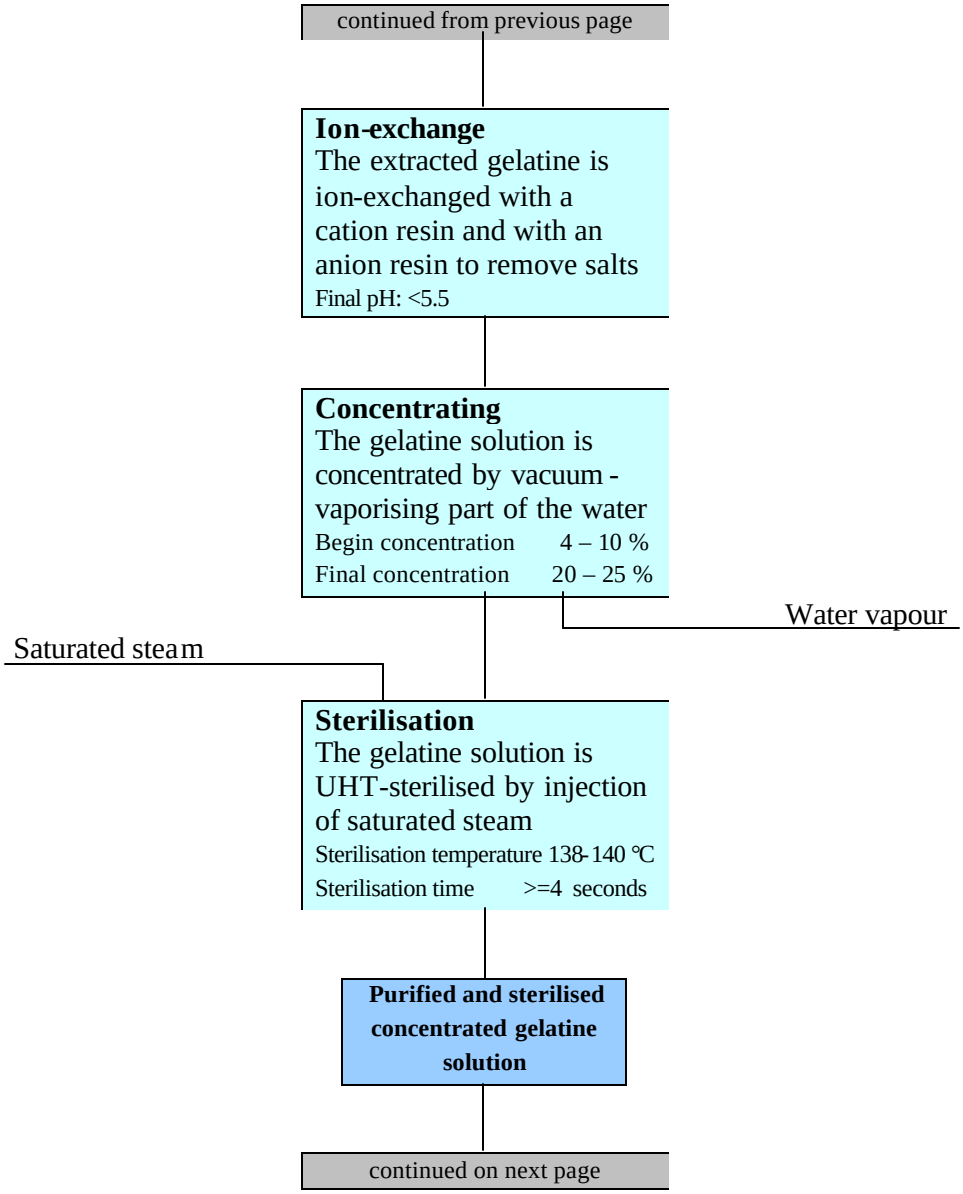
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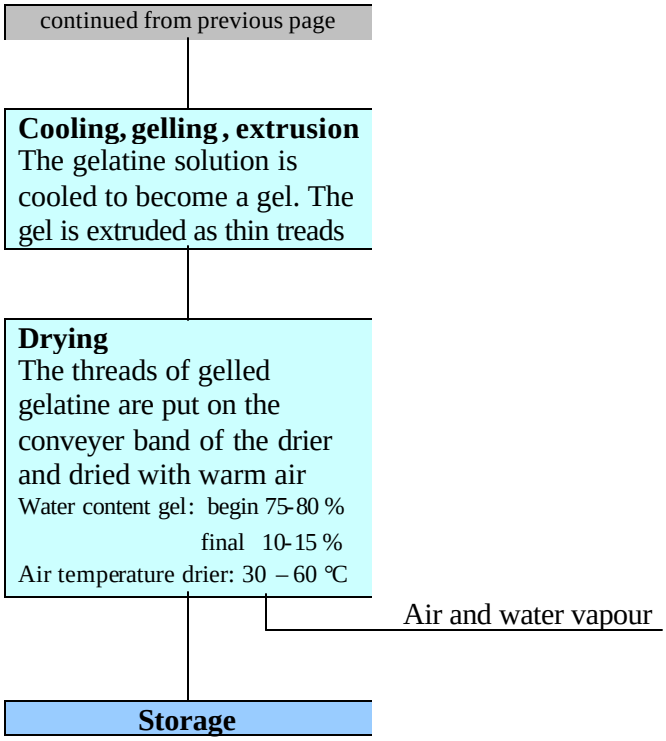
Ion-exchange
The extracted gelatine is ion-exchanged with a cation resin and with an anion resin to remove salts
Final pH: <5.5
Concentrating
The gelatine solution is concentrated by vacuum - vaporising part of the water
Begin concentration 4 – 10 %
Final concentration 20 – 25 %
Water vapour
Saturated steam
Sterilisation
The gelatine solution is UHT-sterilised by injection of saturated steam
Sterilisation temperature 138-140 °C
Sterilisation time >=4 seconds
Purified and sterilised concentrated gelatine solution
continued on next page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Cooling, gelling , extrusion
The gelatine solution is cooled to become a gel. The gel is extruded as thin treads
Drying
The threads of gelled gelatine are put on the conveyer band of the drier and dried with warm air
Water content gel: begin 75-80 %
final 10-15 %
Air temperature drier: 30 – 60 °C
Air and water vapour
Storage

2.3. Summary of the conditions of the acid bone gelatine manufacturing 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.

Acidulation: Batch size: 50 tons bone chips.
Dimensions of reactor 50 tons: height 7 m, diameter 3.5 m
4 reactors in cascade
Duration of process 4 days
Flow of acid 17.8 ton/hour
Concentration of acid 4%(w)
Temperatures: temp. of fresh acid 8 °C
Final temp. after reaction 17 °C (14 – 20 °C due to season)
Time at 4 %: 48 hours.
Washing: 2 times with 50 tons of water.

Acid treatment: Immersing batch of ossein in dilute acid. (Usually sulphuric acid but other acids may be used.)
pH more than 2
Draining of acid after 10 to 12 hours
Stirring at least 2 times with water.

Extraction: Stepwise extraction, each step at at least 10°C higher temperature.
Extraction starts at 50 to 60 °C and continues until concentration falls well below 3 % at a temperature from 70 °C to 90°C or no ossein is left.
Gelatine concentration of extract 3 to 9 %.
Ossein stirred or liquid circulated through the ossein bed.

Filtration: With 5 cm thick cellulose cake filters or with diatomaceous earth.

Ion-exchange: Two columns, one with cation resin and anion resin.

Concentration: Vacuum evaporation.

Sterilisation: Temperature 138 – 140 °C
Time more than 4 seconds

Appendix 2. DOWNSCALING PROCEDURE.

Industrial gelatine is manufactured from batches of between 100 tons and 250 tons of fresh bones. The scaled down process used approximately 2 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 1:50,000 and 1:200,000.

All acid bone gelatine manufacturers use the same basic manufacturing process with the same process steps and conditions, however, the precise process conditions can differ somewhat. For example, in degreasing, the temperature can vary between 80 and 90°C, the degreasing time from 20 minutes to more than half an hour and the proportion of water to bone from 1:2 and 1:8.

This variation in conditions presented the problem to the design of the downscaled process of which process conditions to use. Hence each manufacturer within GME was asked anonymously for its process conditions of every process step. The minimum conditions for each step used by any manufacturer were identified and applied in the model process. This ensured a process, representative of the industrial process used by all GME producers, 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.
- Process conditions, for which no mutual minimum ones can be found, are tested individually throughout all experiments.

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.
Demineralising	Concentration of HCl increases from 0,5% to 4% in 2 days. Two days with 4% HCl Two times washing with equivalent volume of water, pumping in stream of water	1 day 0.5% HCl 1 day 2.5% HCl 2 days 4% HCl 2 times soaking in 500 ml water 1 time stirring with 1000 ml water
Acid treatment	Treatment with dilute acid at pH of more than 2 For at least 10 hours Wash at least 2 times with water until extraction pH	If pH <=2 then stir with water until pH >2 Leave overnight at pH >2 Wash with water until pH between 2.5 and at least 3
Extraction	Stepwise with sufficient water, at 60, 70, 80, … °C. Concentration at draining 3-9%	Stepwise with 800 ml water at 60 °C and every next time 10°C more. Concentration at decanting 3 to 9%
Filtration	Over diatomaceous earth with body feed, or over cellulose cakes.	Over diatomaceous earth cake with some body feed and with cellulose filter cake.
Ion-exchange	Over cation resin and over anion resin. Flow 2 to 6 times bed volume.	Over cation resin and over anion column, Flow 2 to 3 times bed volume.
Concentration	In several steps in vacuum	In a rotavapor to 20-25%
Sterilising	By direct steam injection, temperature of 138-140°C maintained for at least 4 seconds	In a capillary with internal thermocouple for 4 seconds at 138-140°C

Appendix 3. DOWNSCALED MODEL PROCESS.

Acid bone gelatine manufacturing process.

Degreasing

a. Amount of bones.

The starting material for the scaled down process consisted of 2,000 g of bone. From these 2,000 g approximately 400 to 500 g of dried bone chips will be obtained, depending on the quality of the crushed bone. This compares with a typical batch size of 20,000-50,000 kg used in the demineralisation on manufacturing scale

b. Crushing of the bones

The 2,000 g of bone consisted of approximately 1,500 g of industrial crushed bone and 500 g of intact spine. Spine material was added to model the inclusion of raw material from several cows pre-clinically infected with BSE. (See spiking of the starting material, Chapter IV, 5 Appendix 1). 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 ¼ 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 °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 °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 °C, then 40 minutes at 85 °C minimum. Testing had shown that this regime resulted in bones of the same water content as industrial drying. During the first phase most water is evaporated and the chips stay below 85 °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. It was necessary to split the raw materials into two approximately equal aliquots, of crushed and sawn bone, of 1 kg and degrease each separately. 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°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 °C then 40 minutes at 85 °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.

Demineralisation

In the industrial process the dried bone chips are demineralised in the cascade process described previously.
One could imitate the industrial process by building such a cascade but takes several cycles to stabilise and maintaining the cascade is elaborate. An imitation of the range of concentration changes encountered within the cascade carried out batchwise is more simple to achieve. This was done by adding acid solutions of 0.5 % HCl during the first day, 2.5 % HCl during the second day and 4 % HCl during the third and fourth day. Mono-calcium phosphate and calcium chloride were added to the 0.5 % and 2.5 % HCl solutions in amounts equal to those in the industrial process. Hydrochloric acid is usually made in the industrial process by diluting concentrated industrial acid with cold water so the acid entering the process is relatively cold and slowly warms up during the process. For this reason the different acid solutions used in the model process were added at different temperatures.

The solutions used were:

1. A solution containing 5g HCl, 53.5g $CaCl_2$, and 40.5g $Ca(H_2PO_4)_2$ per litre water and a temperature of 18 to 22°C for 24 hours.
2. A solution containing 25g HCl, 23g $CaCl_2$, and 17.5g $Ca(H_2PO_4)_2$ per litre water and a temperature of 15 to 20°C for 24 hours.
3. A solution containing 40g HCl per litre water and a temperature of 12 to 18°C for 48 hours.

Instead of preparing the solutions from mono-calcium phosphate, calcium chloride and hydrochloric acid, these can also be prepared by dissolving the appropriate amount of bone ash in 4% hydrochloric acid. Bone ash is in fact a better model as the composition of the solutions is the same as those originating from the treatment of bone with hydrochloric acid.

The process was scaled down by a factor of 100,000 (compared with the illustration given in I.2). This is approximately equivalent to the use of 500g of bone chips with a volume of 630 ml and a flow rate through the chips of 170 ml/hour.
The reactor vessel was scaled down proportional to this factor to give a vessel diameter of 7.5 cm which was filled with the bone chips to a height of 14 cm. The interstitial volume for this amount of bone was 360 ml, therefore at the specified flow rate, 2.15 hours were required for the acid to flow through the bone, the same time as in the industrial process.
The flow rate in the small scale process was 46 times less than shown in the illustration of the industrial process of about 3 m/hour. The flow in both processes is laminar and depends on diffusion but the downscaled process may be slightly less efficient because of the slower flow rate.

Short description of the downscaled process:
500 g of bone chips put in a reactor of 7.5 cm diameter, filled to 14 cm high were treated for 4 days with three acid solutions detailed above for 24 hours, 30 hours and 48 hours

respectively. Solutions were pumped through the chips with a flow of 170 ml/hour. The equipment used was made of glass which differs from the coated steel industrial equipment.

Acid treatment

In the industrial process the acid treatment can be done in the demineralising reactors or in large separate basins. The ossein is completely immersed in the dilute acid for the entire period. The acid treatment is static. Washing to remove the excess acid is best done by stirring with water.
As model of this process the ossein is left immersed in 600 ml of fresh water in the beaker in which it was washed after demineralisation. After some time the pH must be determined which must be more than pH 2 and less than 4. If the pH is below 2, the ossein must be washed one more time to increase the pH. The ossein is left overnight in the acidic solution. The next morning the acidic water is drained and the ossein is stirred at least two times for 30 minutes to 1 hour with 600 to 1000 ml of water. When the pH is then still below 2.5 washing is repeated until the pH is more than 2.5. Preferred is a pH of approximately 3. When the extraction pH is reached the final washing water is drained.

Extraction

Extraction is the process of treating the neutralised ossein with hot water to dissolve it as gelatine. After reaching a sufficient concentration the solution is drained, replaced with fresh hot water and extraction is continued For each of these extractions the temperature of the water is constant but is raised between extractions to obtain a sufficiently fast increase in gelatine concentration. The industrial process agitates the bone either by pumping of the water or stirring of the vessel. The downscaled model was carried out by gentle stirring of the ossein in a beaker with an amount of hot water between one and two times the weight of the original bone. Water was first added at a temperature of 60°C and the concentration of gelatine in solution was measured at regular intervals using a refractometer. Once the concentration of gelatine reached a sufficient concentration (between 3 % and 9 %, average 5 % in the industrial process) and did not increase on subsequent measurement, the solution was pumped out. Fresh water of 70°C was then pumped in, a rise of 10°C, for the second extraction. A series of extractions were performed in this way until little or no ossein remained, or up to a maximum temperature of between 90°C and 100°C until the concentration did not rise any further.

Filtration

Extracted gelatine requires filtration to remove insoluble particles and is carried out on industrial scale as a continuous process. The gelatine solution is first drained through a cloth filter bag to remove coarse particles before flowing under pressure through a filter bed of

diatomaceous earth and/or cellulose. The scaled down process only produced between 1.5 and 2.5 litres of solution, so it was not practical to reproduce the continuous flow, large surface area type filtration of the industrial process. The same filter bed materials were used however, but in batch filtrations using a laboratory scale pressure filter, often used to evaluate industrial filtration processes. The cloth, cellulose and diatomaceous earth beds were made up as for the industrial process to fit the laboratory filter. The gelatine solution was warmed above 40°C to prevent gelling then filtered through the diatomaceous earth followed by filtration through the cellulose sheet or alternatively filtered in a one step process over a cellulose cake.

The process was carried out in a Schleicher & Schuell Pressure filter MD142, pressurised using a compressor adjustable between 0 and 6 bar. The filter had a sealable lid, removed to allow addition of the filter material and sample. The filter drains at the bottom through an outlet valve. This was used to drain heating water or excess filter emulsion, and to collect the filtrate. The filter was preheated with hot water which was then drained. A cloth or cellulose filter disc was put in place, or a filter layer made by filtering an emulsion of 40 g diatomaceous earth (or 30 g cellulose) in hot water. The warmed gelatine solution was then poured in to the filter, the lid sealed and the outlet valve opened. Pressure was applied and the filtrate collected in an Erlenmeyer flask. The amount of solution filtered per filtration step varied depending on the filter material being used. Between each consecutive filtration the filter was decontaminated for at least one hour with sodium hypochlorite solution containing at least 20,000 ppm free chlorine and subsequently washed 5 times with water.

Ion-exchange

To remove salts from the filtered gelatine it is passed in sequence through cation and anion exchange resins after which the gelatine pH is equal to its iso-ionic point. For limed bone gelatine this lies between 4.6 and 5.3. Industrially, this is carried out in 2 columns of each type with limed bone gelatine normally passing the cation column first and then the anion column. Most installations consist of two columns of each type, one of which is in use while the other is regenerated. Flow rate can vary but should remain between 2 and 6 column volumes per hour. The model process was carried out in a pair of custom-made glass columns of 700 ml volume, equivalent to a downscale by a factor of 5000 One column was filled with cation resin Amberjet 1200H to a bed length of 70 cm, the second with anion resin Ira 94 S to a bed length 65.5 cm. The resins were then regenerated with 5% solutions of hydrochloric acid (cationic) and sodium hydroxide (anionic) before the gelatine solution, warmed to 60°C to allow flow, was pumped through at a flow rate of 3 bed volumes/hour. Water heated to 60°C was circulated through the column water mantle to maintain the temperature of the gelatine. The pH and conductivity of the input and effluent solution were measured until a change was observed, at which point the gelatine solution was pumped out and collected.

Concentration

After ion-exchange, the gelatine solution is concentrated, by evaporation of water.
In the industrial process, gelatine solutions at this stage are concentrated from a solution of 3 to 10%, to one of 25%, equivalent to a reduction in volume by a factor of between 2 and 8. The process is carried out by serial vacuum evaporation in one or several evaporators. The downscaled process used a commercially available laboratory rotating vacuum evaporator heated to 55-60°C. Approximately 250 ml of gelatine solution was put in the evaporator flask, water allowed to evaporate, then another 250 ml of gelatine was added and concentrated to approximately 20%.

Sterilisation

Sterilisation is the last purification step before drying of the concentrated solution to produce solid gelatine. In the industrial process, sterilisation is a continuous process commonly done by passing the solution under pressure through a pipe where it is heated by direct injection of steam under pressure at approximately 140°C for 10 seconds, then cooled rapidly. The design of a laboratory facsimile proved impossible and a compromise process was devised. NIZO (Netherlands Institute of Dairy Research), a group experienced in sterilisation techniques advised on the use of indirect heating in capillaries. Although different from direct steam injection, the heating kinetics are very similar. The gelatine to be sterilised was injected into a stainless steel coil sealed at one end by a thermocouple and a pressure release valve at the other. This coil was then immersed in an oil bath at 140°C and the temperature of the gelatine measured by the internal thermocouple. When the temperature recorded reached 138°C, the coil was held in the oil for a further 4 seconds after which it was cooled in a bath of cold water. The coil assembly was left to cool and cut open then warmed in a water bath and the gelatine was pressed out into a plastic tube connected to one end using pressure from a syringe attached to the other. Approximately 1.5 ml sample was obtained with each test.

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 backbones 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 5g of 301V infected mouse brain was smeared over 1,500g 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 5g of infected mouse brain was injected into the spinal cord of a 500g 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. One whole cow weighs approximately 20 to 29 kg, the backbone 5 to 6 kg; a ratio of 5:1. The ratio in the model process of sawn backbone to crushed bone was 3:1.

The amount of infectious brain material used, approximately 10 g on 2 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 1:200. 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 infectious material used to spike 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.

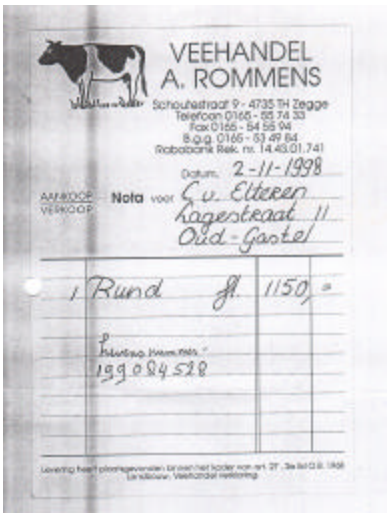
Table 1. Approximate composition of the fresh bone.

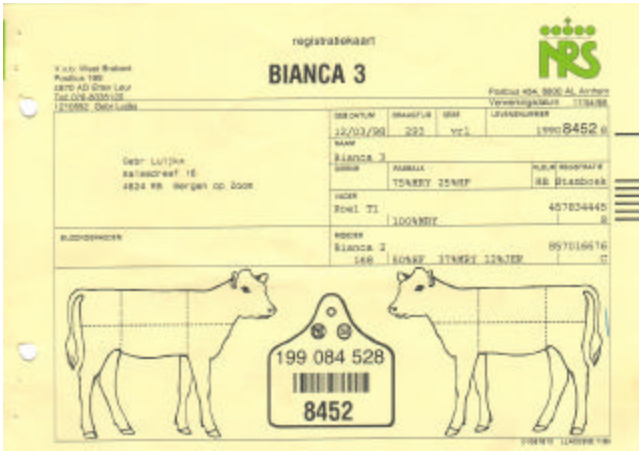
	Composition calculated on dry matter	**Composition calculated on dry matter excluding fat**
Fat	approx. 36 %	
Protein	approx. 32 %	approx. 50 %
Ash 550 ^{o}C	approx. 31 %	approx. 48 %
Ash 1100 ^{o}C	approx. 30.5 %	approx. 47.5 %

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.


VEEHANDEL
A. ROMMENS
Schoutestraat 9 - 4735 TH Zegge
Telefoon 0165 - 55 74 33
Fax 0165 - 54 55 94
B.g.g. 0165 - 53 49 64
Rabobank Rek. nr. 14.43.01.741
Datum 2-11-1998
AANKOOP VERKOOP Nota voor C.v. Eteren
Kagerstraat 11
Oud-Gastel
1 Rund fl. 1150,-
199 084 528



GEZONDHEIDSVERKLARING

NR: 0272485

Diersoort: Kwalificatie
Brucellose: Officieel vrij
Tuberculose: Officieel vrij
Leucose: Officieel vrij
Leptospirose: Vrij
IBR: Niet vrij
Paratuberculose:
BVD: Onbekend
Salmonellose:
BSE: Vrij

Datum afgifte: 16-12-1998

SLAGERIJ VAN ELTEREN V.O.F.

OUD-GASTEL 3-11-98

REKENING voor G.M.E.

KONINKRIJK DER NEDERLANDEN

GEZONDHEIDSCERTIFICAAT

(Veterinair Certificaat)

BONE CHIPS
(DEGREASED BONE PRODUCTS)

BATCH NO. REGARDS RE. A. GHOBADE

IN 1 CONTAINER

GELATINE DELFT BV
ROTTERDAMSEWEG 380-270
2628 AT DELFT
HOLLAND
TEL 5282

APPROVAL NUMBER: NL7321 EEG
CRODA COLLOIDS LIMITED (EWG)
FORMERLY LEWIS DITTON MIDDEX
CHESHIRE WA5 4LB
UNITED KINGDOM

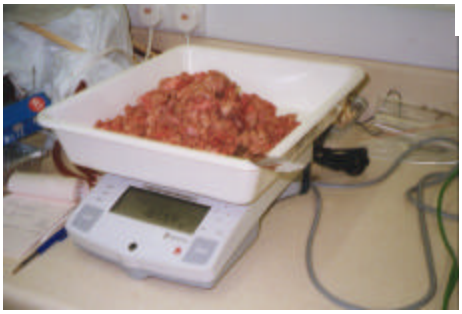
BY SEA OR ON ABOUT 05-02-1999

Appendix 5. DETAILED DESCRIPTION OF EXPERIMENTAL PROCEDURES

Preparation of the starting material

Crushed bone starting material and calf backbone

Two bags of crushed bone and a piece of intact backbone were taken from the freezer and were thawed in the refrigerator. The backbone was weighed on Ohaus Explorer scales (4100±0.1g cat no EOD120). The difference between the weight of the backbone and 2000 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 15ml Schütt homogeniser with a PTFE piston (LS ca no 1931 05143/53). Approximately 12 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 and the stirrer was switched on at 1000 rpm. The diameter of the tube was too narrow to effectively homogenise the macerate so it was transferred to a Schütt homogeniser tube of 30 ml (LS cat no 1931 05145/55) which gave a larger clearance between the wall and the piston. During this transfer some brain material was lost, remaining adhered to the equipment. The homogeniser was fitted to the stirrer and the brain was homogenised at 1000 rpm by passing it three times along the tube wall

Jar with brain- macerate

Transfer to homogeniser tube

Homogenising

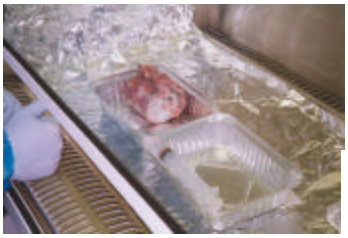
and the piston. The homogenate was transferred to "jar-I" which was weighed. The jar, the homogeniser tubes and the spatulae were set aside. The glove bag was removed from the cabinet together with the pistons of the homogenisers and disposed of. The stirrer and stand were also removed from the cabinet and put in polythene bags until further use.

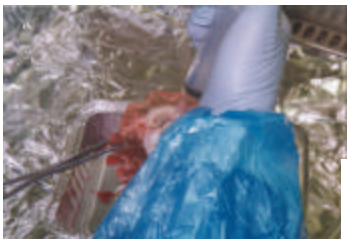
Injecting into the spinal cord of the backbone and spiking of the crushed bone.

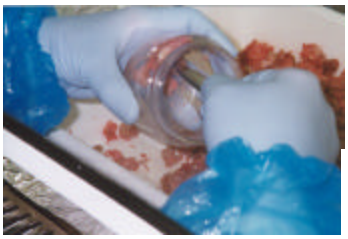
A Braun Omnifix 30 ml syringe (LS cat no 1942 13030) was filled with the brain homogenate from jar-I. A Beckton Dickinson 18G6 needle (cat no 408360) was fixed on it and the syringe was weighted. The homogenate was injected into the spinal cord of the calf backbone after which the syringe was weighted 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 spatula's used previously with adherent brain were weighted before the brain tissue was smeared on the crushed bone. The spatula's were weighted again and are 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 weighted 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 weighted before and after wiping. Finally approximately 4 g of brain macerate was taken from the stock jar and smeared on the crushed bone. 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 was 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	1892 g
Weight of empty tray	355 g
Crushed bone	**1546 g**

Tray and bag with backbone			477.2 g
Empty tray and bag			22.7 g
Back bone			**454.5 g**
Total weight of starting material			**2001 g**
Stock jar before taking out			106.11 g
Stock jar after taking out to homogeniser tube			100.10 g
Taken out of stock jar to homogeniser tube			6.01 g
Stock jar before smearing on the crushed bone			100.10 g
Stock jar after smearing on the crushed bone			96.28 g
Taken from stock jar and smeared on crushed bone			3.82 g
Taken out of stock jar to homogeniser tube			6.01 g
Taken from stock jar and smeared on crushed bone			3.82 g
Total taken from stock jar			**9.83 g**
Syringe with brain homogenate	21.90 g		
Syringe after injection in spinal cord	19.99 g		
Injected in spinal cord			**1.71 g**
Stockjar with brain macerate	100.10 g		
Stockjar after smearing on crushed bone	96.28 g		
Macerate smeared on crushed bone		3.82 g	
Beaker with spatulas with brain	115.97 g		
Beaker with spatulas after smearing	114.83 g		
Brain smeared on crushed bone		1.14 g	
Jar I after filling syringe	24.90 g		
Jar I after smearing on bone	24.48 g		
Brain smeared on crushed bone		0.42 g	

Beaker with tube before smearing	156.29 g		
Beaker with tube after smearing	154.44 g		
Smeared on crushed bone		1.85 g	
Total smeared on the crushed bone			**7.23 g**
Total spike (301V pool 2)			**8.94 g**

Differences:

Syringe after injection	19.99 g		
Syringe before use	19.73 g		
Left in syringe		0.26 g	
Beaker with spatula after smearing	114.83 g		
Beaker with spatulas before use	114.81 g		
Left on spatula		0.02 g	
Jar after smearing	24.48 g		
Jar before use	24.21 g		
		0.27 g	
Left on spatula after smearing from stockjar		0.09 g	
Weight difference homogeniser tube		none	
Total differences			0.64 g

Taken from stockjar	9.83 g
Total spike	8.94 g
Total losses	0.89 g
Total differences	0.64 g
Difference	0.25 g

Infective material was lost during spiking due to material left on surfaces of the used equipment. Part of the difference in weight could 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 be therefore in reality somewhat larger than the stated amount of 8.94 g.

Reference: Note book 19-3-99 to 25-5-99 page75 to 81. Date 5-6-99.

Sawing of the backbone

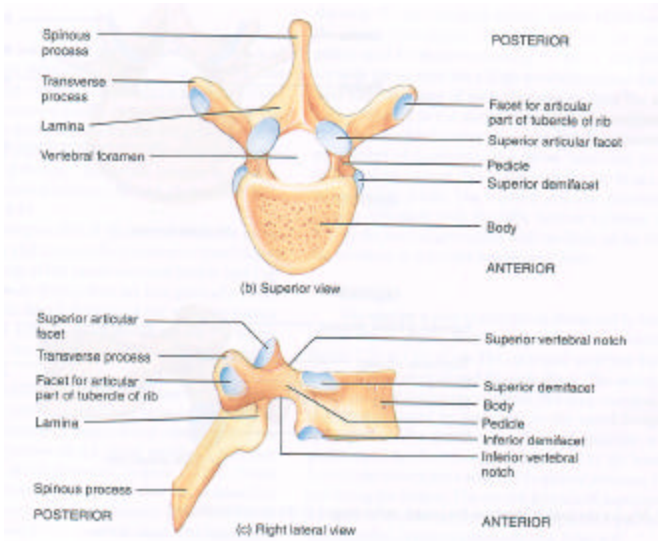
A glove bag was set up in the biological safety cabinet. One plastic and one aluminium tray, two pairs of tweezers (Bochem 18/8 steel LS cat no 3305 01000), two pairs of forceps (arterienklemme stainless steel LS cat no 3351 11038) and two scalpels (scalpel handle LS cat no 3351 18061, blade LS cat no 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 glove bag was intended to protect against the saw dust 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 to 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

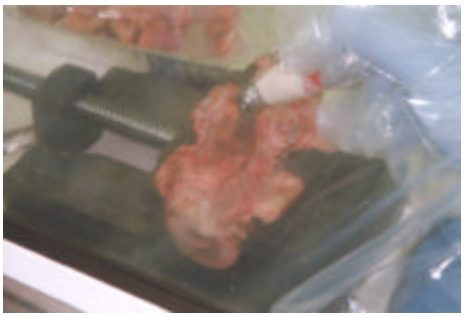
Description of sawing of th e backbone:

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 the 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, such that it did not touch the sides of the sleeve. The sleeve was then cut so the contaminated top of the saw was covered by the sleeve. The sleeve was closed with tape and the saw with sleeve 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 are 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 biological safety cabinet and disposed of. The cabinet was decontaminated with sodium hypochlorite as before.



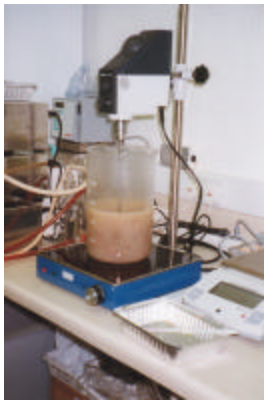
Sawing the backbone

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 back 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 31-12-98 to 24-3-99 page 11. Date 3-1-99.

Degreasing

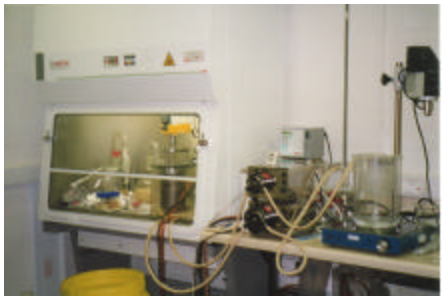
Preparation of the degreasing emulsions.

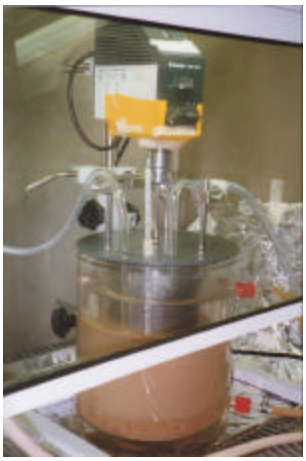

Preparing Degreasing emulsion

2 bags of fresh crushed bone were taken from the refrigerator and after thawing, two equal portions of 600 g were 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 80°C. One of the 600 g portions 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 stored in a 3 litre 2 neck round bottom flask (Schott DURAN). The other portion of 2 litres degreasing emulsion was prepared in the same way from the second 600 g of crushed bone.

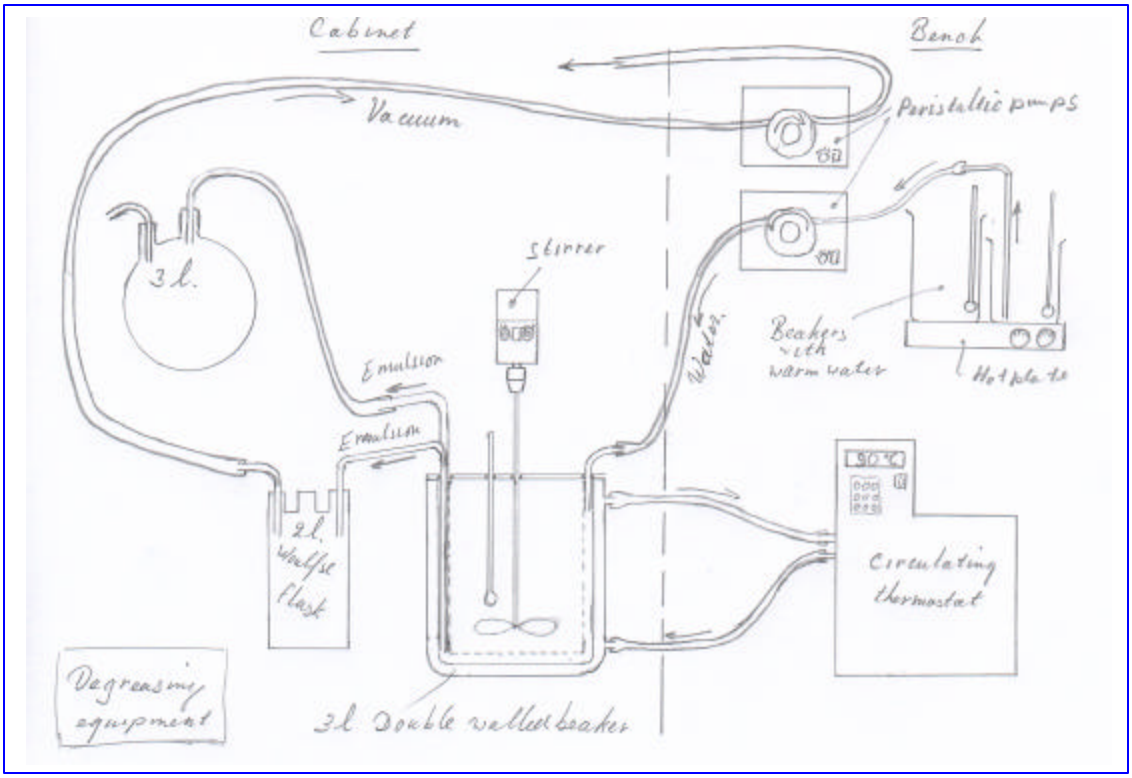
Degreasing of the crushed bone and sawn backbone spiked with 301V infected mouse brain.

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 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.


Degreasing equipment


Beaker and basket

The basket was removed from the double walled beaker and the blade from the stirrer. One 2 litre portion of degreasing emulsion was 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



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)

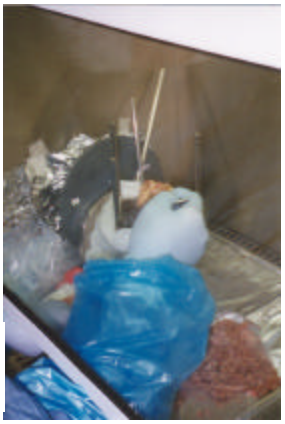
and backbone mixture was taken from the refrigerator and split into two approximately equal amounts in aluminium foil trays and weighed.

1:

Tray+bag +bone	1001 g
Tray+bag	23 g
Bone	978 g

2:

Tray+bag +bone	990 g
Tray+bag	22 g
Bone	968 g



Filling the basket with spiked crushed bone

(The difference of the total weight here and earlier weights was caused by evaporation of water)

The basket was wrapped in a polythene bag and filled with crushed bone portion 1. 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, 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 18 minutes 1 litre of emulsion was pumped out of the beaker and 1 litre of clean water was pumped in. A sample of the degreasing emulsion was taken after 5, 10 and 15 minutes. After 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.

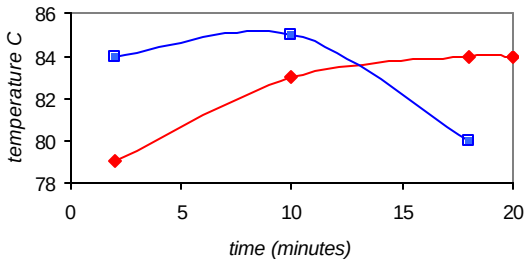
Draining the emulsion

Two litres of water heated to 80°C were pumped in, the bones stirred for 3 minutes then the water pumped out into a second 3 litre flask. The temperature measured for this stage was 83-86°C.

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**
0	79°C	84°C
10	83°C	85°C
18	84°C	80°C
20	84°C	-



Degreased bone

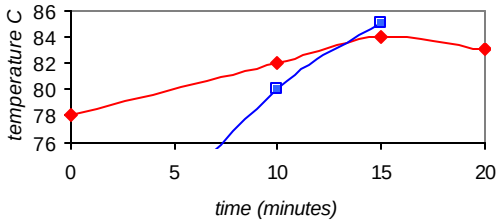
The two 3 litre flasks with spent degreasing liquid were removed from the cabinet and disposed of. Two clean 3 litre round bottom flasks were placed in the cabinet and the necessary hose connections made. The second portion of degreasing liquid was put in the 3 litre double walled beaker and was warmed to 80°C. Two beakers with 1 and with 2 litres of water were heated on the hotplate. The basket was filled with the second half of the spiked crushed bone and sawn backbone, the propeller stirrer was put in the basket, the lid put on it and the basket fitted to the ring. When the degreasing emulsion reached 80°C the basket was immersed in the degreasing liquid and the bones were treated as in the first degreasing. The obtained degreased bone was added and mixed with the first portion prior to further processing.

Details of the second degreasing:

1 litre of emulsion was pumped out and replaced by clean water after 15 minutes.
3 samples of the degreasing emulsion were taken, after 5, 10 and 15 minutes.

Temperature during degreasing.

Time (min)	**temperature degreasing emulsion**	**temperature fresh water**
0	78°C	64°C
10	82°C	80°C
15	84°C	85°C
20	83°C	-



Tray with bag and degreased bone 1400.5 g

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

All equipment which 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.

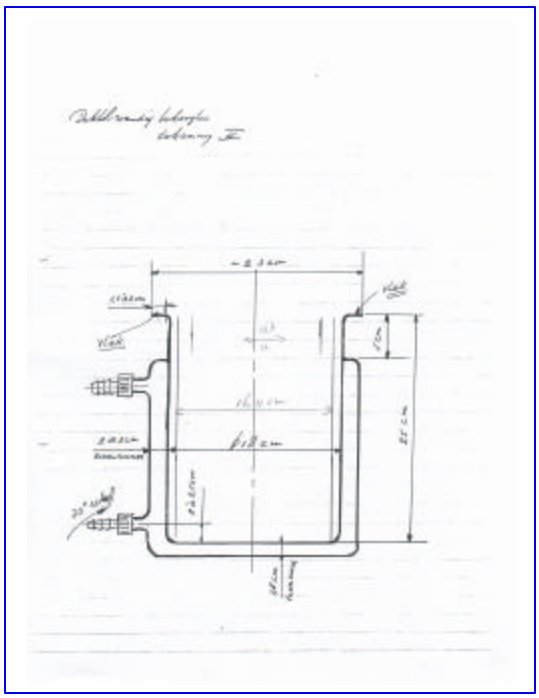
Separating bone and soft tissue

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.

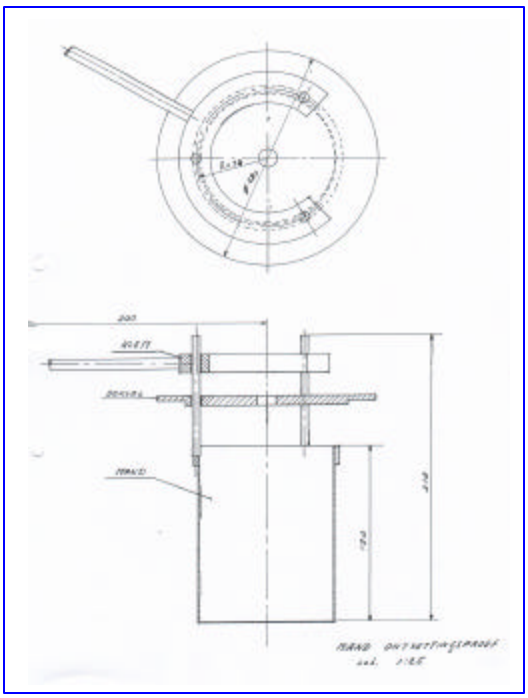
Separating degreased bone and meat

Sorted wet bone 580.8 g

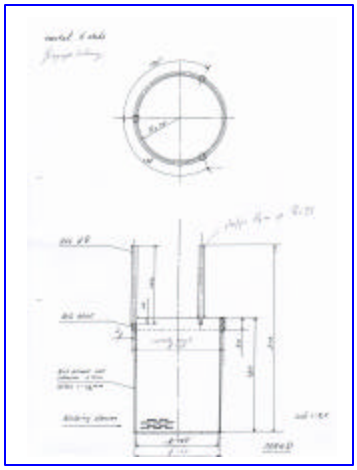
Custom made equipment



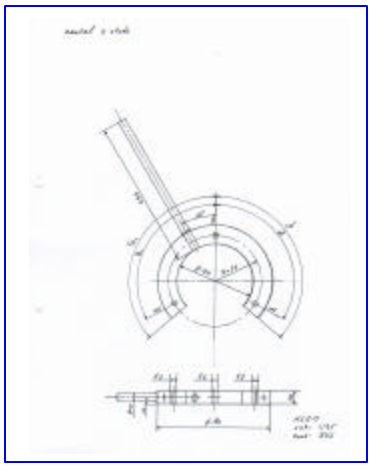
3 litre double walled beaker



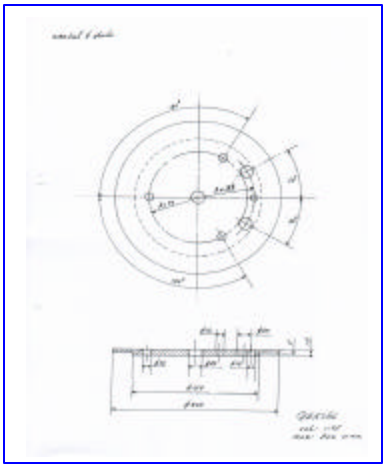
basket assembly



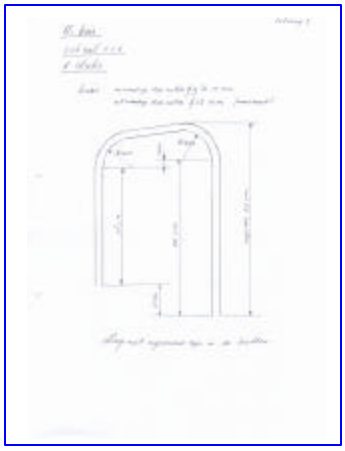
basket

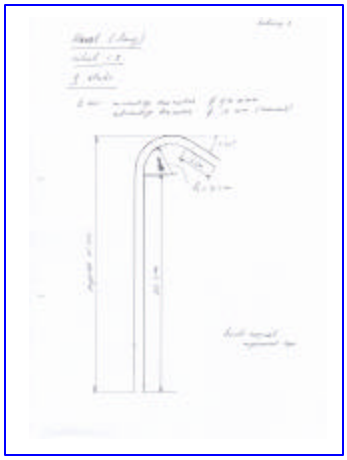


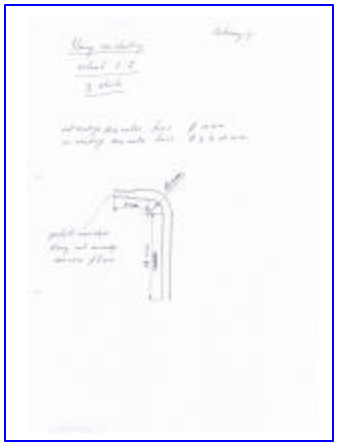
ring



lid







siphons

Drying of the bone

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

Trays with wet bone are put in drier

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 30 minutes at 2,400W. 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 110°C and the maximum temperature between the bone was 83°C.

Drier

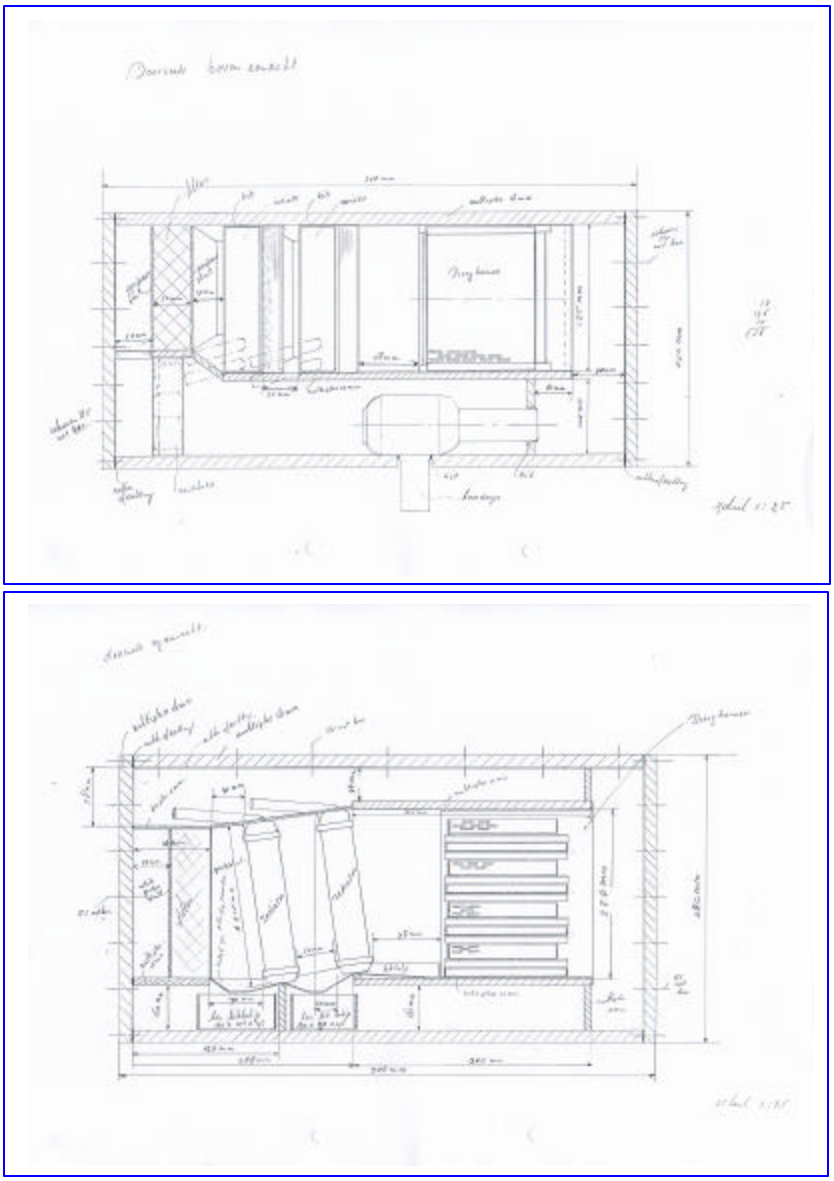
All small equipment was wrapped in polythene bags and disposed of. The drier was closed again, taken from the cabinet and disposed of. The safety cabinet was decontaminated as before.

Inside drier

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 7 hours.

Tumbler jar on roller bank



Drier, side view and top view

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

Tray with bag and bone chips	467.6 g
Empty tray and bag	24.4 g
Sorted degreased dried bone chips	**443.2 g**

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

Sorted degreased dried bone chips	443.2 g
Sample of bone chips	14.9 g
Bone chips for demineralisation	**428.3 g**

The tray with bone chips was put in a polythene bag and stored in the refrigerator until further use.

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

Reference: Notebook 19-3-99 to 25-5-99 page 83-88. Date 10-5-1-99 to 13-5-99.

Demineralising (Acidulation)

Preparation of the demineralisation solutions.

The hydrochloric acid solutions required for demineralisation of the bone material were prepared from the following amounts of bone ash dissolved in 18% acid:

Solution 1: Composition: 0.5% hydrochloric acid, the remainder mono-calcium phosphate and calcium chloride.
6 litre solution: 390 g bone ash
566 ml 36% hydrochloric acid
5440 ml water

Solution 2: Composition: 2.5% hydrochloric acid, the remainder mono-calcium phosphate and calcium chloride.
5 litre solution: 171 g bone ash
566 ml 36% hydrochloric acid
4690 ml water (750 g ice added later)

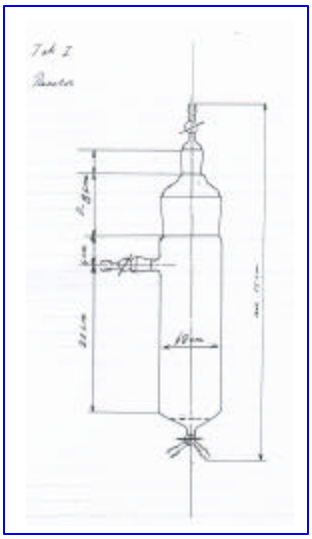
Solution 3: Composition: 4% hydrochloric acid
10 litre solution: 943 ml 36% hydrochloric acid
6650 ml water (2400g ice added later)
Composition: 4% hydrochloric acid.

(Bone ash: Industrial quality bone ash Smits-Vuren BV, Vuren, The Netherlands, Hydrochloric acid: Merck (BDH) Normapur AR min 36% d. 1.19 analytical reagent cat.no 20252.324, lot 268F and 987G)

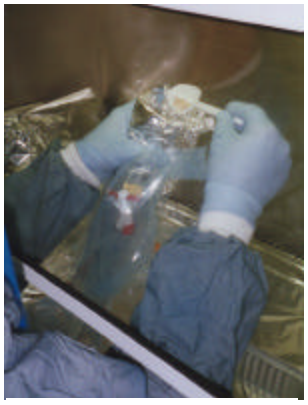
The solutions were stored in 10 litre polythene bottles.

Demineralising of the degreased crushed bone ch ips.

The demineralising equipment was set up (see pictures and drawing on next page). The bone chips were transferred from the refrigerator to the safety cabinet then put into a custom-made glass reactor flask which would allow the acid solutions to be pumped through it across the bone chips. The flask was closed and fitted to a stand on the bench. In case of breakage or leaks, the equipment was contained within a large plastic tray and covered by polythene sheeting. A hose connected the flask inlet valve to the acid stock bottle filled with solution 1. The outlet valve hose drained to an empty 10 litre container to collect the spent acid. The valves were opened and solution 1 pumped through the flask using a peristaltic pump. The pump was adjusted to a flow rate 158 ml/hour.

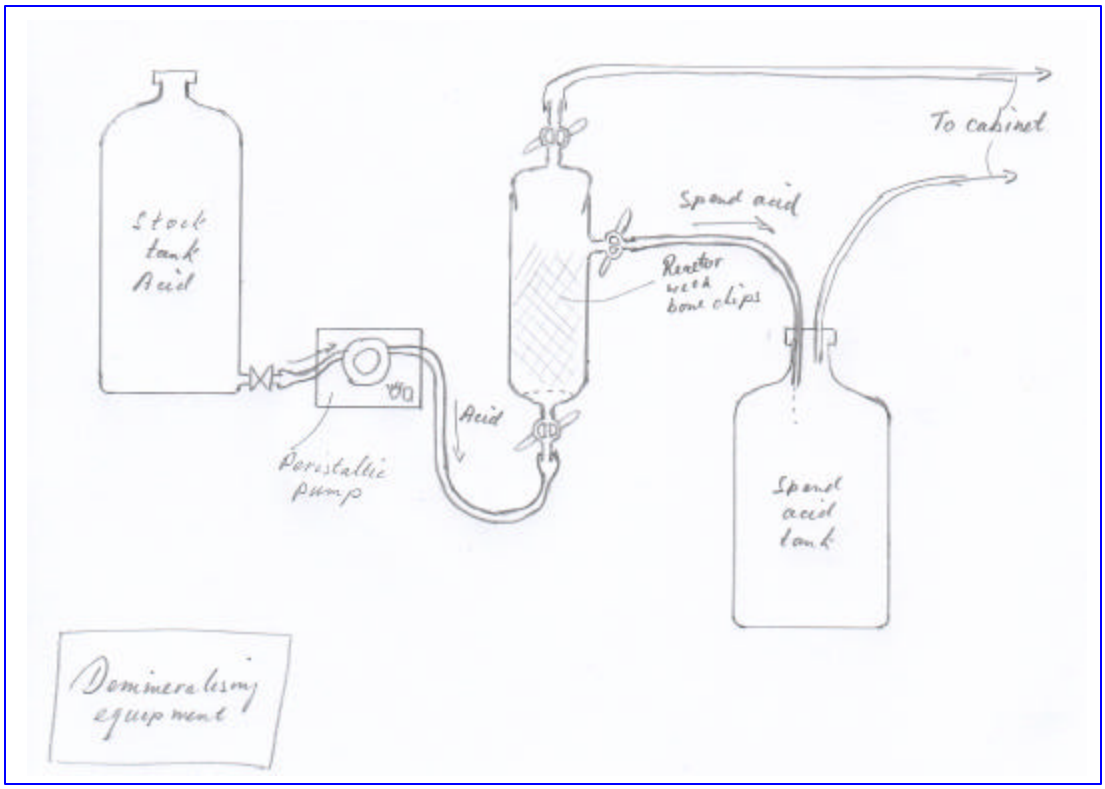


Reactor demineralisation and liming



Filling reactor with bone chips

After 24 hours solution 1 was replaced by solution 2, which was replaced after a further 25h by solution 3. Solution 3 was pumped through for 52 hours at which point the demineralisation was complete. Ice was added to solutions 2 and 3 to keep the demineralisation reaction temperature below room temperature.



Demineralisation equipment

List of equipment:
1 pc. 10 l polythene bottles with valve at bottom for the fresh solution Kautek cat no 22 52 04102
6 pc.10 l polythene bottle as recipient Kautek cat no 22 52 04101
Verder Pericor peristaltic pump 9F-240
Pumping hose Verderprene 0.8x1.6 cat no 58 84 81
Silicon hose of different diameters Deutch & Neumann
Different connectors Bürkle-Laboplast Tubing Fittings Polypropylene

To replace a solution, the pump was switched off and the valves closed. The remaining solution in the stock tank was removed and replaced by the new iced solution. The waste solution was also removed and replaced by an empty container. A sample of the spent acid was taken and the remainder disposed off. The valves were re-opened and the pump switched on.

Demineralisation equipment

Table of use and replacement of demineralisation solutions.

	Started		Stopped	
Solution	**Date**	**Time**	**Date**	**Time**
1	14-5-99	11.00 h	15-5-99	10.00 h
2	15-5-99	10.00 h	16-5-99	9.30 h
3	16-5-99	9.30 h	17-1-99	12.30 h

A 1 litre sample was taken of the effluent of solutions 1 and 2, and a 2 litre sample of solution 3 effluent.

Reaction flask demineralisation

Washing.

The reactor was closed and all hoses removed, bagged, and disposed off. The reactor was removed from the stand, covered with a polythene bag and transferred to the cabinet. In the cabinet the bagged reactor was fitted in a lab stand. To drain the reactor a short piece of tubing was connected from one of the two valves at the bottom, to a beaker covered with foil to prevent splashing. By opening this valve the reactor was drained for 15 minutes. The second valve was connected to a funnel and enough water was poured in to fill the reactor. This was left to stand for 60 minutes before draining as before. The filling and draining of water was repeated once. The demineralised bone, or ossein, was then transferred to a 2 litre glass beaker (Scott DURAN). Samples were taken of all drained liquids and stored frozen. The reactor and all connected tubing was bagged and disposed of. The ossein was stirred for 10 minutes with 1100 ml of water (Heidolph RZR 2020 stirrer with PE two blade stirrer Kartell 431). After taking a sample of the water this was pumped out. Then 600 ml of water was added to the beaker, which was left to stand

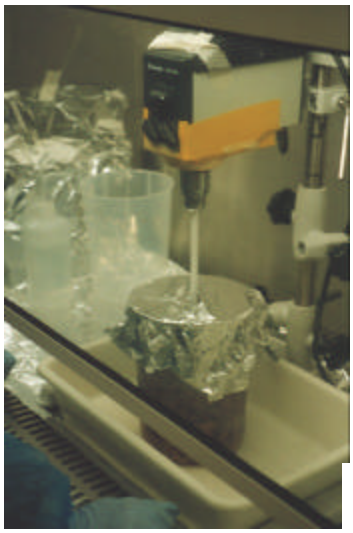
Washing of ossein

overnight. The equipment on the bench which had not been (potentially) in contact with infectivity, the stirrer and stirrer stand in the cabinet were bagged and stored until further use. All other equipment was disposed of.

After standing overnight the pH had gone down to pH 1.8. The water was pumped out. The ossein was then stirred two times for half an hour with 1000 ml of fresh water after which the pH of the water was measured and the water was drained by pumping. The pH was pH 2.5 after the first time stirring and pH 2.7 after the second time.

A sample was taken of the ossein, approximately equal to 20 g of original bone.

The washed ossein was set aside, equipment which had been in contact with infectivity was disposed of. Other equipment was taken from the cabinet in bags and the cabinet was decontaminated as before.



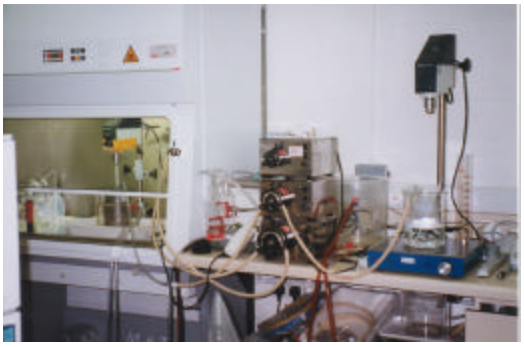
Washing the ossein

Reference: Notebook 19-3-99 to 25-5-99 page 88-92. Date 14-5-99 to 19-5-99.

Extraction.

Building up equipment.

The extraction equipment was built up in the safety cabinet and on the adjacent bench. (see drawing and pictures below). The equipment consisted of a 2 litre double walled beaker with a stainless steel basket and a lid. A two blade polyethylene stirrer and a thermometer were inserted through holes made in the lid. Water was heated in a circulating thermostatic waterbath and pumped into the wall of the beaker to warm the contents and maintain a constant temperature inside the beaker. Water was also heated on a hot plate and pumped into the beaker cavity using a peristaltic pump. The obtained gelatine solution was collected in an Erlenmeyer flask after passing it through a funnel with a 0.1mm mesh to prevent passage of crude solid material.



Extraction equipment

Extraction.

The conditioned ossein was put into the basket in the double walled beaker. The stirrer was put in place, the lid of the beaker closed and the thermometer was inserted. The circulating thermostat was switched on and adjusted to 63°C to maintain a temperature of 60°C in the double walled beaker. A beaker of water was heated to 60°C on the hotplate and on 19-5-99 at 13.30 hrs, 800 ml of it was pumped into the double walled beaker. Stirring was started and continued to 15.30 hrs when the equipment was then switched off and was left to stand

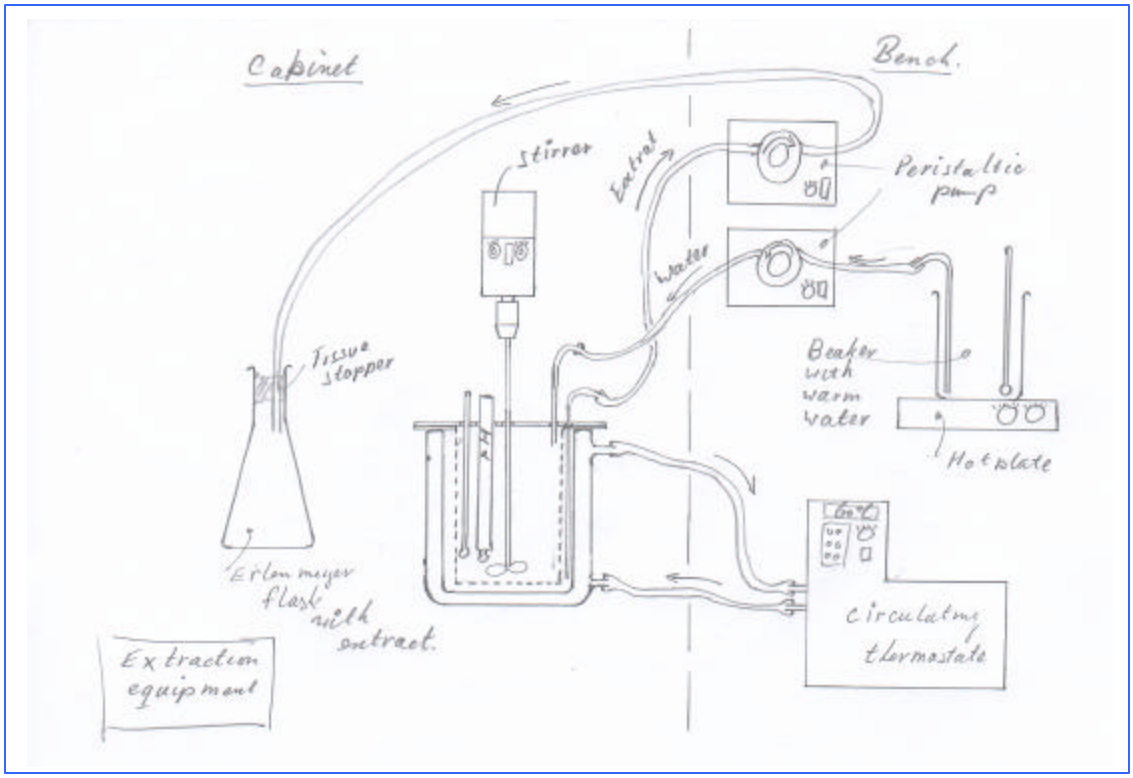


Measuring pH and index of refraction

overnight. At 9.00 hrs on the next day it was switched on and when the gel that had formed overnight had dissolved stirring was started. At intervals the extraction temperature, the extraction pH and the index of refraction (a measure for the gelatine concentration) were measured (see tables below). When the index of refraction reached 6, the extract was pumped into a 1 litre Erlenmeyer flask (Schott DURAN) after passing a funnel with a 100µm nylon mesh, and was left to cool. The siphons and the pumping tube had been pre-warmed with hot water to prevent gelling of the gelatine. Approximately 750 ml gelatine extract was obtained. Not all gelatine solution was pumped out.

Day	Time	Temperature	pH	Brix
19-5-99	13.30	switched on		
	15.30	59°C	2.3	2.0
	15.30	switched off		
20-5-99	9.00	switched on		
	11.30	59°C	2.7	6.0
	12.15	60°C	2.7	6.0

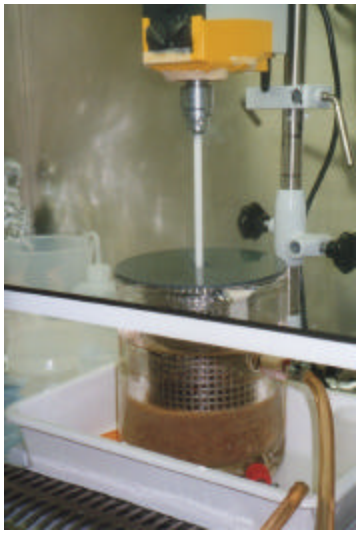
Pumping extract to Erlenmeyer



Extraction equipment

List of equipment:
Heidolph electronic stirrer RZR 2020 cat no 5141 12100, with PP two blade stirrer)
Hanna pHep 2 pocket pH meter)
Circulating thermostat bath Jubalo MW12)
Thermometer –10…110° cat no 67370413
Handrefractometer 0-32 Brix Euromax RF233
Tempering beaker 2 litre custom made
Basket custom made
Ring custom made
Lid custom made
Small siphon custom made
Small tube custom made
2 Verder Pericor peristaltic pump 9F-240
Pumping hose Verderprene 8x1.6 cat no 58 84 81680
Erlenmeyers flasks 1000 ml Scott DURAN cat no AS112121654
Rubber stoppers

The circulating thermostat was left at 63°C, to maintain an extraction temperature of 60°C. 700 ml water of 63°C was pumped in and stirring restarted.
A maximum index of refraction of 5.8 Brix was recorded and the gelatine solution was pumped out and left to cool. Approximately 700 ml extract was obtained which after cooling of was set aside.



Extraction of gelatine

Day	Time	Temperature	pH	Brix
20-5-99	12.30			
	16.00	58°C	3.1	4.0
	17.15	59°C	3.1	4.5
	17.50	switched off		
22-5-99	9.30	switched on		
	10.45	59°C	3.0	5.2
	13.30	59°C	3.1	5.8

The circulating thermostat was set at 78°C, to maintain an extraction temperature of 70°C. 400 ml water of 70°C was pumped in and stirring restarted. A maximum index of fraction of 3.7 Brix was reached at which point no more ossein remained. Therefore the gelatine solution was pumped out of the double walled beaker in the same fashion as before. Approximately 400 ml extract was obtained and cooled. There was no residue left in the double walled beaker.

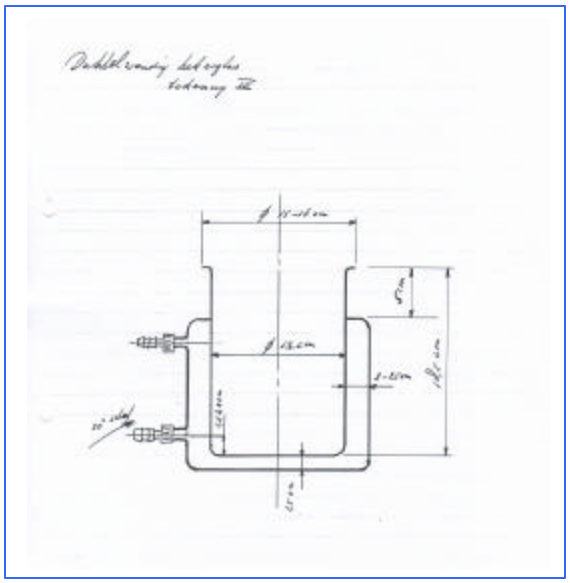
Day	Time	Temperature	pH	Brix
22-5-99	17.00	70°C	3.0	3.7

Of each of the 3 extracts a sample was taken: 40 ml of extract 1, 35 ml of extract 2 and 25 ml of extract 3. A mixed volume proportional sample was taken of all three extracts for inoculation into the mice: 40 ml of extract 1, 35 ml of extract 2 and 20 ml of extract 3.

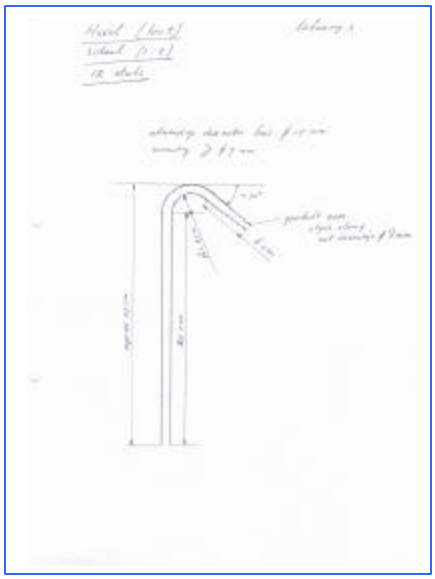
The total volume of obtained extracts was 1850 ml of which 1650 ml was left after the sample had been taken.

The equipment was broken down and the Heidolph RZR 2020 stirrer, the stirrer stand and the refractometer were bagged and stored. All other equipment in the cabinet was disposed of and the cabinet decontaminated.

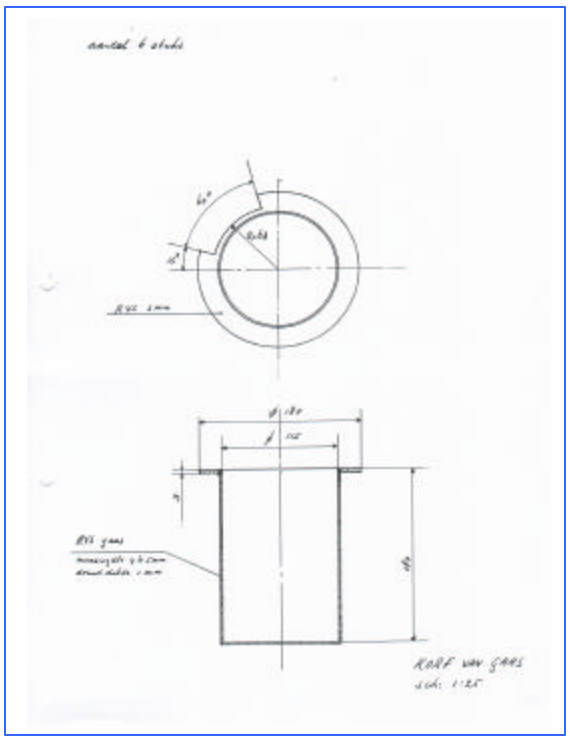
Reference: Notebook 19-3-99 until 25-5-99 page 93 until 96. Date 19-5-99 until 22-5-99.



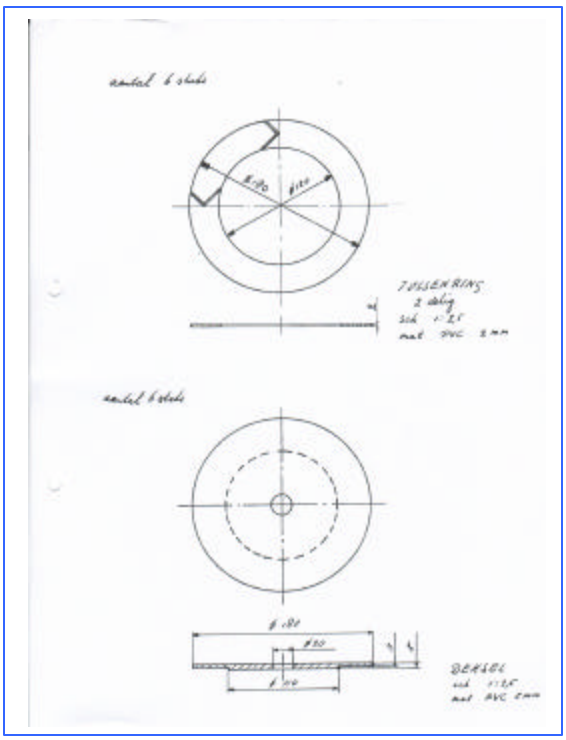
2 litre double walled beaker



siphon



Basket

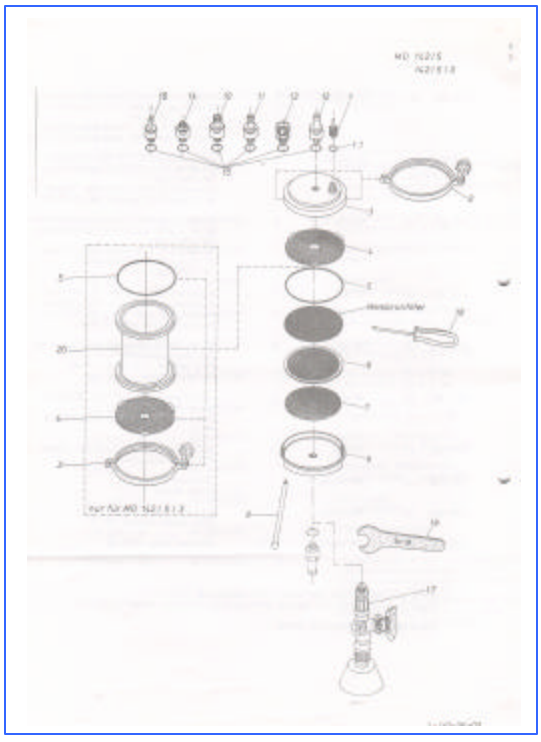


Lid and ring

Filtration.

The filter equipment was set up in the safety cabinet. It consisted of a Stainless steel Schleicher& Schuell Pressure filter MD142. Pressure was applied with a Vacuumbrand Membrane compressor ME4P, which was connected to the filter by a Schleicher & Schuell pressure tube belonging to the filter. A silicon tube was attached at the bottom of the filter which ran into a 10 litre polythene waste fluid bottle. A 2 litre double walled beaker, partly filled with water, was used as a waterbath to warm the gelatine extract. The double walled beaker was connected to a circulating thermostatic waterbath Jubalo MW12. On the bench water was heated in a 3 litre Schott DURAN beaker on a Schott-Geräte CERAN hotplate. The water was pumped into the filter with a Verder Pericor peristaltic pump 9F-240 through a Verderprene 8 x 1.6 pumping tube (LS cat no 5884 81680) equipped with a custom made glass siphon.

Filtering equipment



Schleicher & Schuell Pressure filter MW142

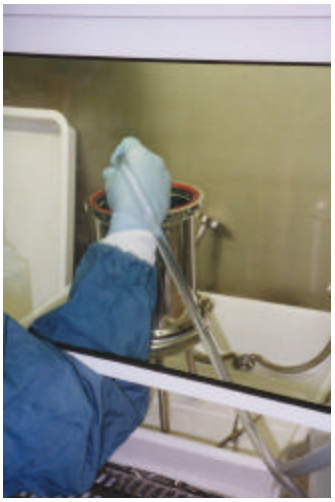
Filtration was done in three steps. First the gelatine extract was filtered through cloth cut from a GAF filter bag (GAF NMO-100-P01S-60L), then through an approximately 1 cm thick layer of diatomaceous earth (Dicalite 4200) and finally through a Schenk AF1000 pre-prepared filter sheet.

After each filtration the filter was filled with a hypo chlorite solution containing 20.000 ppm free chlorine which was held for 1 hour. The filter was then washed 5 times with hot water. The O-rings of the filter were replaced by new ones be fore each next filtration.

Filtration with filter bag cloth.

A circular piece GAF filter bag was cut to fit the perforated base plate of the filter unit. The lid of the filter was removed and water, at a minimum of 80°C, was pumped into the filter to preheat the unit. The water was drained into the polythene bottle, the filter disassembled and the filter cloth installed, after which the filter was re-assembled. The filter cloth was wetted with approximately 3 to 5 ml of water. A silicon tube ran from the bottom of the filter into an Erlenmeyer flask, the neck plugged with a thick wad of tissue. The silicon tube was closed with an artery clamp. The Erlenmeyer flask with the warm gelatine extract was taken from the double walled beaker and the gelatine poured into the filter. The lid was re-placed on the filter and the artery clamp removed from the silicon tube before a tiny amount of pressure was applied and the gelatine was filtered through the filter cloth. The apparatus was opened again, the silicon hose placed in a second Erlenmeyer flask and the second gelatine solution was poured into the filter and was filtered as before. The same was done for the third solution. The filter was disassembled and the piece of filter cloth removed. The filter was re-assembled, rinsed with water and then treated with sodium hypochlorite as described above.

S&S pressure filter

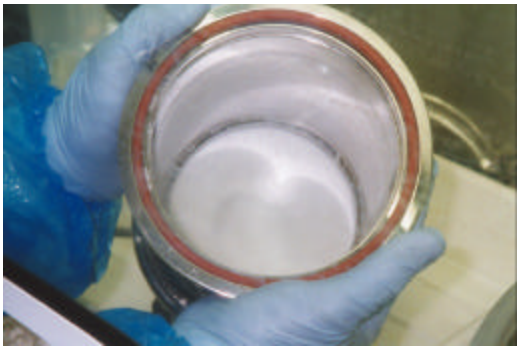

Filling with hot water

Total volume gelatine extract filtered	1650 ml
Total volume filtrate obtained	1600 ml

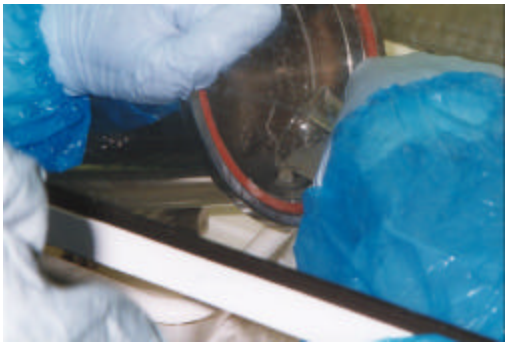
Two 25 ml sample were ta ken from the filtrate.

Filtration with diatomaceous earth.

The filter was preheated with hot water as described above after which it was disassembled. A piece of filter paper (Schleicher and Schuell) was put in place on the perforated base plate before re-assembly. The silicon hose at the bottom was closed with an artery clamp and the filter was filled again with hot water.


Diatomaceous earth filter layer

40g Dicalite 4200 (diatomaceous earth) was suspended in the water by stirring with a plastic disposable spoon. The artery clamp was removed and the water drained slowly to make a filter layer on the filter paper. A small pressure is applied for a few second to remove most of the water from the filter layer. 2 g Dicalite 4200 was added and well suspended in the gelatine extract solution to be filtered. The end of the silicon tube was connected to a new Erlenmeyer flask as before. The tube was closed with an artery clamp. The filter was tilted and the gelatine solution gently poured in to prevent damage to the filter layer. The lid was closed and the artery clamp removed. Filtration was started and maintained by applying pressure on the filter. When all the solution had been filtered, the filtration was repeated in the same way for the remaining two extracts. The apparatus was then taken apart and the filter cake removed. The filter was re-assembled , rinsed with water and then treated with sodium hypo-chlorite as described above.



Filling filter with gelatine extract

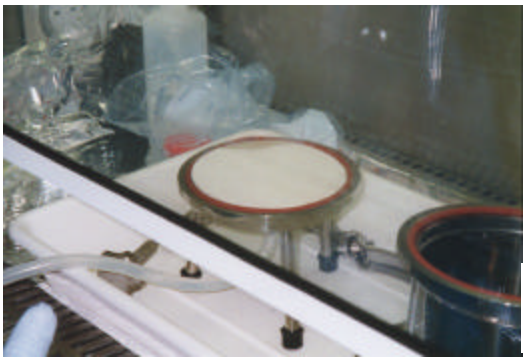
Total volume gelatine extract filtered 1550 ml

Total volume filtrate obtained 1800 ml

Two 25 ml samples were taken from the filtrates.

Filtration with Schenk AF1000.

The filter was preheated with hot water as described above after which it was disassembled. A circular filter was cut from a Schenk AF1000 filter sheet and put in place on the perforated sheet on the bottom of the filter and the filter was assembled. The silicon hose on the bottom was closed with an artery clamp. The end of the silicon tube was put in a new Erlenmeyer flask as before. The first gelatine filtrate from the diatomaceous earth filtration and half of the second one were poured into the filter. The lid was closed and the artery clamp removed. Filtration was started by applying the pressure on the filter. A steady flow was maintained by regulating the pressure with the compressor. When all gelatine was filtered the compressor was switched off. The filtration was repeated in the same way for the remaining filtrate from the



Schenk filter sheet

diatomaceous earth filtration. The apparatus was then taken apart, the filter material removed, re-assembled, rinsed with water and treated with sodium hypo-chlorite as described above.

Total volume gelatine extract filtered 1750 ml

Total volume filtrate obtained 1700 ml

Two 25 ml sample were taken from the filtrates.

When comparing the original gelatine extract with the samples, the gelatine became clearer after each filtration step.

After completion of the entire filtration all equipment in the laminar flow cabinet, including hoses coming in from outside was disposed of. The safety cabinet was decontaminated.

Reference: Notebook 19-3-98 until 25-5-99 page 96 to 100. Date 23-5-99 to 25-5-99
Notebook 25-5-99 until 9-9-99 page 1 to 2. Date 25-5-99

Ion-exchange

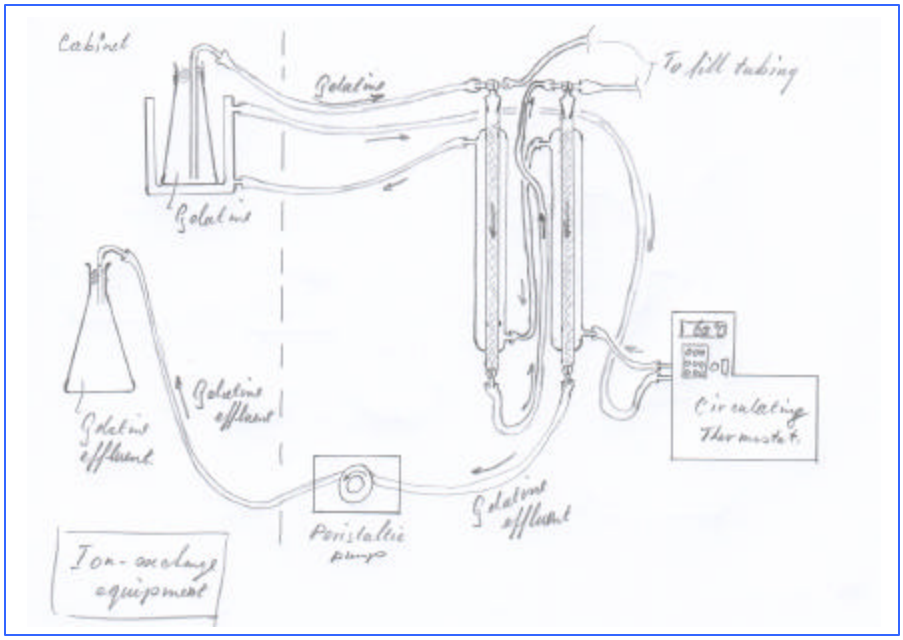
Setting up equipment and regeneration of the resins.

The ion-exchange columns had been used earlier in a blank experiment and were cleaned by filling these with hypo chlorite solution which was kept in for 1 hour, after which the columns were washed 5 times with water.

The scaled down ion-exchange equipment was still too large to fit in the safety cabinet but, as it was completely closed equipment, could be set up on the adjacent bench. The equipment consisted of two columns in sequence. (The pictures show two sets of columns) The outlet of the second column was connected to a Verder Pericor peristaltic pump 9F-240 to draw the gelatine solution through the columns by negative pressure. The open ends of the inlet and outlet tube were run into the safety cabinet.

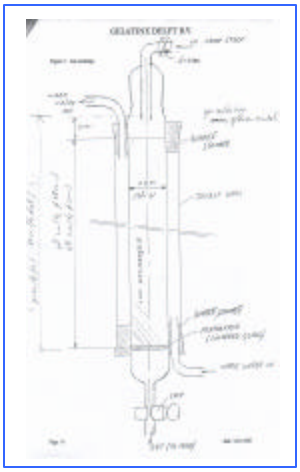
Ion-exchange equipment

The tubes in contact with the gelatine were silicon tubes, except for the Verderprene pumping tube. Water was pumped into the columns from the bottom to approximately half



Ion-exchange equipment

List of equipment:
2 ion-exchange columns custom made
Double walled beaker 2 litres custom made
Pumping thermostat bath Jubalo MW12
Erlenmeyers 1000ml Schott DURAN
Rubber stoppers
Verder Pericor peristaltic pump 9F-240
pumping hose Verderprene 1.6x1.6 cat no 58 84 82
Silicon hose of different diameters Deutch & Neumann)
PVC hose 8x10
Schott-Geräte CERAN hot plate type 930 00 1800W cat no 43 27 93000 (67740401)
Beaker Schott DURAN 3000 ml cat no 11 21 10668)
Small siphon custom made
Small tube custom made
2 Hanna instruments pocket pH meters pHep -2
1 Hanna instruments pocket conductivity meters 100-19900 µS type 4
2 Hanna instruments pocket conductivity meters 10-1990 µS type 3



the column volume. The columns were then filled with the appropriate ion-exchange material by adding the resin as an emulsion in water to the top of the column. Attention was given to prevent formation of air inclusions. The first column was filled with cation resin Amberlite 200CNa (Rohm & Haas lot 6218PE60) to a bed length of 70 cm, the second column was filled with anion resin Amberlite IRA G4S (Rohm & Haas lot 6218AB32) to a bed length of 65.5 cm. After filling, the columns were closed and slowly filled with water pumped up the column. 3 litres of boiled, cooled water was then pumped from top to bottom through each of the columns, with the peristaltic pump, to wash the resins and to remove any dissolved and adhered air. The cation column was regenerated with 3 litre 5% hydrochloric acid followed by washing with water until the pH of the in-flowing water and out-flowing water was equal within 0.5 pH units. The anion column was regenerated with 3 litre 5% sodium hydroxide and rinsed with water in the same way. Approximately 5 litres of water were required to wash each column. The columns were then washed with 3 litres of cold boiled water and the flow adjusted to 2,200 ml/hour.

Ion-exchanging the filtered gelatine extract.

Gelatine forms a solid gel at room temperature, so to allow flow through the ion-exchange equipment, the extract must be heated at a temperature above 50°C. The flask of gelatine extract was placed into a 2 litre double walled beaker, partly filled with water. The wall of the beaker was filled with water heated to 62°C and circulated using a Jubalo MW12 circulating thermostatic waterbath. The water was also circulated through the water mantle of each ion-exchange column to permit flow. To preheat the equipment the circulating thermostatic waterbath was switched on and 3 litres of boiled water cooled to 60°C was pumped through the columns. The filtered gelatine was then drawn through the columns. The pH and conductivity of the filtered gelatine had been measured. When a change in the index of refraction of the effluent was observed, it was collected in new Erlenmeyer flasks. When the input gelatine solution was exhausted, water was pumped through to remove any gelatine from the columns. Collection of the effluent continued until the index of refraction was equal to water, after which the equipment was switched off. The pH and conductivity of the effluent was measured. The Erlenmeyer flasks with effluent were closed with new rubber stoppers and set aside. A 50 ml sample was taken from the first flask of effluent.

Ion-exchange of gelatine extract

Filtered gelatine extract in:

Total volume approximately 1650 ml.

pH = 3.3 Conductivity = 2700µS

Effluent ion-exchange out:

Total volume approximately 2000 ml.
pH = 7.1 Conductivity = 21µS

For each measurement of pH and conductivity a new meter was used.

(pH: HANNA instruments pocket pH meter pHep-2)
(Conductivity: HANNA instruments pocket conductivity meter type 3 10-1999 µS
HANNA instruments pocket conductivity meter type 4 100-19990 µS)

The columns, tubing and all equipment used in the cabinet was disposed of. The cabinet was decontaminated.

Reference: Not ebook 25-5-99 until 9-9-99 page 3 to 6. Date: 25-5-99 to 27-5-99

Concentration.

The gelatine solution obtained from ion-exchange was concentrated using a Heidolph Laborota 4000 film-evaporator with a Heidolph WB waterbath. Vacuum was applied (80-100 mm Hg) with a Verder KNF Laboport vacuum-pump with a Hepafilter attatched to the inlet and outlet. The solution was concentrated as two aliquots of 750ml; 500 ml from Erlenmeyer flask 1 and 250 ml from Erlenmeyer flask 2. The waterbath was heated to 60°C, 250 ml of solution was added to the evaporator flask and evaporation carried out until approximately 200 ml water was collected, before addition of another 250 ml of solution. Finally, the solution was concentrated to approximately 20% and transferred to a 100 ml DURAN sample bottle.

Rotavapor equipment

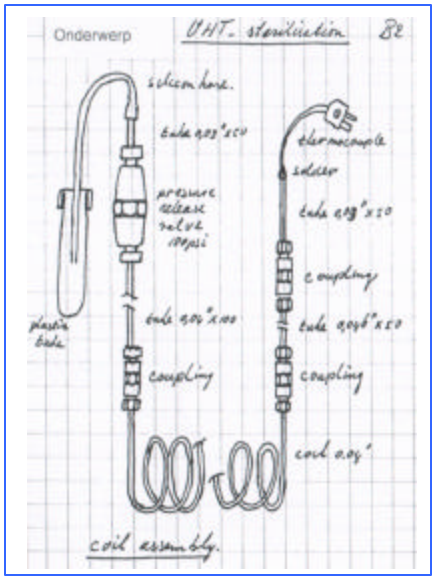
Evaporation 1: Approximately 450 ml from Erlenmeyer flask 1 and 450 from Erlenmeyer flask 2.
Concentrate: 80 ml of 24 Brix.

Evaporation 2: Approximately 450 ml from Erlenmeyer flask 1 and 400 ml from Erlenmeyer flask 2.
Concentrate: 110 ml of 16 Brix.

Reference: Notebook 25-5-99 until 9-9-99 page 7. Date: 27-5-99 to 29-5-99

UHT-sterilisation.

UHT sterilisation was repeated in stainless steel capillaries as described below.
The gelatine to be sterilised was contained in a closed stainless steel coil, closed at the one end by a thermocouple and at the other end by a pressure release valve (PRV). This coil was immersed in oil of 140°C. The temperature of the gelatine was measured by the thermocouple inside the coil in the gelatine. When the temperature recorded by this thermocouple reached 138°C, a 4 seconds time period was measured. After 4 seconds the coil was cooled in a bath with cold water.



Inserting the thermocouple would displace part of the infective gelatine. The PRV at the other end of the coil was to prevent building up of high pressure on heating, for the coefficient of expansion of gelatine solution is larger than of stainless steel. On heating some liquid would escape through the PRV. To prevent infective material to leave the equipment, to the thermocouple end of the coil a 0.046"x 50 mm tube filled with commercial gelatine was connected, and to the other end, between the coil and the PRV, a 0.04"x100mm tube filled for one quarter with commercial gelatine and the remaining three quarters with water. On inserting the thermocouple the commercial gelatine would be displaced. On heating only water, held in place by commercial gelatine would pass the PRV.
Care was taken that the filled assembly did not contain air bubbles.

The complete coil consists of the following parts:

StSt coil 0.04'x 2 m:	SSt Sample loop 2 ml. Upchurch Scientific cat no 1872
3 StSt couplings:	SS True ZDV Union .062". Upchurch Scientific cat no U-438
StSt tube 0.046"x 50 mm:	SS Tube 0.046" x 1/16"x 5 cm Upchurch Scientific cat no U-145
StSt tube 0.03"x 50 mm:	SS Tube 0.030" x 1/16"x 5 cm Upchurch Scientific cat no U-115
StSt th.couple type K, 0.5 mm:	RS Components cat no RS 219-4337
StSt tube 0.04"x 100 mm:	SS Tube 0.040" x 1/16"x 10 cm Upchurch Scientific cat no U-139
StSt tube 0.046"x 50 mm:	SS Tube 0.020" x 1/16"x 5 cm Upchurch Scientific cat no U-101
Pressure release valve 100 psi:	100psi BPR Assembly Upchurch Scientific cat no U-607

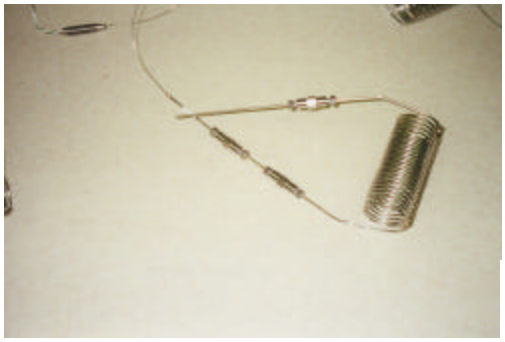
Piece of silicon hose inner diameter 1 mm
Centrifuge tube with orange cap 10 ml

Heating of the tube was done in two oil baths (Moulinex deep fryers filled with Sainsbury corn oil). The first was set to 160°C and was used for quick heating of the capillary to approximately 130°C. The second was set at 140°C. The coil was held in the 140°C bath for 4 seconds. The tube was then quickly cooled in a tray with cold water.

The temperature was measured with an electronic digital multimeter (Voltcraft M3860M) connected to Toshiba Libretto laptop computer.

Assembling the coil.

A coupling was connected to each end of the coil. To one coupling the 0.04"x 100mm tube was connected, to the other the 0.046"x 50 mm tube. The couplings were fitted with care, ensuring the correct distance between the tube-ends and correct tightening of the couplings. To the free end of the 100mm tube the PRV was connected. A 0.02"x 50 mm tube was. connected to the outlet of the PRV. A 50cm x 0.5mm type K thermocouple was soldered into the 0.03"x 50 mm tube with silver solder such that after assembly of the equipment the tip of the thermocouple would be positioned at the start of the first turn of the capillary coil. The other end of the tube with the thermocouple was closed with two-component poly-urethane cement. The 0.02"x 50 mm tube at the PRV was connected to a piece of silicon hose run into a centrifuge tube.



Coil assembly for UHT sterilisation

Determining the offset of the thermocouples.

The offset of the thermocouples was determined in one of the oil baths against the thermocouple on channel 1 of a Kane May 1242 electronic temperature recorder. The thermocouple of the Kane May 1242 had been calibrated against a calibrated thermometer.

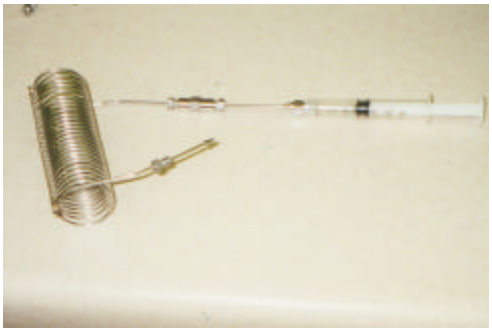
Filling of the coil and tubes, assembling the coil assembly.

A syringe was filled with molten unspiked commercial gelatine, the syringe was then fitted with a blunt 16 G needle connected to a coupling. The syringe and needle were pre-warmed in an electric heating pad to prevent gelling of the gelatine. This allowed the syringe to be screwed to a 0.046" x 50 mm tube which after filling with gelatine was left to cool and gel. Once solidified, the syringe was removed ensuring the cavity of the coupling was filled with gel.
Similarly, a 0.04"x 100 mm tube was connected to the syringe of unspiked gelatine, the tube ¼ filled with gelatine and allowed to gel. A 30G needle with a thin silicon hose was attached to a syringe filled with water. The hose was put in the 0.04"x 100 mm tube. The remainder of the tube was filled with water and fitted to the PRV. The syringe with commercial gelatine was then

UHT-sterilisation equipment

removed from the other end, again such that the cavity of the coupling was filled with gelatine.

A 3 ml syringe was warmed in a heating pad and filled with the concentrated experimental gelatine, ensuring any air bubbles were removed. A pre-warmed needle with a coupling attached was put on the syringe and connected to a pre-warmed steel capillary coil. A short length of tubing draining into a 15 ml plastic tube was connected to the other end of the coil. All connections were then tightened. The coil was replaced in the heating pad then filled with the concentrated gelatine by depression of the syringe plunger. This was continued until gelatine was seen to run into the tube at the far end, indicating the coil was filled. The heat pad was removed and the gelatine allowed to solidify, at which point the plastic tube and tubing were removed and the pressure release fitting was attached. At the filling end, the syringe and coupling was removed from the coil and the 0.046"x 50 mm tube was connected to the coil.



Filling the coil with gelatine

Finally the thermocouple was inserted through the 0.046"x 50 mm tube. Insertion of the thermocouple displaced a small amount of gelatine. The gelatine displaced was carefully removed with a tissue. All connections were checked and secured. The thermocouple was connected to the electronic multimeter. (Voltcraft M3860M)

UHT-sterilisation.

Two oil baths were heated up, one set at 160°C, the other at 140°C. The electronic multimeter and the laptop computer were switched on and tested. When the oil bath temperatures stabilised, the coil was immersed in the 160°C bath until the temperature reached approximately 130 to 135°C, then transferred to the 140°C bath. The coil was immersed and used to stir the bath for approximately 4 seconds then plunged into iced water.

Heating the coil in hot oilbaths

The temperatures of the oil baths were measured with thermocouples recorded with a Kane May 1242 electronic temperature recorder.

Retrieval of the sample from the coil.

The coil assembly was left to cool, allowing the gelatine within to solidify. To open the coil, both ends were partly cut using a file and then broken off with pliers. Firstly, the end with the pressure release valve was removed and a length of sterile silicon tubing connected which

drained into a 15 ml sterile plastic tube. A 10 ml syringe with a blunt needle and coupling was attached to the other end. The coil was then warmed in a water bath of approximately 50°C and the gelatine was pressed out into the plastic tube by depressing the plunger of the syringe. The tube was taken from the assembly, closed and labelled. The sample was stored at –20 °C. Approximately 1.5 ml sample was obtained from each test.

Retrieval of sterilised gelatine from the coil

First experiment with coil assembly.

The UHT sterilisation was repeated in stainless steel capillary assemblies as described above. The temperature was read on the display of an electronic digital multimeter and recorded by a Toshiba Libretto laptop computer connected to the multimeter. The sterilisation time was measured with a stopwatch which was started when the temperature on the display reached 138°C. When the temperatures recorded by the computer were displayed however it appeared that the display of the multimeter was approximately 3 seconds delayed, which resulted in a sterlisation time 3seconds to long and a temperature overshoot of several degrees. Therefore the test had to be repeated.

Reference: Notebook 9-9-99 until 14-12-99 page 59 to 65. Date: 19 to 22-10-99

Second experiment with coil assembly.

Determining the offset of the thermocouples.

The offset of the thermocouples was determined in one of the oil baths against the thermocouple on channel 1 of a Kane May 1242 electronic temperature recorder. The thermocouples of the Kane May 1242 were calibrated against a calibrated thermometer.

Calibrated thermometer:	135.9°C
Thermocouple on Ch1 Kane May:	136.9°C.
Result:	Thermocouple measurement: + 1°C

Measurement offset thermocouples of coil assemblies.

Tested thermocouple number	Temperature of calibrated thermocouple	Temperature according tested thermocouple	Calculation offset	Offset
K10	142.3	136	142.3 - 1 – 136	**5.3**
K21	139.3	134	139.3 - 1 – 134	**4.3**
K22	137.0	133	137.0 – 1 – 133	**3.0**
K23	137.9	133	137.9 – 1 – 133	**3.9**

UHT-sterilisation.

The test was done as described, but the coil was kept in the 160°C oil bath for approximately 2 seconds, then transferred to the 140°C oil bath in which it was kept for 4 seconds, and then plunged into iced water.
The test was repeated 4 times, the details of which are shown below.

Results

Summary of the results

E8K10 and E8K22 both showed an overshoot to 146°C

E8K21:	rise time to 133°C	3.3 seconds
	Temp above 120°C	6.1 seconds
	Temp above 133°C	4.6 seconds
	Temp above 137°C	3.3 seconds
	Rise time to 137°C	4.3 seconds
E3K24:	rise time to 133°C	1.9 seconds
	Time above 120°C	7.4 seconds
	Time above 133°C	6.1 seconds
	Time above 137°C	4.4 seconds
	Rise time to above 137°C	2.5 seconds

Although the time above 137°C is 0.7 second to short, this test was the best approximation of the 4 seconds UHT sterilisation.

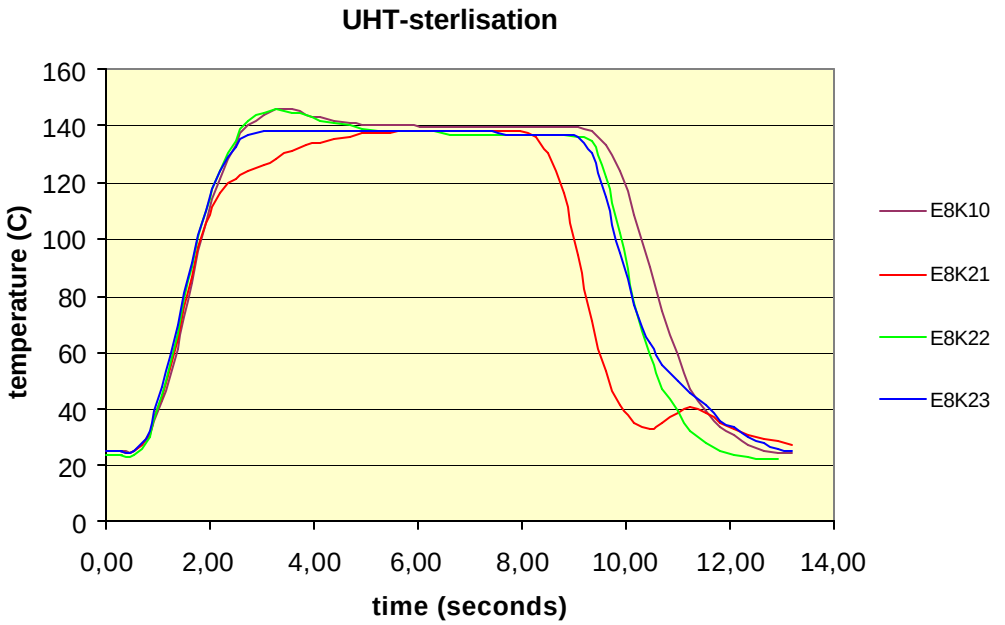
Sterilised sample E8K21 was injected.

Table of sterilisation temperatures

Sample E8K10 **Sample E8K21** **Sample E8K22** **Sample E8K23**

UHT sterlisation of E8			UHT sterlisation of E 8			UHT sterlisation of E8			UHT sterlisation of E8		
test E8K10		offset= 5.3	test E8K21		offset= 4.3	test E8K22		offset= 3.0	test E8K23		offset= 3.9
time (sec)	temp C	corr. temp	time (sec)	temp C	corr. temp	time (sec)	temp C	corr. temp	time (sec)	temp C	corr. temp
0,00	20	25,3	0,00	21	25,3	0,00	21	24	0,00	21	24,9
0,28	20	25,3	0,28	21	25,3	0,28	21	24	0,28	21	24,9
0,55	20	25,3	0,55	21	25,3	0,55	21	24	0,55	21	24,9
0,83	25	30,3	0,83	27	31,3	0,83	27	30	0,83	28	31,9
1,10	37	42,3	1,10	40	44,3	1,10	42	45	1,10	44	47,9
1,38	56	61,3	1,38	60	64,3	1,38	64	67	1,38	66	69,9
1,65	79	84,3	1,65	82	86,3	1,65	87	90	1,65	88	91,9
1,93	100	105,3	1,93	101	105,3	1,93	107	110	1,93	106	109,9
2,20	116	121,3	2,20	112	116,3	2,20	121	124	2,20	120	123,9
2,48	128	133,3	2,48	117	121,3	2,48	132	135	2,48	129	132,9
2,75	135	140,3	2,75	120	124,3	2,75	139	142	2,75	133	136,9
3,03	139	144,3	3,03	122	126,3	3,03	142	145	3,03	134	137,9
3,30	141	146,3	3,30	124	128,3	3,30	143	146	3,30	134	137,9
3,58	141	146,3	3,58	127	131,3	3,58	142	145	3,58	134	137,9
3,85	139	144,3	3,85	129	133,3	3,85	141	144	3,85	134	137,9
4,13	138	143,3	4,13	130	134,3	4,13	139	142	4,13	134	137,9
4,40	137	142,3	4,40	131	135,3	4,40	138	141	4,40	134	137,9
4,68	136	141,3	4,68	132	136,3	4,68	137	140	4,68	134	137,9
4,95	135	140,3	4,95	133	137,3	4,95	136	139	4,95	134	137,9
5,23	135	140,3	5,23	133	137,3	5,23	135	138	5,23	134	137,9
5,50	135	140,3	5,50	133	137,3	5,50	135	138	5,50	134	137,9
5,78	135	140,3	5,78	134	138,3	5,78	135	138	5,78	134	137,9
6,05	134	139,3	6,05	134	138,3	6,05	135	138	6,05	134	137,9
6,33	134	139,3	6,33	134	138,3	6,33	135	138	6,33	134	137,9
6,60	134	139,3	6,60	134	138,3	6,60	134	137	6,60	134	137,9
6,88	134	139,3	6,88	134	138,3	6,88	134	137	6,88	134	137,9
7,15	134	139,3	7,15	134	138,3	7,15	134	137	7,15	134	137,9
7,43	134	139,3	7,43	134	138,3	7,43	134	137	7,43	134	137,9
7,70	134	139,3	7,70	134	138,3	7,70	134	137	7,70	133	136,9
7,98	134	139,3	7,98	134	138,3	7,98	134	137	7,98	133	136,9
8,25	134	139,3	8,25	132	136,3	8,25	134	137	8,25	133	136,9

8,53	134	139,3	8,53	126	130,3	8,53	134	137	8,53	133	136,9
8,80	134	139,3	8,80	112	116,3	8,80	134	137	8,80	133	136,9
9,08	134	139,3	9,08	90	94,3	9,08	133	136	9,08	132	135,9
9,35	133	138,3	9,35	67	71,3	9,35	132	135	9,35	126	129,9
9,63	128	133,3	9,63	49	53,3	9,63	119	122	9,63	111	114,9
9,90	119	124,3	9,90	37	41,3	9,90	99	102	9,90	91	94,9
10,18	103	108,3	10,18	31	35,3	10,18	75	78	10,18	73	76,9
10,45	85	90,3	10,45	28	32,3	10,45	56	59	10,45	59	62,9
10,73	69	74,3	10,73	31	35,3	10,73	44	47	10,73	52	55,9
11,00	54	59,3	11,00	34	38,3	11,00	36	39	11,00	46	49,9
11,28	42	47,3	11,28	36	40,3	11,28	29	32	11,28	42	45,9
11,55	34	39,3	11,55	34	38,3	11,55	25	28	11,55	37	40,9
11,83	28	33,3	11,83	31	35,3	11,83	22	25	11,83	32	35,9
12,10	25	30,3	12,10	28	32,3	12,10	21	24	12,10	29	32,9
12,38	22	27,3	12,38	26	30,3	12,38	20	23	12,38	26	29,9
12,65	20	25,3	12,65	25	29,3	12,65	19	22	12,65	24	27,9
12,93	19	24,3	12,93	24	28,3	12,93	19	22	12,93	22	25,9
13,20	19	24,3	13,20	23	27,3				13,20	21	24,9
			13,48	22	26,3				13,48	21	24,9
			13,75	22	26,3						
			14,03	21	25,3						
			14,30	21	25,3						



Reference: Notebook 9-9-99 until 14-12-99 page 98 and 99. Date: 14-12-99
Notebook 14-12-99 until 17-1-99 page 1, 2, 13 to 24. Date: 14-12-99

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.

Thermometers and thermocouples.

Thermometers and thermocouples were calibrated against a calibrated mercury thermometer by placing both in the heated water or oil bath being measured and comparing the respective temperatures indicated. Thermocouples were calibrated while attached to the recording instrument. When temperatures were recorded with calibrated thermometers or thermocouples, this was noted in the reports.

Laboratory thermometers for general temperature measurements were disposed of after every test. These thermometers were not calibrated on every use, but a number of recordings were taken and checked against the calibrated thermometer.

Results of checking general use thermometers against a verified thermometer

Verified:	**26.5**	**30**	**62**	**70**	**78**	**81.2**	**92.5**
100°C:							
1	26	30	62	69	75	80	90
2	26.8	31	62	70	76	81	91
3	26.5	30.5	61	69	75.5	80	90
4	26.5	30.5	61	69	75.5	80	90
5	26.5	30	61	68	75.5	79	90
Max 150°C:							
1		31	63	71		81.2	92
2		30	62	70		82	93
250°C:							
1	27	31	63	71	77	81	92
2	27	30.5	62.5	70	76	82	93

Certificate verified thermometer:

LANDESAMT FÜR MESS- UND EICHWESEN THÜRINGEN

THURINGIAN STATE OFFICE OF METROLOGY AND VERIFICATION

EICHAMT FÜR MESSGERÄTE AUS GLAS
VERIFICATION OFFICE FOR MEASURING INSTRUMENTS MADE OF GLASS

Eichschein 144 09

Gegenstand der Eichung: Flüssigkeits-Glasthermometer

Identifikation: Laborthermometer

Auftraggeber/ Hersteller

Anzahl der Seiten des Eichscheines: 2

Ort und Datum der Eichung: Ilmenau, 1999.01.13

Das Messgerät entspricht den Eichvorschriften (EO 14.1 und PTB-A 14.1).

Gültigkeit der Eichung: 10 Jahre

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Ort und Datum: Ilmenau, 1999-01-14

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pH meters

The pH meters were calibrated before and after each measurement with standard buffer solutions of pH4 and pH 7 or pH7 and pH10.

Conductivity meters.

Conductivity meters were verified against each other with demineralised water and demineralised water to which a small amount of tap water was added. All meters gave the same reading in each test.

General observation.

The water used in all experiments was laboratory quality demineralised water, unless otherwise indicated.

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 less.
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	8.94 g
	====
Crushed bone	1546.0 g
Backbone	454.5 g
301V infective mouse brain	8.94 g
Total amount of spiked bone starting material	2010 g
	====

Correction factor for extracted gelatine

I.	Amount of sorted dried bone chips	443.2 g
	Sample taken of sorted dried bone before weighting	14.9 g
	Remaining amount of sorted dried bone	428.3 g
	Amount of sorted dried bone chips	428 g
	Bone weight equivalent of sample ossein	20 g
	Bone weight equivalent extracted	408 g

Calculated correction factor = (443/408) = 1.085

Correction factor for extracted gelatine 1.1

Correction factor for sterilised gelatine.

The correction factor is based on the amount of obtained gelatine and is corrected for samples taken.

Samples taken from extracts, filtrates and effluent ion-exchange:

Extracts:

	Initial volume	Samples	Final volume
Extract 1	750 ml	80 ml	670 ml
Extract 2	700 ml	70 ml	630 ml
Extract 3	400 ml	45 ml	355 ml

Filtrates:

All samples have been taken from the filtrates

Filter bag	2 x 25 ml from total 1650 ml
Diatomaceous earth	2 x 25 ml from total 1800 ml
Schenk filter sheet	2 x 25 ml from total 1750 ml

Estimated back to original extracts, corrected for change of volume:

	Extract 1	Extract 2	Extract 3
Filter-bag	19	19	12
Diatomaceous earth	17	17	10
Schenk filter	21	20	11
Totals	57	56	33

Apart from samples, gelatine was also lost in the filtration because of absorption by the filter media and by gelatine solution staying behind in the equipment. Water from the equipment added to the volume of the filtrate. These differences are compensate for by using the final amount of gelatine obtained in the calculation below.

Effluent ion-exchange:

The effluent is considered as one pool of material

Obtained effluent	2000 ml
Sample	50 ml
Effluent to concentration	1950 ml

Amount of concentrated gelatine: 0.80 x24 + 1.1 x 16 = 36.8 Brix

Correction factor = Corr.fact.$_{\text{samples before extraction}}$ x Corr.fact.$_{\text{losses in purification equipment}}$ x
x Corr fact.$_{\text{samples during purification}}$

= 1.085 x ((((7-0.8-0.57)x6+(6.3-0.7-0.56)x5.8+(4-0.45-0.33)x3.7)x1950/2000)/36.8)x
x1850/1655x1650/1600x1800/1750x1750/1700x2000/1950

= 2.57

Correction factor sterilised gelatine 2.6

Summary of important data for the calculation of the clearance factors

A. Total amount of 301V infected mouse brain on the bone starting material 8.94 g

B. Extracted gelatine

Total volume of extracted gelatine	**1850 ml**
Correction factor for extracted gelatine	**1.1**

C. Sterilised gelatine

Total volume concentrated gelatine	**190 ml**
Correction factor sterilised gelatine	**2.6**

Appendix 7: PREVENTION OF CROSS-CONTAMINATION.

The whole series of experiments was done in a new build laboratory room, 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 laminar flow cabinet, which was decontaminated at 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 different experiments, between the different steps of one experiment and within one step of an experiment.

Cross-contamination between different experiments was prevented by performing operations from only one experiment at a time and by keeping the different experimental equipment separate in the laboratory.
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 then decontaminated after every use with sodium hypochlorite solution (20,000 ppm for 1 hour min.).

Cross-contamination between steps of one 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 hypochlorite 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 dsposing 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. The filtration process used the same apparatus for all filtrations of the same experiment. It was however decontaminated using sodium hypochlorite solution after every filtration. (Care was taken to remove all remaining hypochlorite by intensive rinsing with water).

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 hypochlorite when there had been any risk of contamination.

Appendix 8: BIOASSAY PROCEDURES.

All samples for bioassay were produced in a Category 3 containment laboratory within IAH-E. These samples were then taken to the experimental animal unit also within IAH-E. All samples were administered by the intracerabral inoculation of 20 µl into weanling mice of the VM strain. To measure the infectivity of each sample, these were serially diluted and injected, (a.k.a. a titration).

Samples for bioassay in this study were:

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

Acid process - crude gelatine extract. Undiluted gelatine extract (10^0 log dilution) serially diluted to produce a series of log 10 dilutions. Each mouse was injected with 20µl of the appropriate dilution (see table).

Acid process - sterilised concentrated gelatine. Undiluted sterilised concentrated gelatine (10^0 log dilution) serially diluted to produce a series of log 10 dilutions. Each mouse was injected with 20µl of the appropriate dilution (see table).

To be prepared as follows:

	301V -infected mouse brain	Acid process - crude gelatine extract	Acid process - sterilised concentrated gelatine
10^0	N/A	18	
10^{-1}	2	18	18
10^{-2}	2	18	18
10^{-3}	2	18	18
10^{-4}	6	18	18
10^{-5}	6	18	
10^{-6}	6		
10^{-7}	12		
10^{-8}	12		
10^{-9}	6		

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 aluminium foil. 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). Titrated 23/5/01.

A 10% brain homogenate was prepared from the same undiluted macerated 301V-infected brain tissue used to spike the model gelatine process. 100 mg of macerate was weighed using a White's torsion balance then transferred to a new, sterile, glass, tissue homogenising tube. 0.9 ml of sterile 0.85% saline was then added to the tube and the tissue homogenised by grinding with a new, sterile glass pestle. The homogenate produced was then transferred to a glass test tube labelled as 10^{-1}. Using the syringe labelled 10^{-1}, 0.1 ml of homogenate was removed from the tube labelled 10^{-1} and deposited in the tube labelled 10^{-2}. Using the syringe labelled for saline, 0.9 ml of 0.85% saline was added to the tube labelled 10^{-2}. The resultant solution in the 10^{-2} tube was mixed by drawing up and down in the syringe labelled 10^{-2}. Using the same syringe, 0.1 ml of the 10^{-2} solution was removed to the tube labelled 10^{-3}. 0.9 ml of 0.85% saline was added to the 10^{-3} tube and the solution in this tube mixed using the syringe labelled 10^{-3}.
This process was continued up to and including the production of a 10^{-9} dilution.

Acid process - crude gelatine extract. Titrated 4/11/99 (10^{0} group 5/11/99).

The undiluted 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^{-5}. The warmed undiluted (10^{0} group) was also inoculated into mice.

Acid process - sterilised concentrated gelatine. Titrated 11/7/00.

The undiluted concentrated gelatine 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}. Due to the viscosity of this sample, it was not possible to inoculate mice with the warmed undiluted (10^{0} group), as it would not pass easily through the inoculation needle.

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. Duplicates were made of each individual mouse data card identifying the experimental group each mouse belonged to. These were kept separate to the cage to allow blind assessment of the mice throughout the experiment.

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. One foil-covered syringe rest was placed in the cabinet.

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. 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.

Appendix 9: TABLES.

Titration data of brain pools.

301V mouse brain pool 2
Titration 372S-1T/2
Final 358 days post injection.

-log dillution	Number of mice	Mice positive on 301V	Average incubation period (days)
1	2	2	109
2	2	2	112
3	2	2	126
4	6	6	141
5	6	5	161
6	6	4	193
7	11	1	205
8	12	0	-
9	6	0	-

Titration data of gelatine samples.

Sample of extracted gelatine
Titration 372J-1H
Final 658 days post injection.

-log dillution	Number of mice	Mice positive on 301V	Average incubation period (days)
0	15	15	163
1	16	10	213
2	14	0	-
3	17	0	-
4	18	0	-
5	17	0	-

Sample of sterilised gelatine
Titration 372J-1N
Final 604 days post injection.

-log dillution	Number of mice	Mice positive on 301V	Average incubation period (days)
-	-	-	-
1	15	0	-
2	16	0	-
3	17	0	-
4	18	0	-
-	-	-	-