Validation of the clearance of TSE agent by the alkaline 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 limed 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 $10^{7.7}$ ID_{50}/g, the crude extract $10^{1.8}$ ID_{50}/g, while no infectivity was detected in the sterilised gelatine (< $10^{1.3}$ ID_{50}/g). Calculated clearance factors were: $10^{3.7}$ ID_{50} for the process steps up to extraction, and >=$10^{4.9}$ 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 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 alkaline process (usually called limed bone process), a description of the experiments done, and all other data associated with this study.

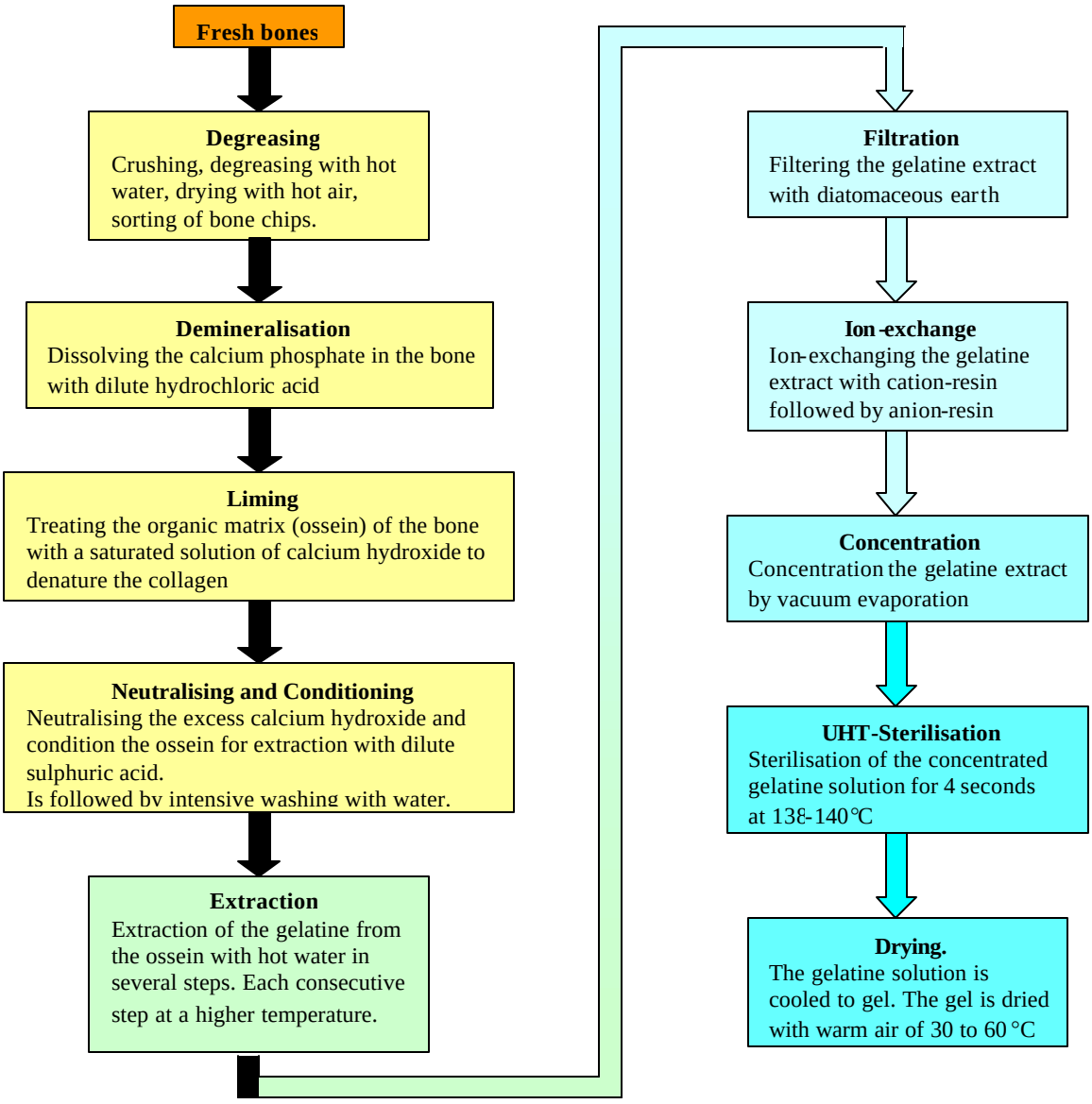
Industrial manufacturing process.

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

The Alkaline 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.

- Liming, during which the ossein is treated for at least 20 days with an oversaturated solution of calcium hydroxide, which is refreshed at regular intervals. After liming the ossein is washed to remove adhering lime.
- Neutralisation, in which the ossein is treated with dilute sulphuric acid to condition it for extraction. Excess acid is removed by washing several times with water.
- 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 limed bone gelatine manufacturers of the GME use the same manufacturing process. However, the process conditions of individual process steps can vary slightly between manufacturers (Appendix 1). The mildest industrial conditions identified for each process step were applied in the downscaled model process, hence reflecting real conditions but not favouring inactivation. The process was downscaled such that all steps of the industrial process were performed in the downscaled model process, using small amounts of material in laboratory-scale equipment. The laboratory process was thus representative for the industrial process used by GME members. The downscaling followed these principles:

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

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

Downscaled Alkaline manufacturing process with 301V spike.

The raw materials consisted of 2013 g of fresh crushed bone and backbone, which contained 10.14 g 301V infected mouse brain. The entire process was executed from degreasing up to UHT-sterilisation. The ossein produced by demineralisation was limed for 48 days. 1470 ml of crude gelatine was extracted from the limed ossein, then purified by filtration and ion-exchange, and concentrated to 125 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 infectivity titres.

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

symptoms of neurological disease. Animals which did not develop clinical disease were culled approximately 400 days (spike material) or 600 days (output samples) post-injection. The brains of all animals injected were removed and fixed in formol-saline. Sections were subsequently cut and stained with haematoxylin and eosin. These were examined microscopically for the spongiform lesions that are pathognomonic for 301V infection in mice. Using the ratios of positive and negative animals in each dilution group, the titre of infectivity in each sample was calculated by the statistical method of Kärber. (1931) (*Archives of Experimental Pathology and Pharmacology* **162**, 480-483)

Results

Table 1 contains the infectivity titres measured for the 301V spike material (titrated twice) 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
301V brain titration 1		$10^{7.74}$	420 days
301V brain titration 2		$10^{7.62}$	385 days
Limed bone process; extracted gelatine	Study 1 - Sample 1	$10^{1.82}$	666 days
Limed bone process; sterilised gelatine	Study 1 - Sample 2	No infectivity detected	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 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
Alkaline process - extracted gel.	Study 1 - sample 1	10.14	$10^{7.7}$	1470	1.1	$10^{1.8}$
Alkaline process - sterilised gel.	Study 1 - sample 2	10.14	$10^{7.7}$	125	2.6	$<=10^{1.3}$

Table 3. Clearance factors.

Sample name	Total clearance factor ID_{50}
Alkaline process - extracted gelatine	$10^{3.7}$
Alkaline process - sterilised gelatine	$>=10^{4.9}$

Discussion

The titration values recorded show a significant reduction of 301V infectivity of at least $10^{4.9}$ by the downscaled alkaline manufacturing process. A titre reduction of $10^{3.7}$ was observed following degreasing, demineralisation, liming and extraction. Filtration, ion-exchange and UHT-sterilisation added a further reduction of $\geq 10^{1.2}$. 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 meet 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 limed bone gelatine manufacturing process removed/inactivated 301V infectivity to below the level of detection.
2. A 301V infectivity reduction of more than $10^{4.9}$ ID_{50} was obtained by the downscaled model of the limed bone gelatine manufacturing process.
3. 301V infectivity was decreased substantially, with a factor $10^{3.7}$, by the first steps of the process; degreasing, demineralisation, liming and final washing. Most of the infectivity was removed/inactivated by these process steps.
4. Purification of the gelatine extract; filtration, ion-exchange and UHT-sterilisation, contributes to the removal/inactivation of infectivity.
5. The gelatine manufacturing process was successfully scaled down; gelatine was prepared from industrial starting material.
6. The study complies with the requirements of a validation study.

In conclusion, these data provide actual measurements of clearance factors for the alkaline bone gelatine manufacturing process that can be used to facilitate risk assessment of the safety of bovine derived gelatine with regard to BSE and human safety.

Appendix 1. INDUSTRIAL MANUFACTURING PROCESS

Short description of the limed 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 ossein, are washed with water and then treated for several weeks with a solution of saturated lime to partially break down the collagen and purify by breaking down and removing other organic components of the ossein. The lime is refreshed at regular intervals. The limed ossein is washed and then neutralised with dilute acid. After neutralisation the ossein is again washed several times. From the neutralised limed 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 stream 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 %

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

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

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

c. Fat emulsion of fat and small floating particles are decanted. The bone is immersed in a fast stream of hot water, in which the soft tissue and the bone chips 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 fact coupled with the cooling effect of the evaporation of water ensures the temperature of the chips will not normally exceed 85°C, although the air entering the drier can be heated to over 400°C. The drying time varies from 20 to 60 minutes and the size of the equipment and the amount of bones that are processed per hour varies between the different installations.
Modern installations are made of stainless steel whereas older installations are made of normal steel.
The amount of dried degreased bone chips obtained from 1 kg of crushed fresh bone is usually about 200g (20 %). The typical composition of these bone chips is:

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

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 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

Hence, 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.

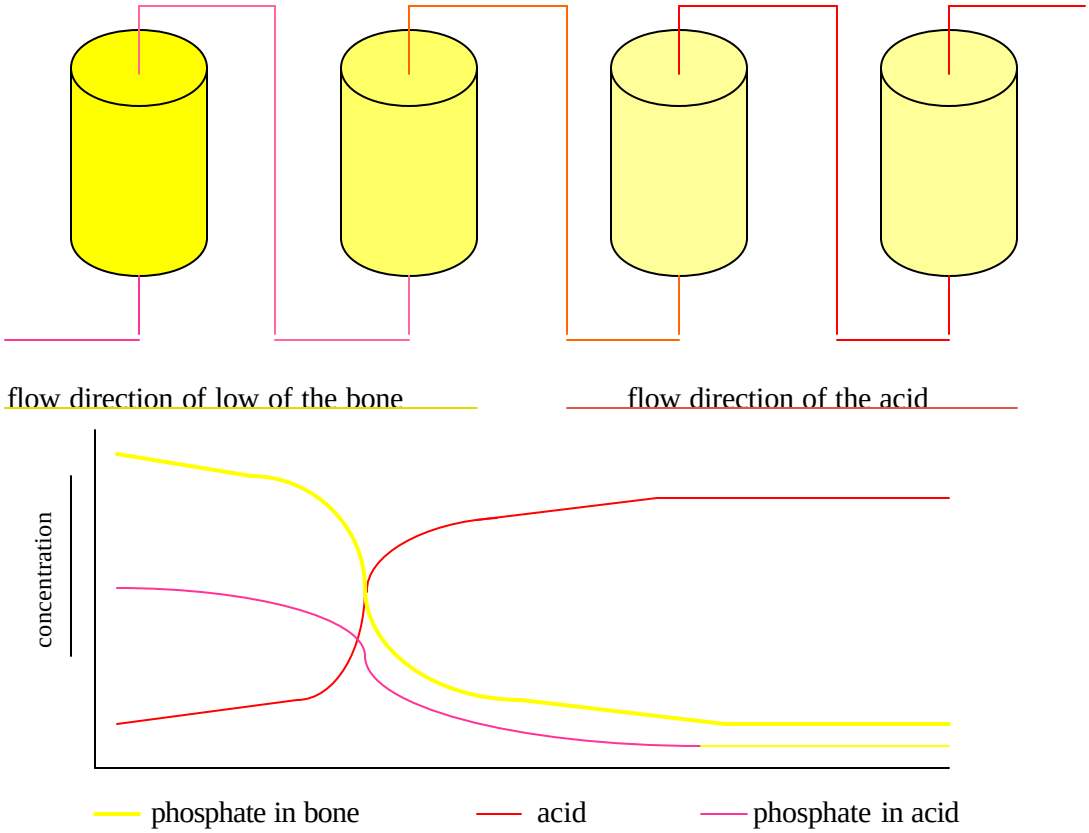


fig. 1 cascade process

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%.

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 of 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 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 take also an hour. Hereafter the ossein is pumped in a flow of water to the liming department.
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.

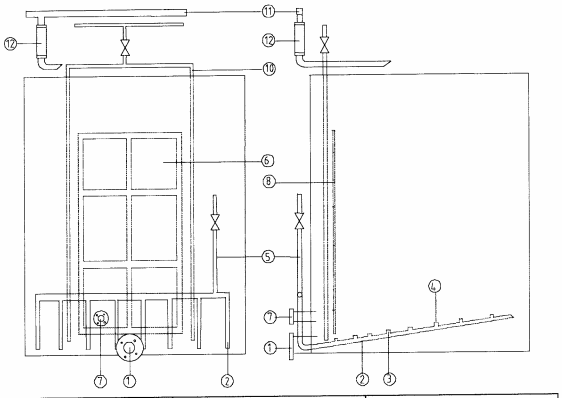
Liming.

During liming the ossein is immersed in a supersaturated calcium hydroxide solution with a pH >= 12.5. Being supersaturated the pH remains 12.5, because any consumed dissolved lime is replaced by excess precipitated lime. The ossein is kept immersed in this solution for a period of at least 20 days to approximately 80 days. During this period the lime is replaced by fresh lime at regular intervals, short in the beginning of the treatment and approximately every ten days during the remainder. Several times a week air is blown through to stir the lime and ossein to prevent pockets of low lime concentration and pH, and to saturate the solution with air to prevent anaerobic growth.

Table 1. Schedule of the liming process

Day	Adding fresh lime	Decanting	Pumping air	Washing
1	X			
2	X	X		
3			X	
4	X	X		
8			X	
9	X	X		
12			X	
15			X	
17	X	X		
21			X	
25			X	
27	X	X		
31			X	
35			X	
37	X	X		
41			X	
43			X	
46		X		X

During liming certain components of the ossein like fatty acids, glycoproteins, and glycosaminoglycans are broken down by alkaline hydrolysis. The collagen in the ossein is also partly denatured. Covalent bonds which interconnect the bar-like collagen molecules, and also those between the single molecules in the collagen triple helix, are broken down. The collagen triple helix structure itself is not affected. The extent to which this hydrolysis takes place is dependent on the length of liming time. The liming time selected also depends on the quality of the bones, a short liming time can result in the collagen being incompletely denatured. Liming is usually done in large concrete basins, or pits that can contain and submerge the ossein obtained from one batch of demineralised bone chips. The lime is refreshed and air blown through according to a schedule as in table 1.



1. verlaagde aansluiting voor osseine leiding	5. centrale luchtleiding	9. pijp kalkmelk oplaten
2. kunststof luchtleidingen	6. rooster	10. centrale kalkmelkleiding
3. draadaansluitingen	7. aansluiting achter rooster kalkmelk aflaten	11. osseine leiding
4. nozzles	8. geperforeerde plaat	12. draaibare aftakkingen osseine leiding

The temperature of the fresh lime is approximately equal to, or below room temperature.

After the last decanting of the lime the ossein is washed, the minimal treatment consisting of washing the ossein twice in the lime pit, each time with an amount of water approximately equal to the weight of the original bone chips. The washed ossein is then pumped in a stream of water to the neutralisation installation.
The liming process described is a typical example of one carried out in industry. Liming can however differ to a certain extent between different batches, depending on the quality of the bone and e.g. the average ambient temperature. The important parameters and processing steps however remain the same between batches and manufacturers i.e. use of oversaturated lime, pH ≅12.5, the minimum amount of lime used, regular refreshing of lime, agitating with air, and washing. Differences can include:

- The number of liming days. This depends on the quality of the bone chips, the average temperature of the lime and the desired physical properties of the gelatine.
- The frequency of refreshing the lime , depending on individual batch requirements and on what is done in an individual installation.
- The frequency and time of blowing air depends on the dimensions of the pit.
- The number of times the ossein is washed and the method used. Washing the ossein with twice its weight of water is a minimum. Individual manufacturers might wash the limed ossein more times, might use agitation or wash the ossein by spraying with water.

Neutralisation.

The washed limed ossein, which still contains lime and has a high pH in the centre of the particles, is treated with dilute acid to neutralise and remove this lime. This is followed by conditioning of the ossein at a neutral or slightly acidic pH which is necessary for the extraction of the gelatine. In the neutralising installation the limed ossein is completely immersed in water which is stirred vigorously while dilute sulphuric acid is added. The pH of the process is continuously measured and controls the acid flow. Neutralisation and conditioning is continued until the ossein pH stabilises within the desired limits without further addition of acid.
To remove excess acid and salts, the ossein is vigorously washed several times with a total amount of water equal to at least five times the weight of the original bones. After completion of washing, the ossein is pumped in a stream of water to the extraction installation.

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 ^{o}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 Van der Waals forces maintaining the collagen helix are broken, allowing individual collagen molecules to dissolve. The process is not ideal and, especially at higher temperatures, peptide bonds in the collagen molecules are broken. Further, not all covalent connections have been removed during liming. The first gelatine extracted mainly consists of single collagen chains and therefore has the highest gel strength and the lowest viscosity. Part of it will however consist of broken chains, double chains and even triple chains. Gelatine that is extracted later in the process will generally have a more irregular composition and consequently a lower gel strength and higher viscosity. In these later extracts the role of thermal decomposition of the collagen starts to become more important.
Industrially, extraction is executed as follows. In the extraction 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.

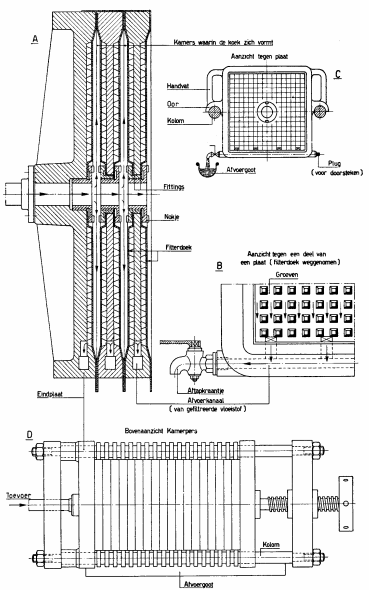
Filtration

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 commercially 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 off two or more

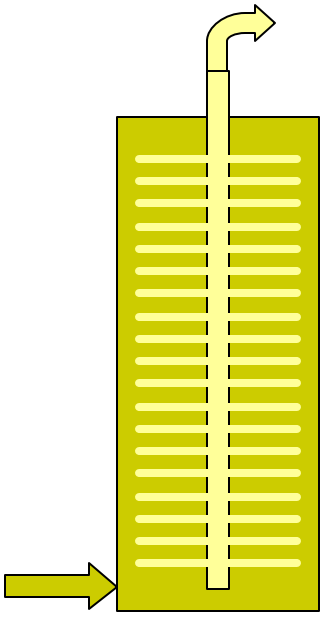
parallel filters. When the one in use becomes saturated the flow is switched to the fresh parallel filter.

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.

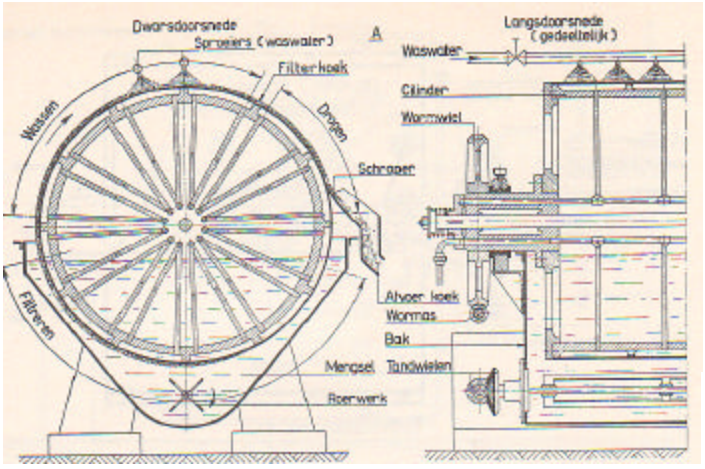
The diatomaceous earth filter layer is made freshly before use, by suspending the filter material in water and running this suspension through the filter installation. The equipment contains a mesh on which the filter material settles, forming a filter layer several millimetres thick. More earth is added 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. 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 below atmospheric pressure.



Filter press



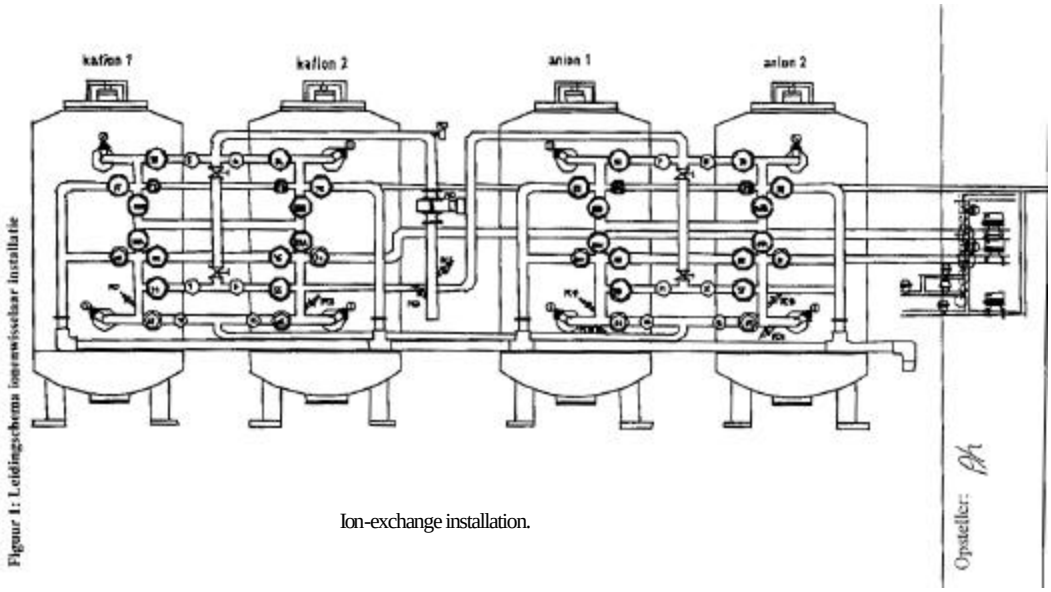
disk filter



Rotating vacuum filter

Ion-exchange.

The filtered gelatine solution is ion-exchanged over cation and anion resin. 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.

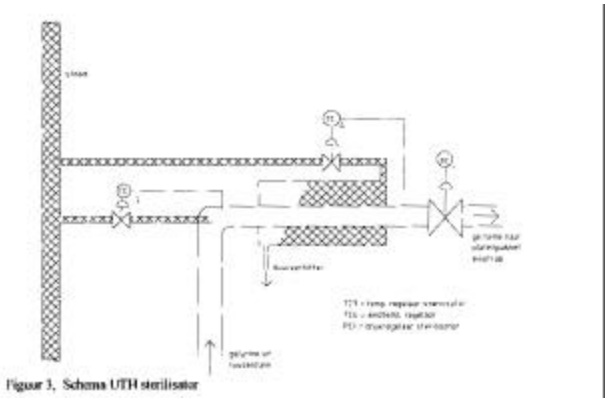
When pH and conductivity get out of the pre-adjusted range, the gelatine flow is automatically switched to a parallel column and the first column is regenerated. Cation resin is regenerated with 5 % hydrochloric acid, the anion resin with 5 % sodium hydroxide. After regeneration the columns are rinsed 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 between 4.6 and 5.3 for limed bone gelatine and usually more than 7 for acid bone gelatine. The low iso-ionic point for limed bone gelatine is caused by the conversion of glutamine and asparagines into the corresponding acids and of part of the arganine into ornitine by the liming.
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 ºC. 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

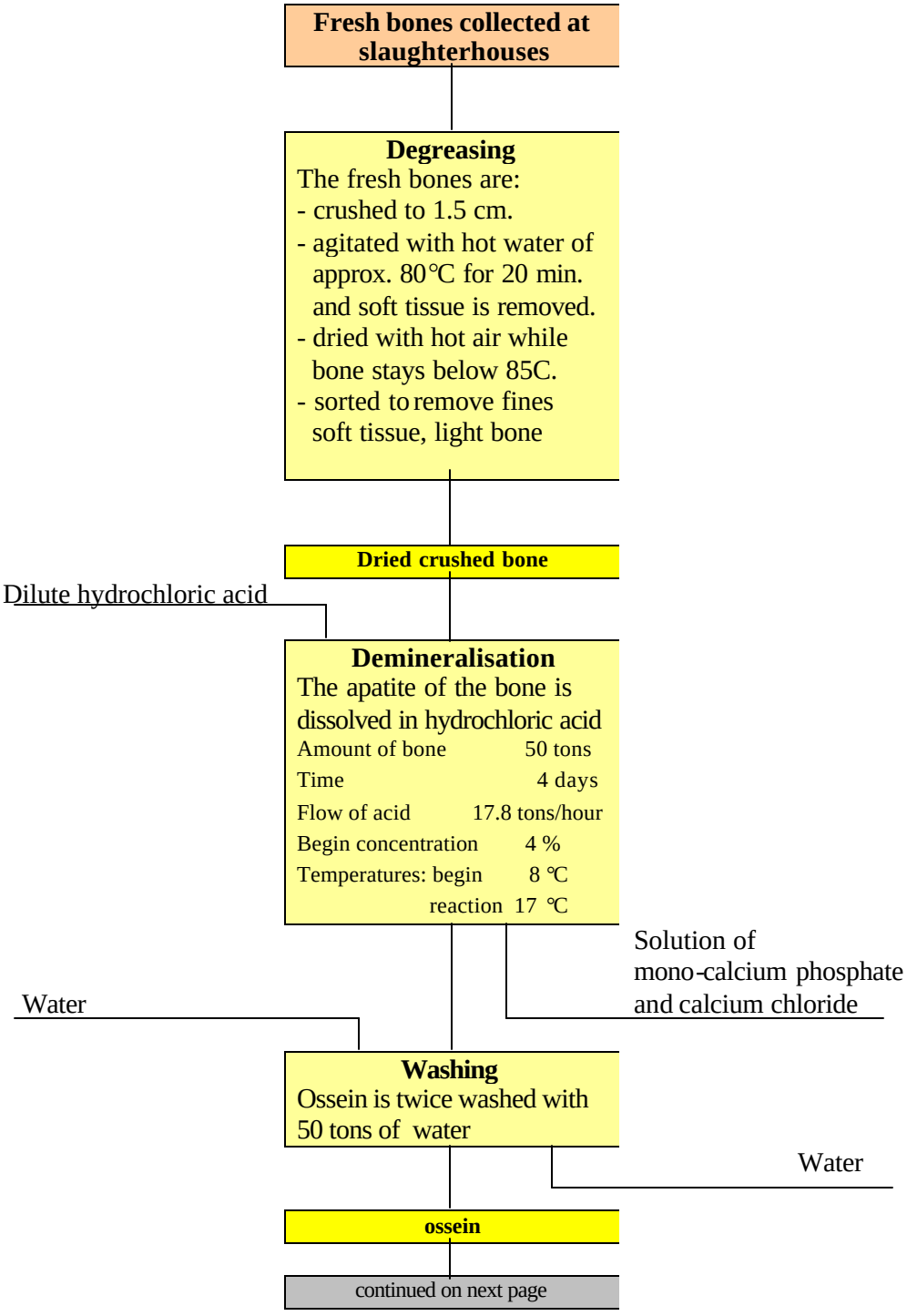
The sterilising equipment is made of stainless steel.

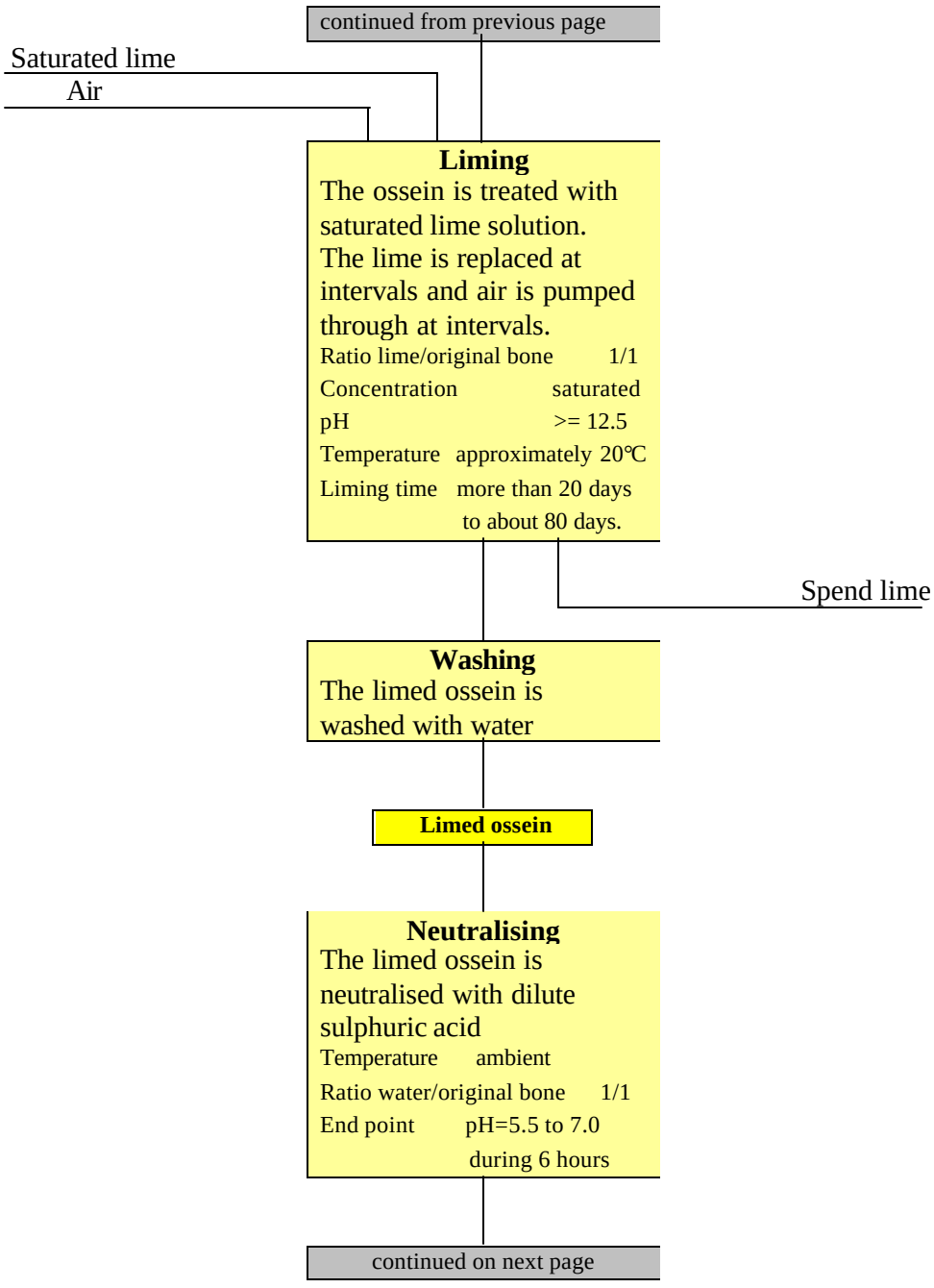
Drying, cutting, blending and packing of the gelatine.

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. The gel that enters 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 limed bone gelatine manufacturing process.

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Saturated lime
Air
Liming
The ossein is treated with saturated lime solution. The lime is replaced at intervals and air is pumped through at intervals.
Ratio lime/original bone 1/1
Concentration saturated
pH >= 12.5
Temperature approximately 20°C
Liming time more than 20 days to about 80 days.
Spend lime
Washing
The limed ossein is washed with water
Limed ossein
Neutralising
The limed ossein is neutralised with dilute sulphuric acid
Temperature ambient
Ratio water/original bone 1/1
End point pH=5.5 to 7.0 during 6 hours
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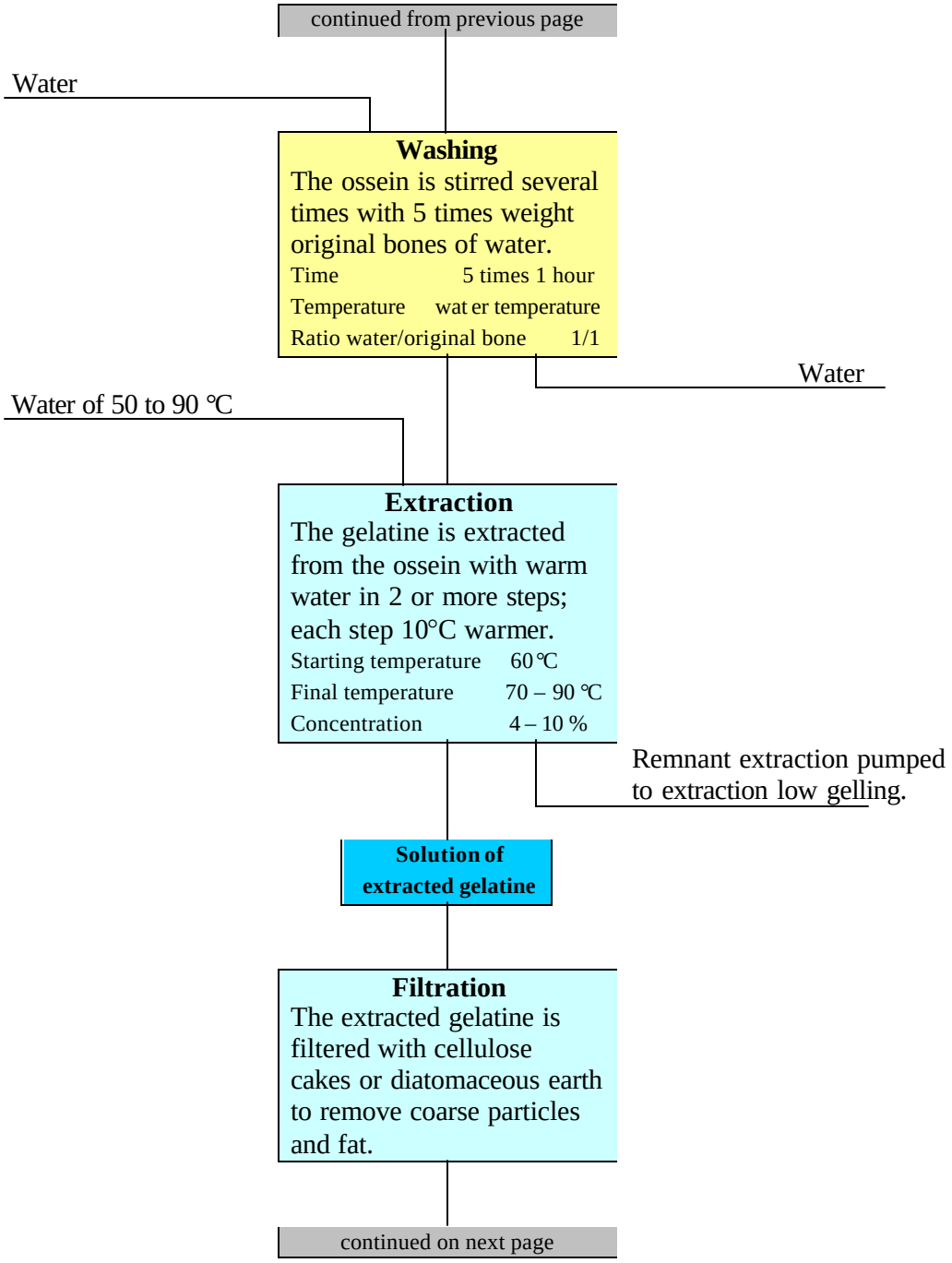
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Water
Washing
The ossein is stirred several times with 5 times weight original bones of water.
Time 5 times 1 hour
Temperature wat er temperature
Ratio water/original bone 1/1
Water
Water of 50 to 90 °C
Extraction
The gelatine is extracted from the 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 extracted gelatine is filtered with cellulose cakes or diatomaceous earth to remove coarse particles and fat.
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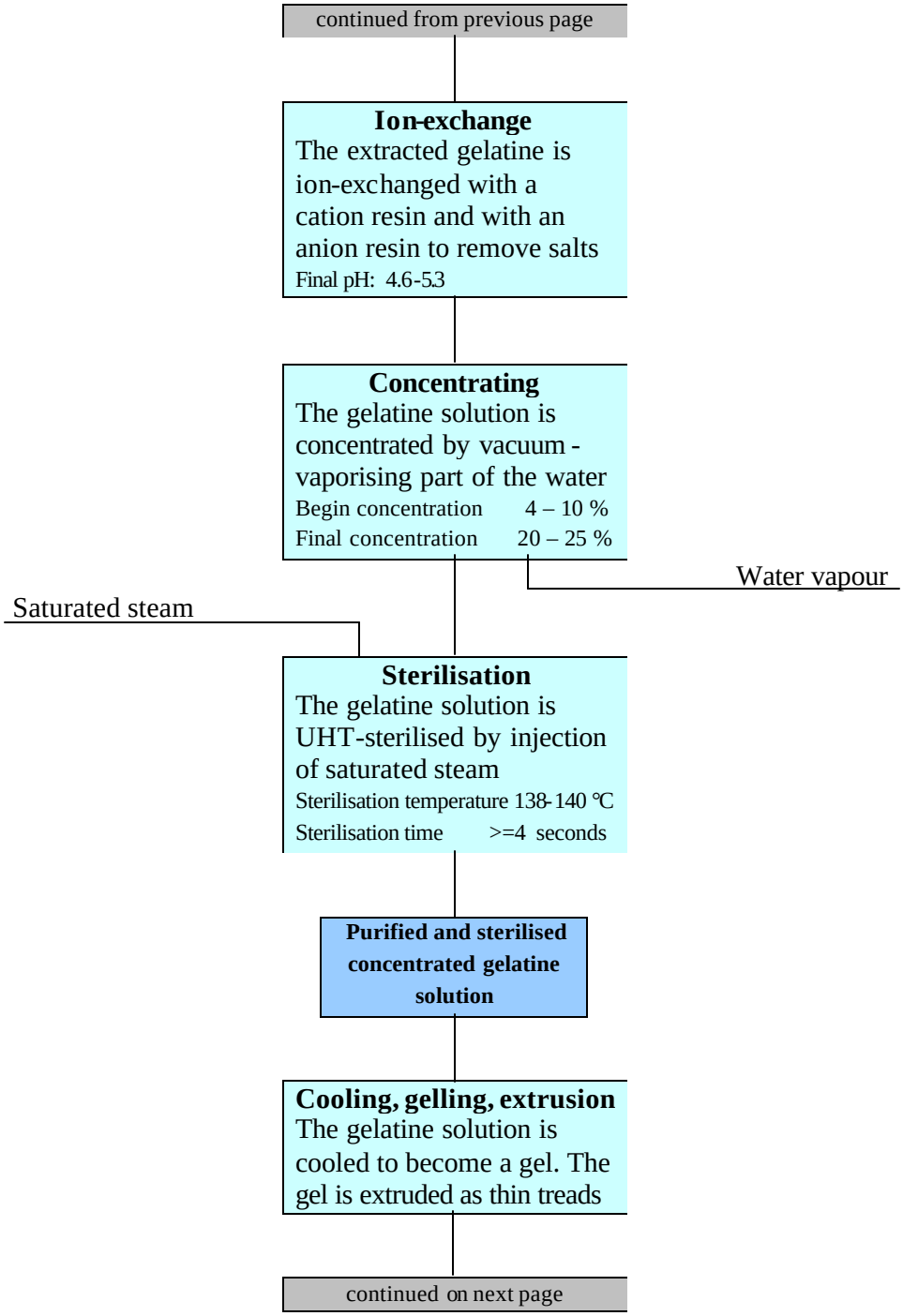
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Ion-exchange
The extracted gelatine is ion-exchanged with a cation resin and with an anion resin to remove salts
Final pH: 4.6-5.3
Concentrating
The gelatine solution is concentrated by vacuum - vaporising part of the water
Begin concentration 4 – 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
Cooling, gelling, extrusion
The gelatine solution is cooled to become a gel. The gel is extruded as thin treads
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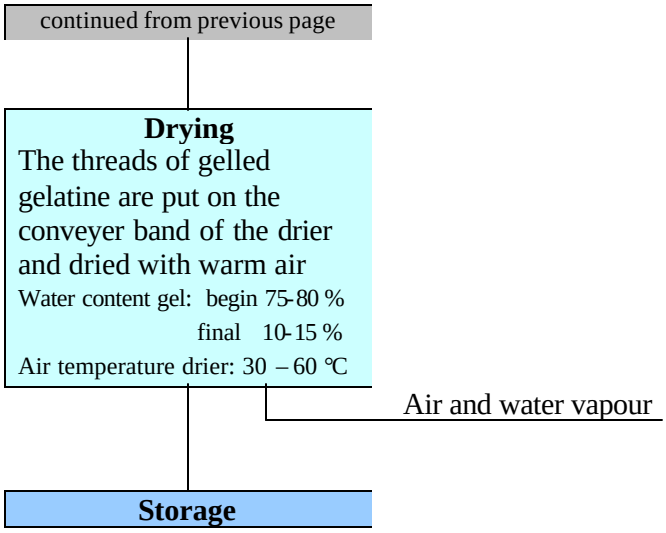
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Drying
The threads of gelled gelatine are put on the conveyer band of the drier and dried with warm air
Water content gel: begin 75-80 %
final 10-15 %
Air temperature drier: 30 – 60 °C
Air and water vapour
Storage

2.3. Summary of the conditions of the limed 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.

Liming: Liming time: 20 to 80 days
Refreshing lime and blowing air according table 1
Washing 2 times with about 50 tons of water.

Neutralising: With dilute sulphuric acid until end point pH: appr. 5.5
Washing by stirring i.e. 5 times 30 minutes with 50 tons of 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 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 limed 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 a 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
Liming	Saturated lime for 20 to 80 days, refreshed with intervals, air blown through regularly. Washed with clean water Pumped in stream of clean water	Saturated lime for approximately 45 days, refreshed according table, air blown through according table. 2 times with 500 ml water 1 time stirred with 1000 ml water
Neutralising Extraction	Neutralising under stirring with sulphuric acid. Washing in several steps with 5 times original weight of bones of water. Stepwise with sufficient water, at 60, 70, 80, … °C. Concentration at draining 3-9%	Neutralising by adding dilute sulphuric acid until pH stays between 5 and 6 for 3 hours or more. Washing 5 times with 500 ml of water. Stepwise with 800 ml water at 60 °C and every next time 10°C more. Concentration at decanting 3 to 9%
Process step	**Minimum process conditions of industrial process**	**Process conditions of downscaled process**
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.

Limed 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 was obtained, depending on the quality of the crushed bone. This compares with a typical batch size of 20,000-50,000 kg used in demineralisation on an industrial 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.

Liming

Industrial liming takes place in large basins, in which the ossein is completely immersed in a saturated lime solution, as a static system. The action of the lime on the ossein is achieved by diffusion. To keep the concentration of the lime constant throughout the lime pit, and to maintain an aerobic environment, air is blown through at regular intervals. To remove soluble impurities, such as non-collagen proteins, the lime is replaced on a regular basis.
The reaction takes place by diffusion so the process should not be affected by downscaling; a large or small amount treated separately under the same conditions of pH and temperature will react in the same way.
The ossein obtained from the acidulation was immersed in a solution of saturated lime and refreshed according to the same schedule as the industrial process. The ratio of lime to ossein was kept typical of that for the industrial process as was the schedule of pumping air through the vessel to maintain even lime concentration and aerobiosis. Because of the smaller vessel used in the scaled down process, the time during which air is pumped through, at a flow equivalent to the industrial process, was limited to approximately an hour each time.

The ossein obtained from the downscaled demineralisation step, approximately equivalent to 500 g of original bone was put in the same type of reactor flask as used in the demineralisation step. Sufficient saturated lime solution, of 25 g/litre, was added to immerse the ossein completely and left to stand in the lime. The lime was replaced and air pumped through according to the schedule mentioned above. The flow of air was at least 100 ml/minute. After the desired number of days (20 days or 45 to 50 days), the limed ossein was washed twice by immersing the ossein in the reactor flask in 500 ml of water for 1 hour. Finally, the ossein was removed from the flask and stirred in 1 litre of water for 10 minutes.

Neutralisation

The main function of neutralisation is to stabilise the limed ossein at a desired pH and is achieved by addition of dilute acid. During this treatment, any remaining lime will be neutralised and washed away in subsequent washing steps. The pH of the neutralisation solution is measured and recorded continuously and is used to control the addition of acid.
The process was scaled down by gentle stirring of the limed and washed ossein in water. This was carried out in a beaker in which a pH electrode was immersed. The pH was measured continuously during the addition of small amounts of acid until a stable desired pH was achieved. Smaller and less frequent additions of acid were made as the desired pH value approached, reflecting practice in a controlled industrial process. The liquid was then decanted and the ossein stirred several times with an equal weight of clean water for a minimum of 30 minutes.

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 reality. It was decided to use samples collected from the bone crusher of a European industrial gelatine plant. Eight 10 litre amounts were collected, thoroughly mixed then frozen in 1kg aliquots. The bone collected did not contain cattle heads, and because backbone would be added separately at spiking, any cattle backbone present were 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.

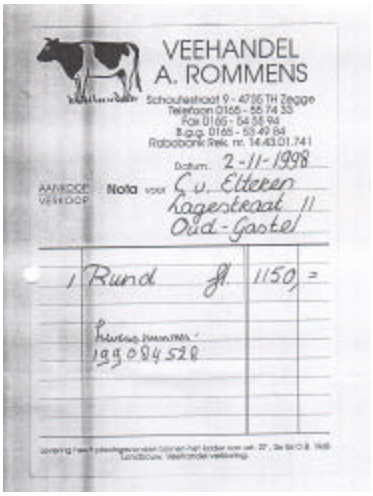
Table 1. Approximate composition of the fresh bone.

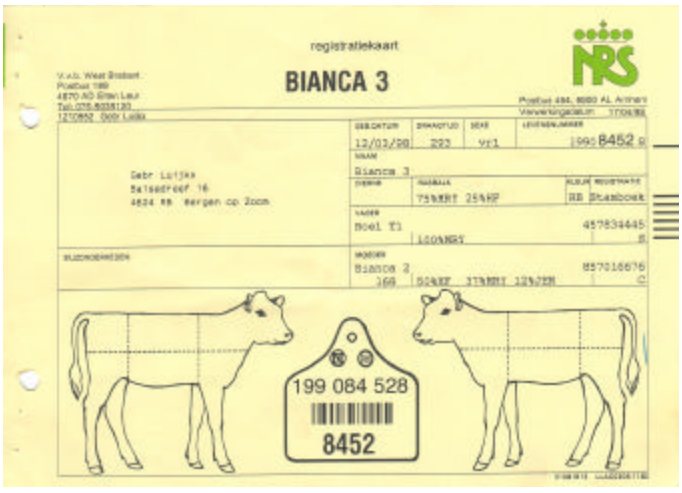
	Composition calculated on dry matter	**Composition calculated on dry matter excluding fat**
Fat	approx. 36 %	
Protein	approx. 32 %	approx. 50 %
Ash 550 ^{o}C	approx. 31 %	approx. 48 %
Ash 1100 ^{o}C	approx. 30.5 %	approx. 47.5 %

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
Schoutheithoek 9 - 4756 TH Zegge
Telefoon 0165 - 55 74 33
Fax 0165 - 54 58 94
Bijg. 0165 - 53 49 84
Rabobank Rek. nr. 14.43.01.741
Datum 2-11-1998
Nota voor G.v. Elteren
Kogerstraat 11
Oud-Gastel
1 Rund fl. 1150,=
199 084 528

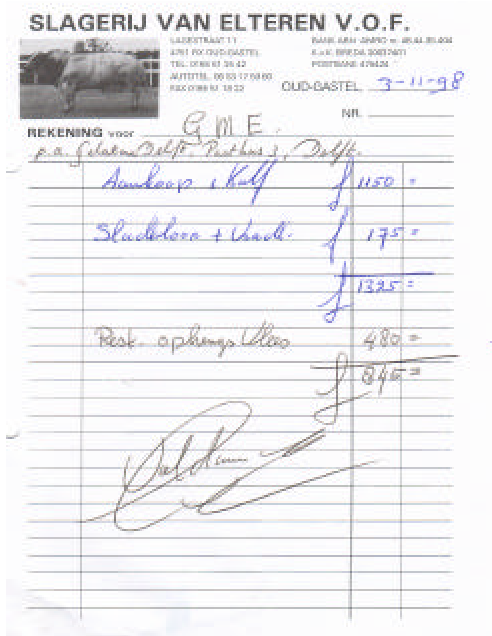


GEZONDHEIDSVERKLARING

NR: 0272485

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

Datum afgifte: 16-10-1998



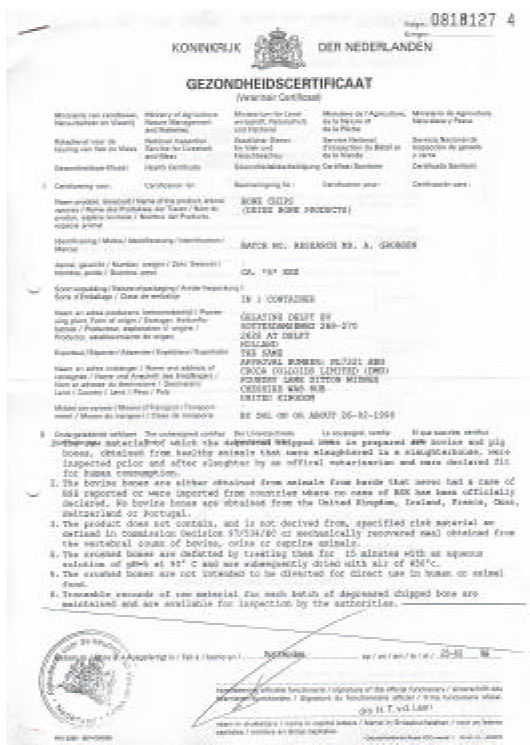
SLAGERIJ VAN ELTEREN V.O.F.

OUD-GASTEL 3-11-98

REKENING voor G M E

p.a. Gelatine Delft [illegible]

Aankoop Kalf	ƒ 1150 =
Slachtloon + [illegible]	ƒ 175 =
	ƒ 1325 =
Rest. ophalen [illegible]	480 =
	ƒ 845 =



0818127 4

KONINKRIJK DER NEDERLANDEN

GEZONDHEIDSCERTIFICAAT

(Veterinair Certificaat)

BONE CHIPS
(DRIED BONE PRODUCTS)

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 calcula ted and this amount of crushed bone was aliquoted into a tray.

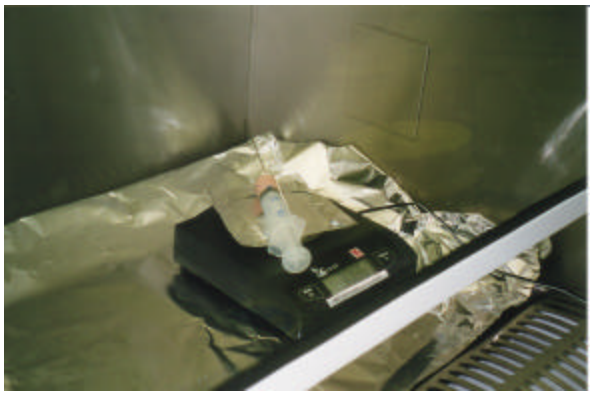
Preparation of the 301V mouse brain homogenate for spiking

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 15 ml Schütt homogeniser with a PTFE piston (LS cat 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 adherent 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 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 f urther use.

Equipment for preparing the brain homogenate for injection in the spinal cord

Left: Preparing homogenate

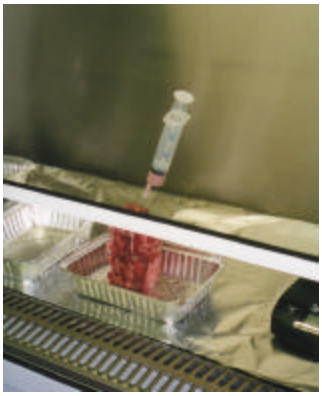
Right: Weighting syringe with homogenate.



Injecting the backbone spinal cord 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 weighed. The homogenate was injected into the spinal cord of the calf backbone after which the syringe was weighed again. The tray with the spiked backbone was put in a polythene bag and put in the refrigerator.

Injecting spinal cord



Syringe and backbone

The tray with crushed bone was taken from the refrigerator and placed in the biological safety cabinet. The spatulae used previously with adherent brain tissue were weighed before the brain tissue was smeared on the crushed bone. The spatulae were weighted again and disposed of. The brain tissue left in jar-I was also smeared on a few pieces of the crushed bone by wiping the inside of the jar with pieces of crushed bone. Jar-1 was weighed before and after the wiping. The brain tissue left in the homogeniser tube was transferred to the crushed bone in the same way. The homogeniser tube was also weighed before and after wiping. Finally, approximately 2 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 chlorites solution of 20,000 ppm free chlorine for 1 hour minimum.

Weight of crushed bone, backbone and brain tissue.

Weight of tray with polythene bag with crushed bone	1894 g
Weight of empty tray with polythene bag	383 g
Crushed bone	**1501 g**
Tray with backbone	516.2 g
Empty tray	14.5 g
Back bone	**501.7 g**
Stock jar before taking out	122.46 g
Stock jar after taking out to homogeniser tube	110.10 g
Taken out of stock jar	12.36 g
Stock jar before smearing on the crushed bone	97.99 g
Stock jar after smearing on the crushed bone	95.97 g
Taken from stock jar and smeared on crushed bone	2.02 g
Taken out of stock jar	12.36 g
Taken from stock jar and smeared on crushed bone	2.02 g
Total infectious brain taken from stock jar	**14.38 g**
Jar-I with homogenate	30.33 g
Jar-I empty	23.64 g
Homogenate	6.69 g
Jar-I with homogenate before filling syringe	30.28 g
Jar-1 after filling syringe	24.96 g
Homogenate taken out	5.32 g

Syringe with 301V brain homogenate	24.08 g
Empty syringe	19.24 g
Homogenate in syringe	4.84 g
Syringe with 301V brain homogenate	24.08 g
Syringe after injection in backbone	19.54 g
Infectious brain injected in backbone	**4.54 g**

Jar-I with remaining 301V brain homogenate	24.94 g
Jar-1 after wiping with crushed bone	23.88 g
Wiped out with crushed bone	1.06 g
Beaker with two spatulas and homogeniser tube with 301V brain	192.75 g
Beaker after smearing on and wiping out with crushed bone	190.23 g
From spatulas and homogeniser tube on crushed bone	2.52 g
301V brain from jar-I	1.06 g
From spatulas and homogeniser tube	2.52 g
From stock jar	2.02 g
Infectious brain smeared on crushed bone	**5.60 g**

Injected in backbone	4.54 g
Smeared on crushed bone	5.60 g
Total spike	**10.14 g**

Loss of brain during spiking: 14.38 – 10.14 = 4.24 g

(partly due to transfer from 15 ml homogeniser tube to 30 ml homogeniser tube, partly left on pistons homogeniser tubes, partly due to evaporation of water from macerate and homogenate)

Weight difference jar-1: 23.88 - 23.64 = 0.24 g (fat from crushed bone?)

The amount of spike used could be in reality somewhat larger than the amount calculated above because of evaporation of water from the brain tissue, and because of fat transferred to the surfaces during contact of these with the crushed bone.

Reference: Note book 31-12-98 to 24-3-99 page 1, 2 and 4 to 9. Date 31-12-98.

Sawing of the backbone

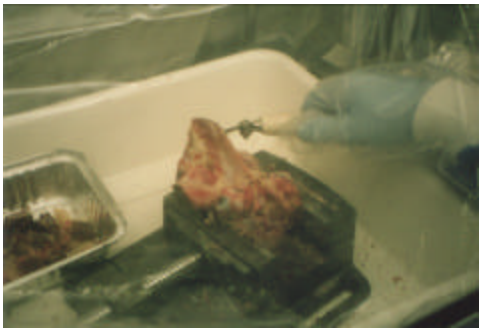
A glove bag was set up in the biological safety cabinet. One plastic and one aluminium tray, two pairs of tweezers (Bochem 18/8 steel LS cat no 3305 01000), two pairs of forceps (arterienklemme stainless steel LS cat no 3351 11038) and two scalpels (scalpel handle LS cat no 3351 18061, blade LS cat no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

Glove bag with backbone and sawing equipment

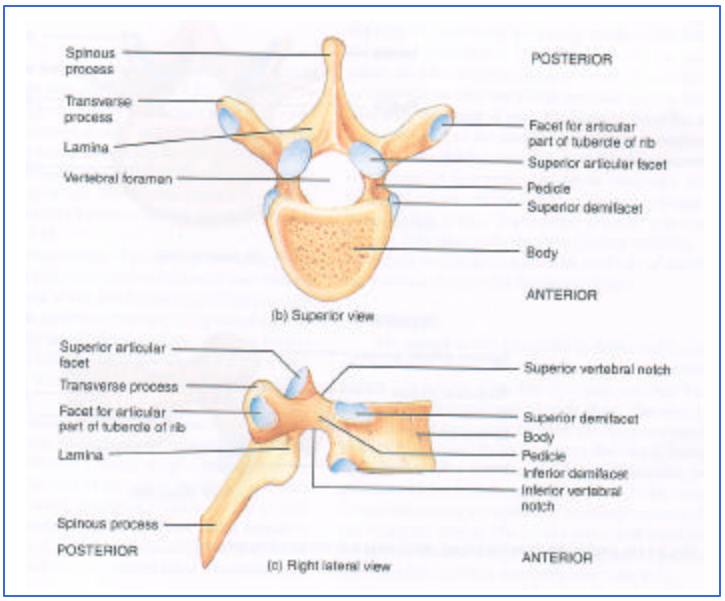
The tray with the backbone was taken from the refrigerator and put in the glove bag. The backbone was placed into the vice and sawn to 1.5 cm pieces as described below. The soft tissue on the backbone was cut away with scalpels and the backbone material was handled using the tweezers and forceps. Care 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.

Description of sawing of the backbone:

The backbone was firmly clamped in the vice to facilitate easier and safer sawing. Before sawing, any muscle or fat surrounding the vertebrae was cut with a scalpel. The bone was sawn with short bursts of the saw, of approximately 10 seconds, to prevent overheating of the saw motor and 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



Sawing the backbone



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.

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.

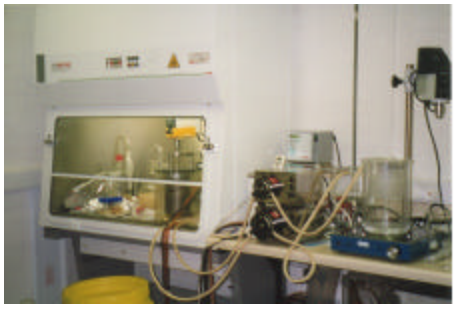
Degreasing

Preparation of the degreasing emulsions.

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). Another 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.

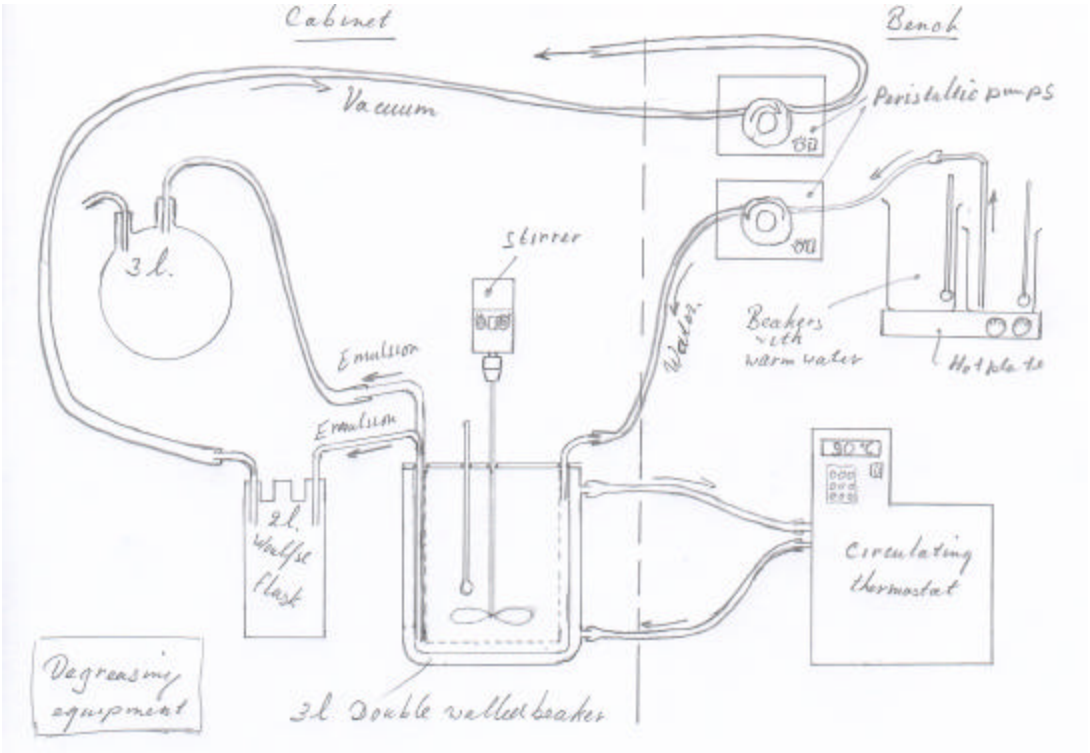
The degreasing equipment was set up. (See pictures and drawing of degreasing equipment below). A Heidolph stirrer stand and Heidolph RZ2020 stirrer with a 3 blade stainless steel propeller of 66mm, was put in the cabinet. A 3 litre double walled tempering beaker was put in the cabinet and fixed to the 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

Degreasing equipment in safety cabinet

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



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)

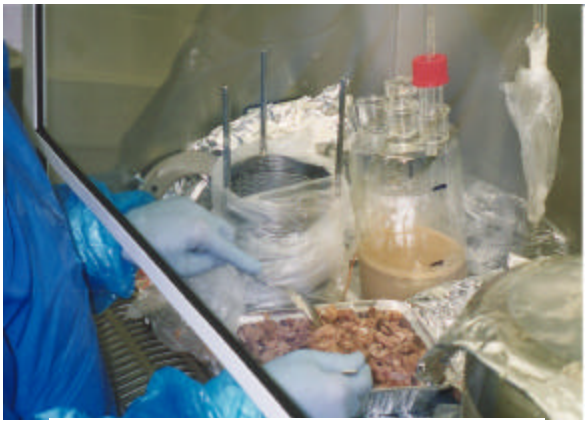
1:

Tray+bag +bone	985.2 g
Tray+bag	20.7 g
Bone	964,5 g

2:

Tray+bag +bone	1001.7 g
Tray+bag	20.7 g
Bone	981,0 g

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



Filling the basket with spiked crushed bone

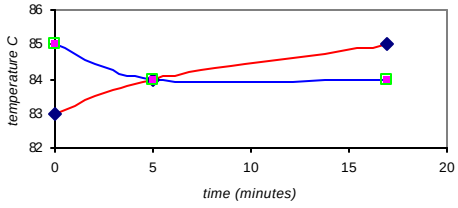
The basket was wrapped in a polythene bag and filled with crushed bone portion 1. The stirrer was placed in the basket and the lid fitted on top. The basket was fixed to the ring and the whole assembly was fitted on to the stand. The polythene bag was removed and the basket assembly

degreasing

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 continued vigorously for 20 minutes. During this period, at regular intervals, 4 x 250 ml of emulsion were pumped out of the double walled beaker into a Woulfse flask, each time being replaced by the same volume of warm water. After 17 minutes 1 litre of emulsion was pumped out of the beaker and 1 litre of clean water pumped in. A sample of the degreasing emulsion was taken at the start and at 5 minute intervals over the 20 minute period.

Temperature during degreasing.

Time (min)	**temperature degreasing emulsion**	**temperature fresh water**
0	83°C	85°C
5	84°C	84°C
17	85°C	84°C



After 20 minutes all degreasing liquid was pumped from the double walled beaker into a 3 litre flask. The pump and the stirrer were then switched off.
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 84°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.

Degreased bone

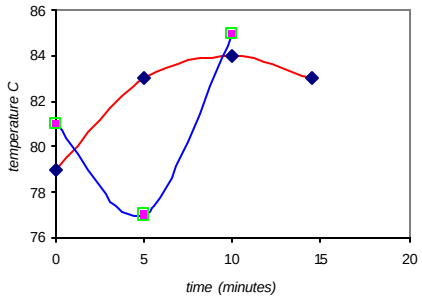
The two 3 litre flasks with spent degreasing liquid were removed from the cabinet and disposed of. Two clean 3 litre round bottom flasks were placed in the cabinet and the necessary hose connections made. The second portion of degreasing liquid was put in the 3 litre double walled beaker and was warmed 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 14.5 minutes.
2 samples of the degreasing emulsion were taken, one after 10 minutes and one after 20 minutes. One sample was taken from the 3 minutes rinse with clean water.

Temperature during degreasing.

Time (min)	**temperature degreasing emulsion**	**temperature fresh water**
0	79°C	81°C
5	83°C	79°C
10	84°C	85°C
14.5	83°C	-



Temperature during 3 minute rinse.

Time (min)	**temperature emulsion**
0	83°C
3	86°C

The degreased bone from the first degreasing had a darker appearance than that of the second. It was also darker than material observed during testing which was similar to that of the second degreasing. In some bone chips, marrow remained in the pores.

Tray with bag and degreased bone	1735.3 g
Tray with bag	389.9 g
Bone chips and meat	1345.4 g

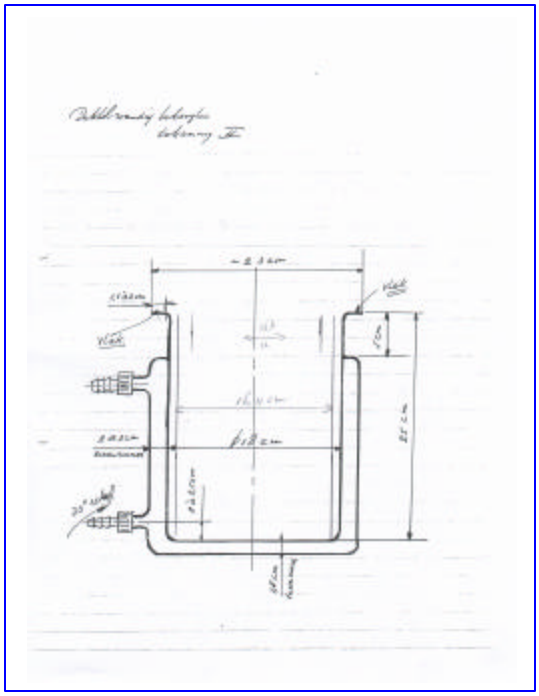
A sample was taken of the degreased bone.

Jar with degreased bone	175.0 g
Jar	146.7 g
Sample degreased bone	28.3 g

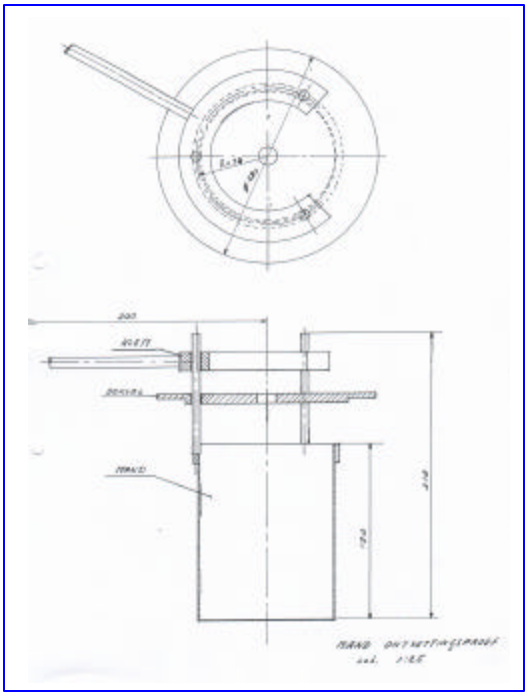
The tray with degreased bone was put in a polythene bag and put in the refrigerator.

All equipment used in the cabinet except the stirrer stand, the clamps and the stirrer motor, was wrapped in polythene bags and disposed of. The stirrer stand, the clamps and the stirrer were put in polythene bags and removed from the cabinet until further use. The cabinet was decontaminated as before.

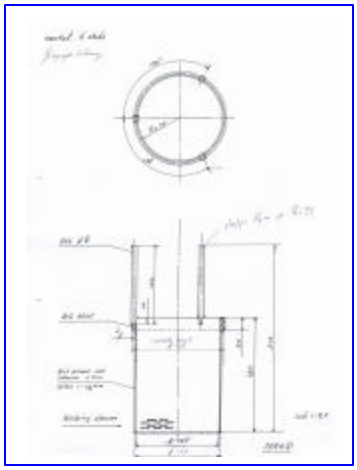
Custom made equipment



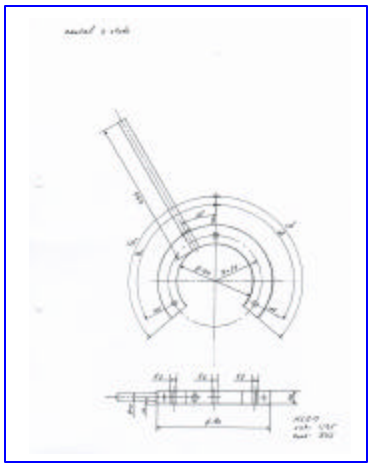
3 litre double walled beaker



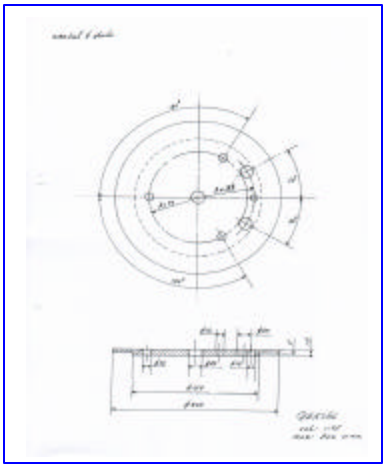
basket assembly



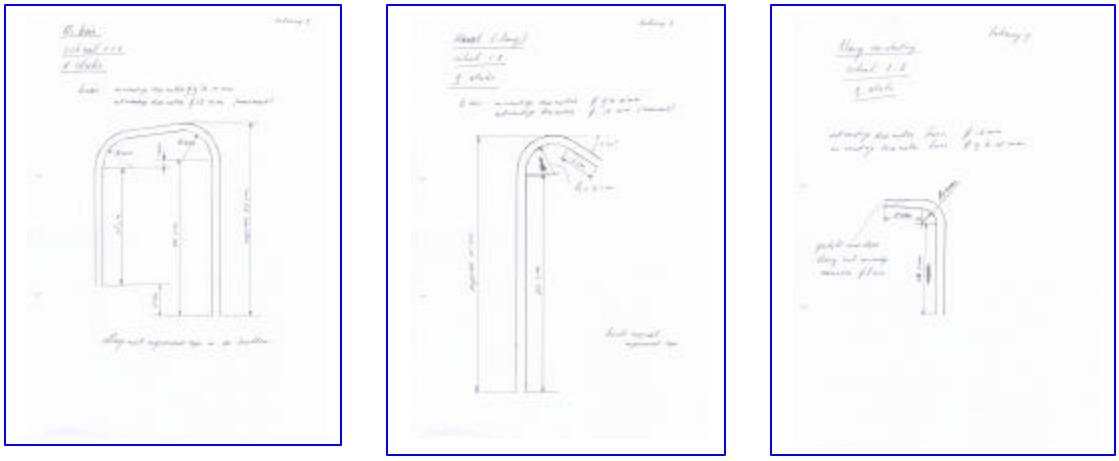
basket



ring



lid



siphons

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.

Tray with bag and sorted bone chips	695.9 g
Empty tray and bag	24.5 g
Sorted wet bone	671.4 g

Separating bone and soft tissue

Drying of the bone

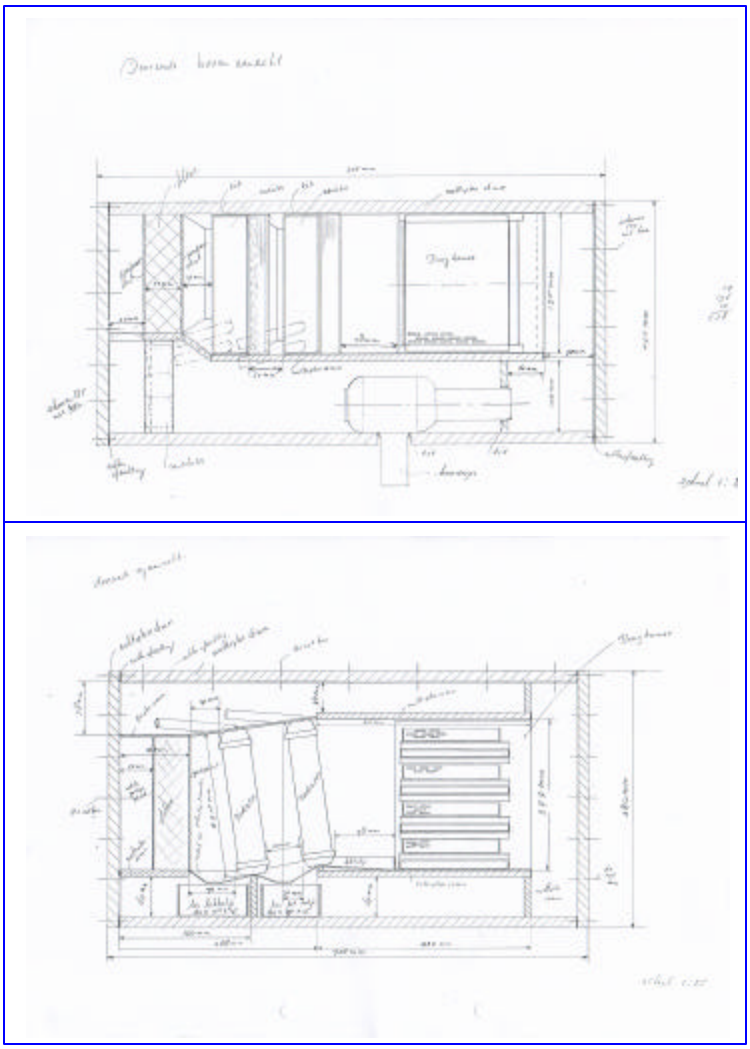
The custom-made bone drier was placed in the safety cabinet. The bone drier was a closed circuit drier using a system of 3 hairdryers, to heat and circulate the air over stainless steel mesh trays used to contain the wet bone. Finally, two water-cooled heat exchangers cooled down the air and condensed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

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 109°C and the maximum temperature between the bone was 70°C.

Drying of the bone

Tumbler jar with dried bone	899.1 g
Tumbler jar empty	359.6 g
Dried bone	539.5 g
Loss of water	131.9 g

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



Drier, side view and top view

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 6 hours.

Tumbling of the dried bone chips in tumbler jar on roller bank

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

1

2

3

Transfer of bone chips to sieve..

4

5

6

Sieving of the bone chips

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.

Sieving of the bone chips

Sorting of the bone chips.

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

Tray with bag and bone chips	498.3 g
Empty tray and bag	24.5 g
Sorted degreased dried bone chips	**463.8 g**

A sample was taken of the bone chips before these were weighted.

Jar with sample	170.4 g
Empty jar	146.7 g
Sample of bone chips	23.7 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 31-12-98 to 24-3-99 page 11-18. Date 4-1-99 to 8-1-99.

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.
5 litre solution: 325 g bone ash
471.5 ml 36% hydrochloric acid
4530 ml water

Solution 2: Composition: 2.5% hydrochloric acid, the remainder mono-calcium phosphate and calcium chloride.
5 litre solution: 142.5 g bone ash
471.5 ml 36% hydrochloric acid
3905 ml water (625 g ice added later)

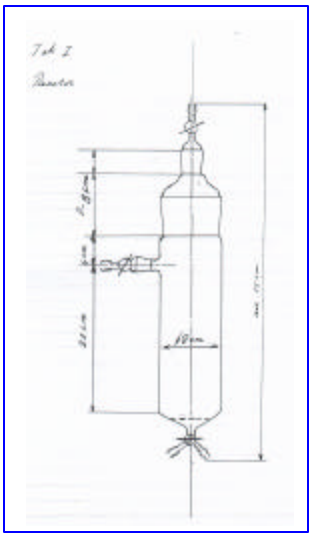
Solution 3: Composition: 4% hydrochloric acid.
10 litre solution: 943 ml 36% hydrochloric acid
6650 ml water (2400g ice added later)

(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)

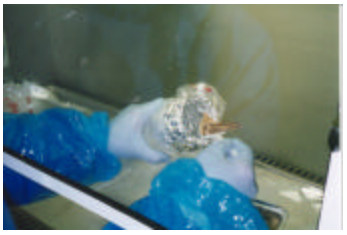
The solutions were stored in 10 litre polythene bottles.

Demineralising of the degreased crushed bone chips.

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 and 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 flask demineralisation



filling reactor with bone chips

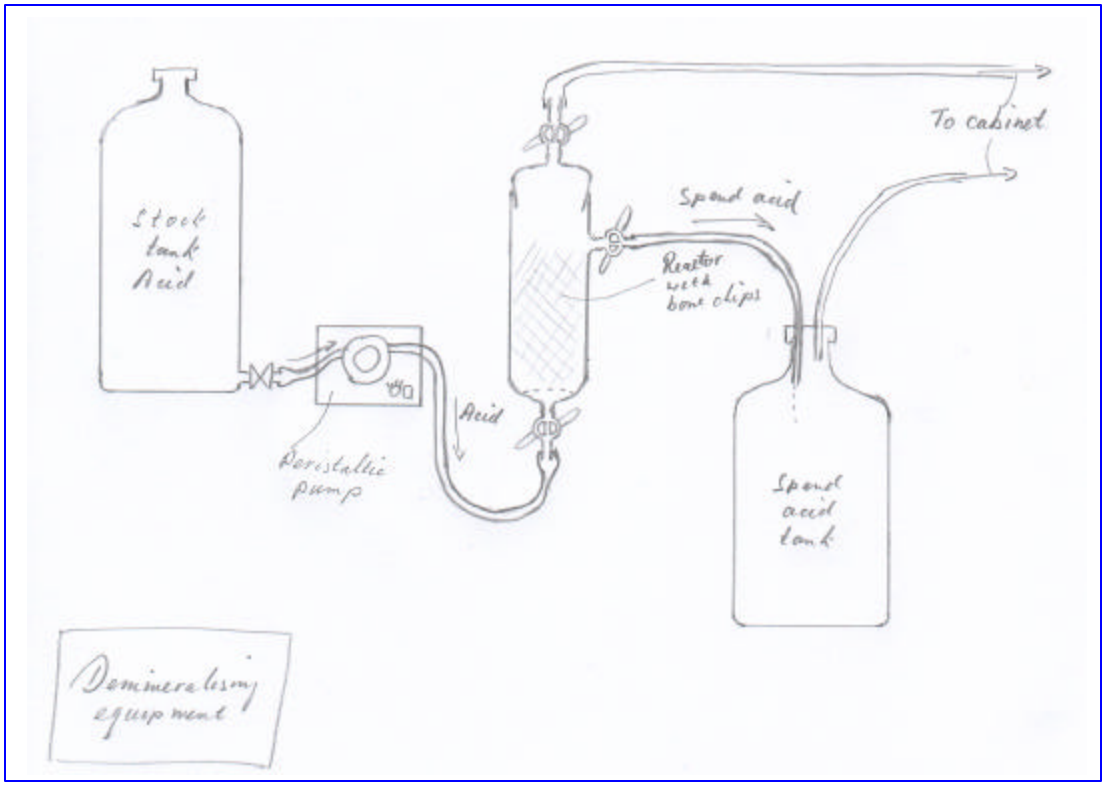
reactor with bone chips

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.
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

Reactor flask with bone chips

acid was taken and the remainder disposed off. The valves were re-opened and the pump switched on.

Solution 1:	Started 13-1-99 at 9.30 h.	Stopped 14-1-99 at 9.30 h.
Solution 2:	Started 14-1-99 at 9.45 h.	Stopped 15-1-99 at 11.00 h.
Solution 3:	Started 15-1-99 at 11.00 h.	Stopped 17-1-99 at 15.00 h.



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

Pumping through of solution 2 stopped approximately 1 hour earlier than intended, as due to the position of the valve on the stock tank, the remaining 1.5 litres in it were not pumped though.
The flow rate of solution 3 would have been slightly lower than desired for approximately 16 hours due to a slight leak in the inlet hose which occurred overnight.


Demineralisation of bone chips.

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

Washing.

The reactor valves were closed and all hoses removed, bagged, and disposed of. 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 peristaltic pump and enough water was pumped 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 polypropylene 2 litre beaker (Griffin beaker, VIT-Lab, PP. LS cat no 2385 13196) and stirred for 10 minutes with 1 litre of water (Heidolph RZR 2020 stirrer with PE two blade stirrer, Kartell 431). The wash water was then pumped out, the stirrer blade removed and disposed of. The washed ossein was kept in the beaker for immediate further treatment. A sample was taken of the ossein, approximately equal to 20 g of original bone. Samples were taken of all drained washing waters.


Washing of ossein after demineralisation

The reactor was cleaned with water then treated with sodium hypochlorite (20,000ppm free chlorine) for 1 hour then rinsed with water. Equipment on the bench which had not been in contact with any infectivity, the stand, stirrer and stirrer stand in the cabinet, were bagged and stored until further use. All other equipment was disposed of.


Washing after demineralisation

Reference: Notebook 31-12-98 to 24-3-99 page 19-22. Date 12-1-99 to 17-1-99.

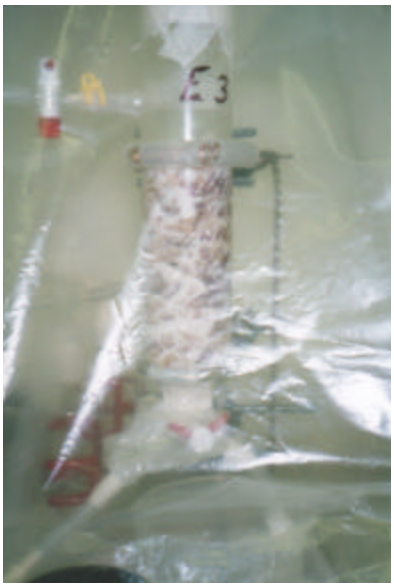
Liming

The reactor flask was filled in the safety cabinet with the washed demineralised ossein, closed then removed to the bench where it was fitted to a stand. One of the valves at the bottom of the flask was connected with tubing to an air pump (Rena air type 200 aquarium air pump) and flow meter (ShoRate flow meter model 1355). The second valve was fitted with a piece of tubing which ran into a covered beaker. The top outlet valve was connected to a washing bottle containing a sodium hydroxide solution and then further to a Hepa filter placed inside the safety cabinet. The side outlet was closed. The assembly was covered with a large polythene bag.


Liming

400 ml of saturated lime was made by adding 10 g calcium oxide to 400 ml of water. This lime emulsion was poured in the reactor from the top through a funnel. The lime was drained off in the morning of the second day and replaced with the same volume and concentration of fresh lime. That evening, air was pumped through the reactor for 1 hour. Draining and replacing of the lime, and pumping of air was repeated several times during the coarse of the liming period (see table below).

date	replacing lime	Pumping air through for 1 hour
17-1-99	first lime added	
19-1-99	X	X
20-1-99	X	
24-1-99		X
25-1-99	X	
31-1-99	X	
6-2-99		X
10-2-99		X
12-2-99	X	
13-2-99		X
17-2-99		X
19-2-99		X
23-2-99		X
24-2-99	X	
26-2-99		X
1-3-99		X
5-3-99		X
7-3-99	final draining of lime	



Liming of the demineralised ossein

Samples were taken of the drained lime and stored in a freezer. The pH of the fresh and drained lime was measured a number of times. Fresh lime had a pH of 12.5 to 12.6, the pH of the drained lime was 12.5 to 12.7. The pH meters (Hanna pHep-2 pocket pH meter) were calibrated before every measurement on a pH 7 and pH 10 buffer solution. These pH meters were kept separate and used only for these solutions. Buffer solutions were disposed of after calibration.

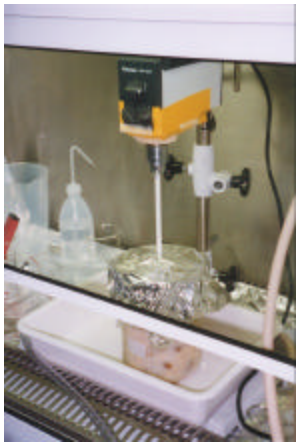
The final draining of the lime was carried out in the safety cabinet. After draining, the limed ossein was removed from the reactor flask with a polythene spoon and transferred to a 2 litre polypropylene beaker(Griffin beaker, VIT-Lab, PP. LS cat no 2385 13196).
The reactor flask and all equipment which had been potentially in contact with test materials were disposed of.

Transferring limed ossein to beaker for washing

Washing

Washing was done in the safety cabinet in a beaker (Griffin beaker, VIT-Lab, PP. LS cat no 2385 13196) with a 2 blade polyethylene stirrer (Heidolph RZR 2020 stirrer with PE two blade stirrer, Kartell 431). The limed ossein was washed with stirring, two times for 5 minutes with 550 ml of water and once for 10 minutes with 1100 ml of water. Fresh water was pumped in and spent liquid was pumped out with 2 Verder Pericor peristaltic pumps 9F-240 using new Verderprene tubes. After the second and the third washing the measured pH was 11.9 and 11.7 respectively.

The washed ossein was kept in the beaker for the next process step.

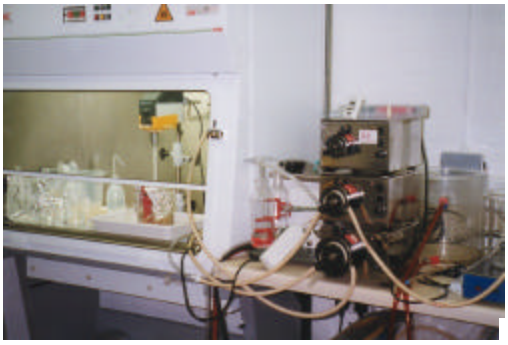


Final washing after liming

References: Notebook 31-12-98 until 24-3-99 page 22, 23.51 and 84. 17-1-99 until 7-3-99

Neutralising and conditioning.

Neutralisation of the lime and conditioning of the gelatine was done with the same equipment as used for washing of the limed ossein, with the addition of a pH meter attached to the beaker (Hanna pHep-2 pocket pH meter). 600 ml of water was added to the ossein and stirring was started. Small amounts of sulphuric acid were added to the ossein while the pH was observed. Addition of acid was at first in larger, regular amounts becoming smaller and less frequent until the pH remained at 5.2 to 5.3 for three hours.
During neutralisation, the pH meter was calibrated at least once a day at pH4 and pH7 and further checked at intervals, from which a deviation of 0.1 was allowed.


Neutralisation equipment

Neutralisation data are in the table below.

date	time	pH	Amount of 2M H_2SO_4 added	pH after addition of H_2SO_4.
7-3-99	17.40	11.5	2.5	2.9
	17.45	7.0	2.5	2.4
	17.50	4.1	2.5	2.3
	18.20	5.1		
	18.50	5.9	2.5	2.7
	19.00	4.2	2.5	2.4
8-3-99	10.30	5.3		
	11.20	5.6	1	3.9
	11.25	4.7	1	3.0
	11.30	4.2		
	12.00	5.0		
	13.05	5.1		
	14.15	5.3		
	15.35	5.4		
	16.50	5.5		
	17.05	5.0		
	18.00	5.0 - 5.1		

date	time	pH	Amount of 2M H_2SO_4 added	pH after addition of H_2SO_4.
9-3-99	10.00	5.3		
	11.10	5.4		
	12.00	5.4		
	12.45	5.5		
	13.45	5.5	1	4.7
	13.46	4.7	1	3.7
	13.50	4.3		
	14.00	4.8		
	14.20	5.0		
	14.35	4.9		
	17.05	5.0		
	17.10	5.1		
	17.55	5.1		
	18.15	5.1		
10-3-99	9.45	5.2		
	11.30	5.2 - 5.3		
	12.00	5.3		
	12.30	5.3		
	13.20	5.3		

Following stabilising of the pH, the liquid was pumped out and the ossein washed 5 times in 550 ml of water for 30 minutes with stirring. Washing removed any excess acid and impurities left from neutralising. The pH of the drained washing water was measured after each washing.

Washing of the neutralised ossein

Washing	pH
1	5.4
2	5.5
3	5.8
4	5.6
5	5.6

The pH meter was calibrated before every measurement.

After the last washing the ossein was set aside until further use

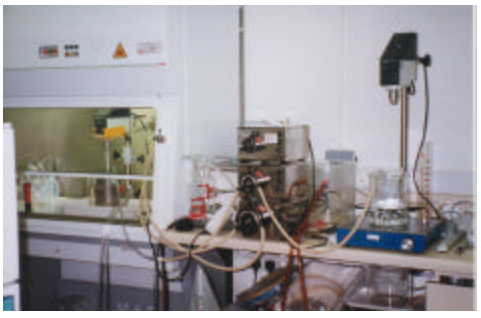
The stirrer and stand were removed from the cabinet and set aside in polythene bags while the cabinet was decontaminated. Any other equipment in the cabinet was bagged and disposed of.

Reference: Notebook 31-12-98 until 24-3-99 page 84 to 89. Date 7-3-99 until 10-3-99.

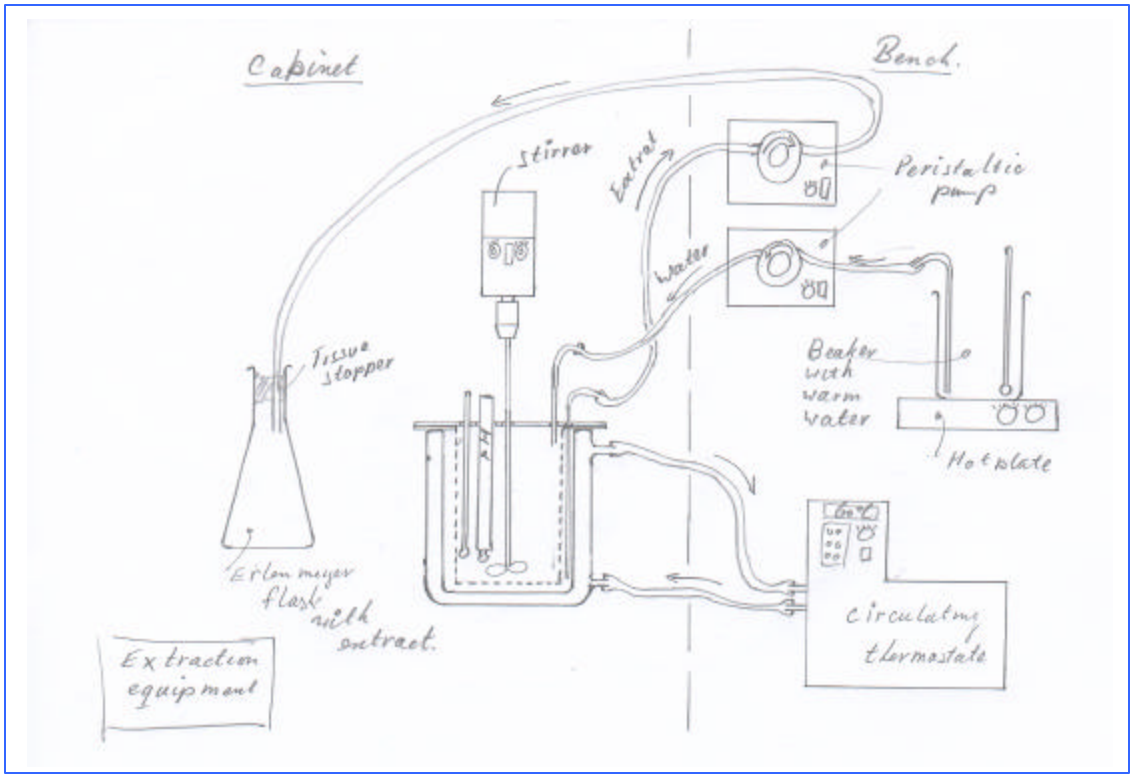
Extraction.

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.

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 65°C to maintain a temperature of 60°C in the double walled beaker. A beaker of approximately 2 litres of water was heated to 60°C on the hotplate of which 700 ml was pumped into the ossein. All equipment was then switched off and left to stand overnight. The heating equipment was switched on at 9.15 hrs



Extraction equipment



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 next day and, when the gel that had formed overnight had dissolved, stirring was started. At regular intervals, the extraction temperature, pH and the index of refraction (a measure of concentration of gelatine), were measured (see tables below). When the index of refraction

pH

index of refraction

reached 8.4 the extract was pumped into the Erlenmeyer flask and left to cool. The siphons and the pumping tube had been pre-warmed with hot water to prevent gelling of the gelatine. Approximately 420 ml gelatine extract was obtained.

Day	Time	Temperature	pH	Brix
10-3-99	9.15	switched on		
	13.00	60°C	6.0	5.6
	16.00	60°C	5.9	6.4
	17.05	59°C	5.9	7.0
	17.10	switched off		
13-3-99	11.30	switched on		
	12.30	60°C	6.0	7.8
	14.30	-	-	8.4

Pumping out the gelatine extract

The circulating thermostat was set at 77°C, to maintain an extraction temperature of 70°C. 750 ml of water at 70°C was pumped into the beaker and stirring restarted. A maximum index of refraction of 4.2 Brix was recorded and the gelatine solution was pumped out and left to cool. Approximately 520 ml extract was obtained.

Day	Time	Temperature	pH	Brix
13-3-99	14.50	63°C	6.2	1.8
	16.15	61°C	6.2	2.6
	17.30	69°C	6.0	3.2
	17.30	switched off		
14-3-99	9.45	switched on		
	11.00	69°C	6.3	3.4
	13.30	71°C	6.1	4.2

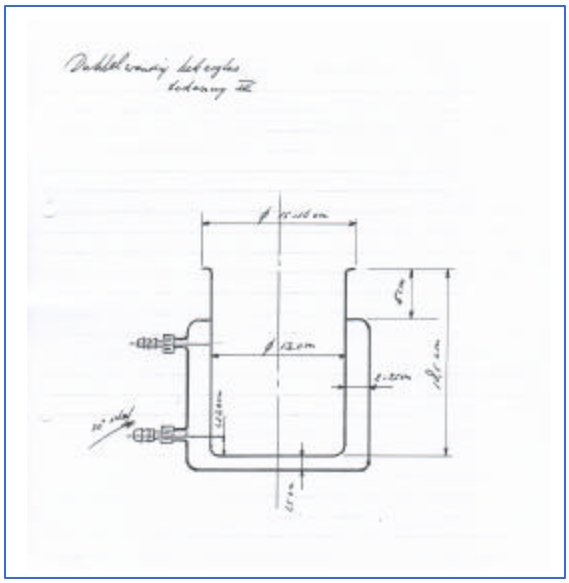
The circulating thermostat was set at 88°C, to maintain an extraction temperature of 80°C. 750 ml of water at 80°C was pumped in and stirring restarted. A maximum index of refraction of 2.4 Brix was measured at which point no ossein remained. The gelatine solution was pumped out of the double walled beaker in the same fashion as before. Approximately 530 ml extract was obtained and cooled. There was no residue left in the double walled beaker.

Day	Time	Temperature	pH	Brix
14-3-99	15.00	80°C	6.3	1.2
	16.15	83°C	6.3	2.4

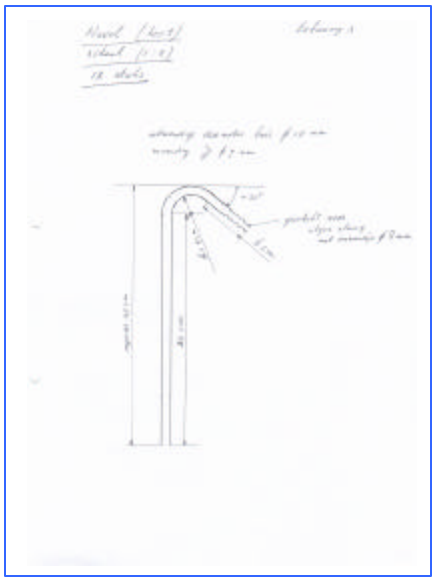
A 40 ml sample of each extract was taken and a mixed sample of all three extracts taken for infectivity assay in mice. The volumes used of each extract in the mixed sample were proportional to the volume of solution obtained, i.e. 12 ml of extract 1, 15 ml of extract 2 and 15 ml of extract 3.

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.

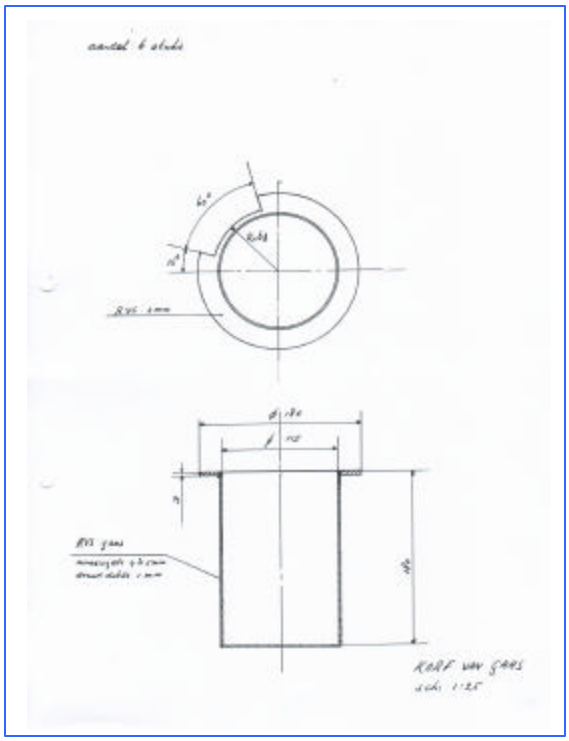
Reference: Notebook 31-12-98 until 24-3-99 page 89 until 92. Date 10-3-99 until 14-3-99.



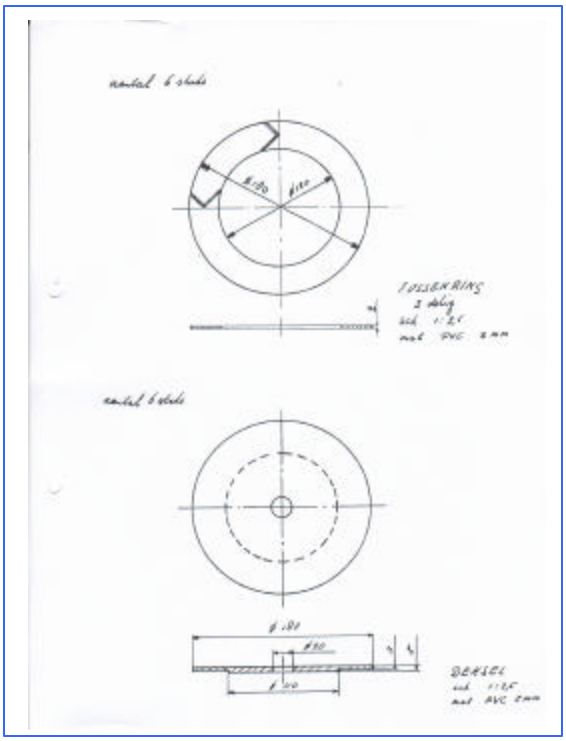
2 litre double walled beaker



siphon



Basket

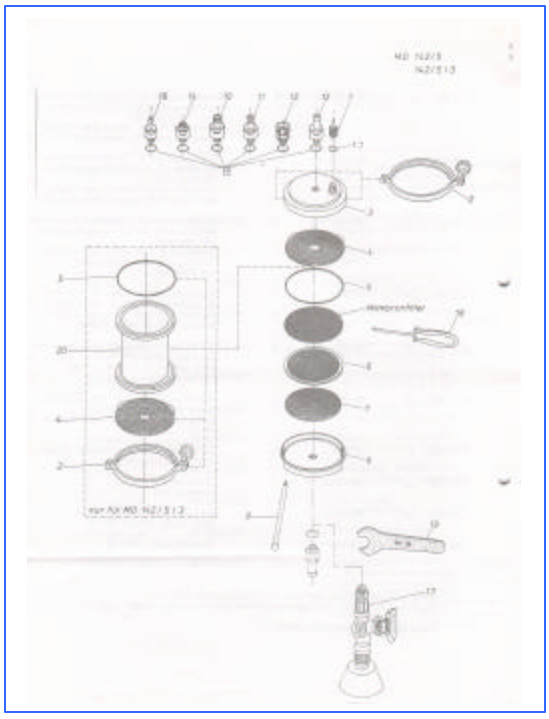


Lid and ring

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 588481680) equipped with a custom made glass siphon.

Filter



Schleicher & Schuell Pressure filter MW142

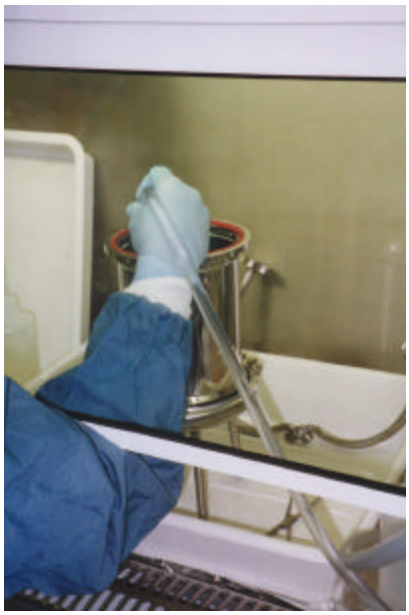
Filtration was done in three steps. First the gelatine extract was filtered through cloth cut from a GAF filter bag (GAF NMO-100-P01S-60L), then through an approximately 1 cm thick layer of diatomaceous earth (Dicalite 4200 and Dicalite speedflow) and finally through a Schenk AF1000 pre-prepared filter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

After each filtration, the filter unit was filled with a hypo chlorite solution containing 20,000 ppm free chlorine which was held for 1 hour. The filter was the washed 5 times with water. The O-rings of the filter were replaced with new ones before the 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.



Filling the filter with hot water

Total volume gelatine extract filtered	1300 ml
Total volume filtrate obtained	1300 ml

A 40 ml sample was taken of the filtrate

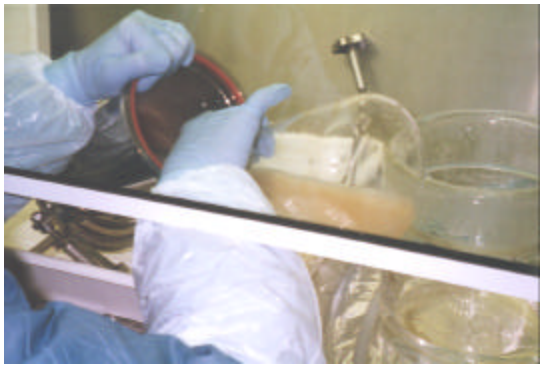
Filtration with diatomaceous earth.

The filter was preheated with hot water as described above after which it was disassembled and a piece of filter paper (Schleicher and Schuell) was put in place on the perforated base plate before re-assembly. The silicon hose on the bottom was closed with an artery clamp and the filter was filled again with hot water. 40 g Dicalite 4200 (diatomaceous earth) and 2.5 g Dicalite Speedplus (diatomaceous earth) were suspended in the water by stirring with a plastic disposable spoon. The artery clamp was removed and the water drained slowly to leave a filter layer on the filter plate. A small pressure was applied for a few seconds to remove most of the water from the filter layer. 1 g Dicalite 4200 and 0.3 g Dicalite Speedflow 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

Suspending diatomaceous earth in water

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

Diatom aceous earth filter layer



Filling the filter with gelatine extract

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 hypochlorite as described above.

Total volume gelatine extract filtered	1260 ml
Total volume filtrate obtained	1260 ml

A 40 ml sample was 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 diatomaceous earth filtration. The apparatus was then taken apart, the filter material removed, re-assembled, rinsed with water and treated with sodium hypochlorite as described above.

Total volume gelatine extract filtered 1225 ml

Total volume filtrate obtained 1225 ml

A 40 ml sample was taken from the filtrates.

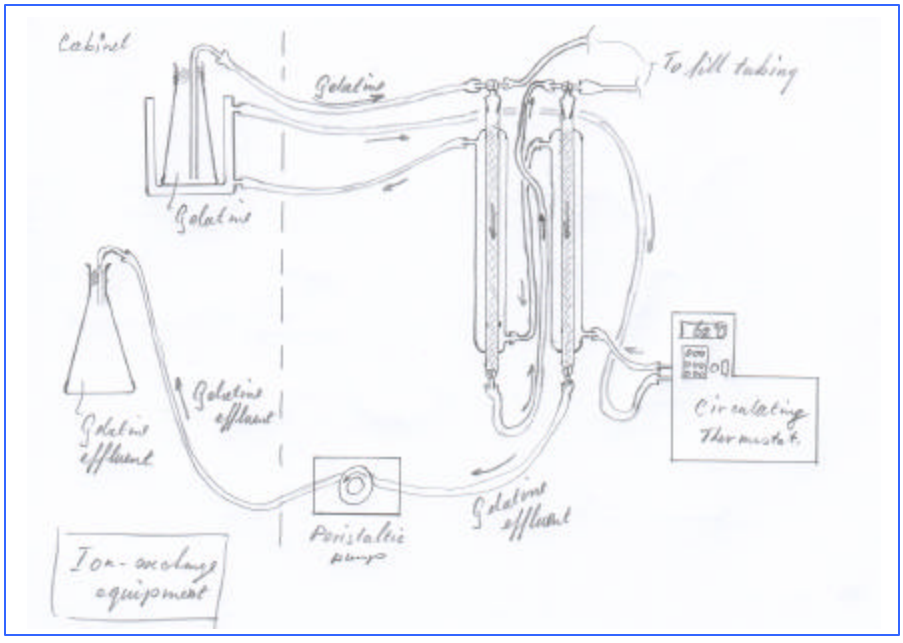
After completion of the entire filtration all equipment in the safety cabinet, including hoses coming in from outside, was disposed of. The safety cabinet was decontaminated.

Reference: Notebook 31-12-98 until 24-3-99 page 93. Date 15-3-99 to 17-3-99
Notebook 19-3-99 until 25-5-99 page 3 to 6. Date 19-3-99

Ion-exchange

Setting up equipment and regeneration of the resins.

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. The tubes in contact with the gelatine were silicon tubes, except for the Verderprene pumping tube.



Ion-exchange equipment

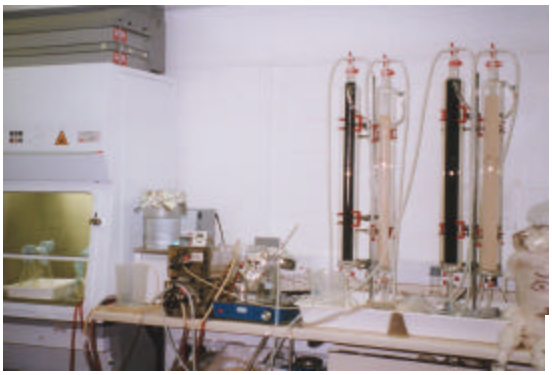
Water was pumped into the columns from the bottom to approximately half the column volume. The columns were then filled with the appropriate ion-exchange material by adding the resin as an emulsion in water to the top of the column. Attention was given to prevent formation of air inclusions. The first column was filled with cation resin Amberjet 1200H to a bed length of 70 cm, the second column was filled with anion resin Ira 94S 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-exchange columns

Filling with resin

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



Ion-exchange equipment

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 40 ml sample was taken from the first flask of effluent.

Filtered gelatine extract in:

Total volume approximately 1200 ml, time to pump in 35 minutes.

Erlenmeyer flask 1:	pH = 6.7	Conductivity = 520µS
Erlenmeyer flask 2:	pH = 6.8	Conductivity = 410µS

Effluent ion-exchange out:

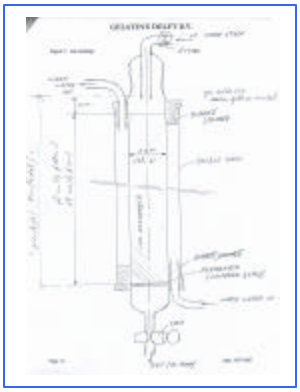
Erlenmeyer flask 1:	1000 ml	pH = 4.7	Conductivity <15 µS	2.0 Brix
Erlenmeyer flask 2:	500 ml	pH = 4.7	Conductivity <15 µS	0.7 Brix

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.

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
4 HANNA instruments pocket pH meters pHep -2
2 Hanna instruments pocket conductivity meters 100-19900 µS type 4
4 Hanna instruments pocket conductivity meters 10-1990 µS type 3



Ion-exchange column

Reference: Notebook 19-3-99 until 25-5-99 page 9. Date: 20, 24 and 25-3-99

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 attached 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.

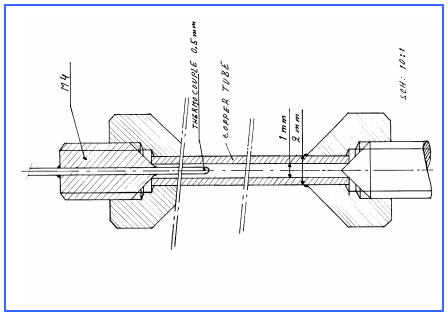
Evaporation 1: Approximately 250 and 200 ml from Erlenmeyer flask 1 and between 220 and 250 from Erlenmeyer flask 2.
Approximately 650 ml water was collected.
Concentrate: 60 ml of 18.8 Brix.

Evaporation 2: Approximately 250 and 200 ml from Erlenmeyer flask 1 and between 250 from Erlenmeyer flask 2.
Approximately 650 ml water was collected.
Concentrate: 65 ml of 21 Brix.

Reference: Notebook 19-3-99 until 25-5-99 page 10. Date: 24 and 25-3-99

UHT-sterilisation.

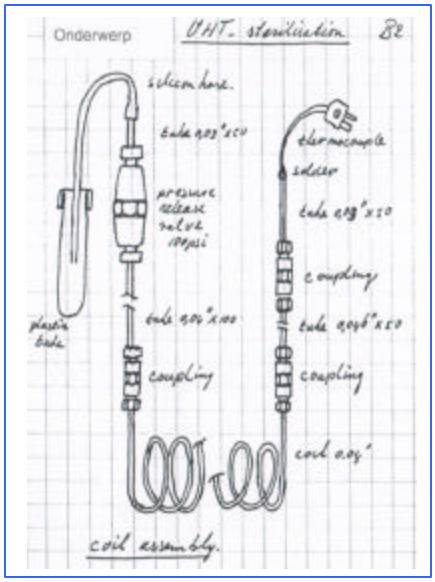
UHT-sterilisation was first carried out in copper capillaries of 1 mm inner-diameter and 2 mm outer-diameter. These were filled with the concentrated gelatine and soldered closed except one, containing a thermocouple, which was closed by tight screwed pointed nuts. Sterilisation of the gelatine was successfully achieved by immersing the copper capillaries in hot oil baths at approximately 140°C. However, mice intracerebrally injected with a 20 µl sample of the sterilised gelatine died shortly after inoculation. This was thought to have been caused by copper toxicity as fine copper deposits were visible in the gelatine following sawing open the capillaries, and the gelatine was also faintly blue-coloured blue most likely because of dissolved copper.



Copper capillary

Reference: Notebook 25-5-99 until 9-9-99 page 8, 9 and 11. Date: 1 to 4, 10 and 11-6-99

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

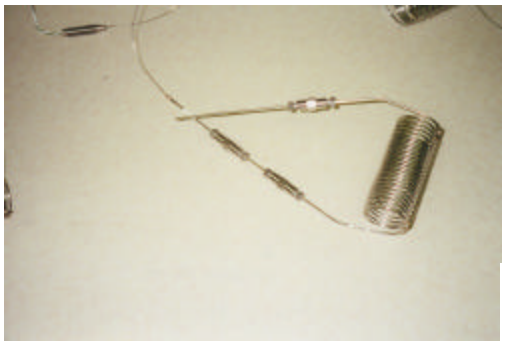
Piece of silicon hose inner diameter 1 mm
Centrifuge tube with orange cap 10 ml

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.

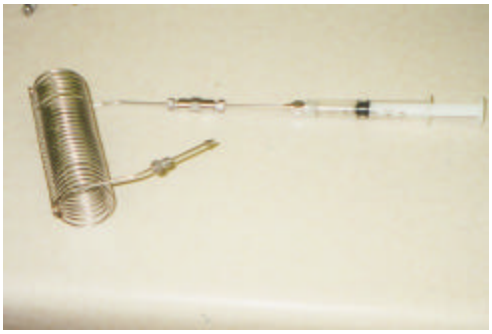
The offset of the thermocouples was determined in one of the oil baths against the thermocouple on channel 1 of a Kane May 1242 electronic temperature recorder. The thermocouple of the Kane May 1242 had been calibrated against a calibrated thermometer.

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

UHT-sterilisation equipment

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 3 seconds 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

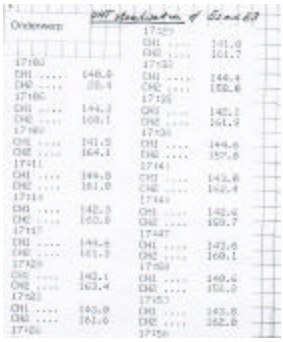
Measurement offset thermocouples of coil assemblies.

Tested thermocouple number	Temperature of calibrated thermocouple	Temperature according tested thermocouple	Calculation offset	Offset
K8	138.9	133	138.9 - 1 – 133	**4.9**
K9	142.4	137-138 (137.5)	142.4 - 1 – 137.5	**4.0**
K24	140.6	135	140.6 – 1 – 135	**4.6**

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 3 times, the details of which are shown below.



Results

Sample E3K8 **Sample E3K9** **Sample E3K24**

UHT sterilisation of E3			UHT sterilisation of E3			UHT sterilisation of E3		
Test E3K8		offset = 4.9	Test E3K9		offset = 4	Test E3K24		offset = 4.6
time(sec)	Temp©	Corr Temp	time(sec)	Temp©	Corr Temp	Time(sec)	Temp©	Corr Temp
0	21	25,9	0	20	24	0	21	25,6
0,28	21	25,9	0,27	20	24	0,28	21	25,6
0,56	21	25,9	0,54	20	24	0,56	21	25,6
0,84	23	27,9	0,81	23	27	0,84	22	26,6
1,12	33	37,9	1,07	33	37	1,12	22	26,6
1,40	51	55,9	1,34	50	54	1,40	29	33,6
1,68	73	77,9	1,61	74	78	1,68	45	49,6
1,96	95	99,9	1,88	96	100	1,96	66	70,6
2,24	112	116,9	2,15	113	117	2,24	88	92,6
2,52	125	129,9	2,42	124	128	2,52	107	111,6
2,80	133	137,9	2,69	130	134	2,80	121	125,6
3,08	137	141,9	2,95	132	136	3,08	129	133,6
3,36	138	142,9	3,22	133	137	3,36	132	136,6
3,64	137	141,9	3,49	133	137	3,64	132	136,6
3,92	136	140,9	3,76	134	138	3,92	132	136,6
4,20	135	139,9	4,03	134	138	4,20	132	136,6
4,48	135	139,9	4,30	135	139	4,48	133	137,6
4,76	134	138,9	4,56	135	139	4,76	133	137,6
5,04	134	138,9	4,83	135	139	5,04	134	138,6
5,32	134	138,9	5,10	136	140	5,32	134	138,6
5,60	134	138,9	5,37	136	140	5,60	134	138,6
5,88	134	138,9	5,64	136	140	5,88	134	138,6
6,16	134	138,9	5,91	136	140	6,16	134	138,6
6,44	134	138,9	6,18	136	140	6,44	134	138,6
6,72	134	138,9	6,44	136	140	6,72	134	138,6
7,00	134	138,9	6,71	136	140	7,00	133	137,6
7,28	133	137,9	6,98	136	140	7,28	133	137,6
7,56	133	137,9	7,25	136	140	7,56	133	137,6
7,84	133	137,9	7,52	136	140	7,84	130	134,6
8,12	133	137,9	7,79	136	140	8,12	117	121,6
8,40	133	137,9	8,06	135	139	8,40	94	98,6
8,68	131	135,9	8,32	134	138	8,68	68	72,6
8,96	124	128,9	8,59	122	126	8,96	49	53,6
9,24	105	109,9	8,86	99	103	9,24	39	43,6
9,52	80	84,9	9,13	71	75	9,52	35	39,6
9,80	58	62,9	9,40	52	56	9,80	33	37,6

10,08	47	51,9	9,67	46	50	10,08	31	35,6
10,36	43	47,9	9,94	44	48	10,36	31	35,6
10,64	40	44,9	10,20	40	44	10,64	31	35,6
10,92	35	39,9	10,47	34	38	10,92	31	35,6
11,20	30	34,9	10,74	28	32	11,20	32	36,6
11,48	26	30,9	11,01	24	28	11,48	32	36,6
11,76	23	27,9	11,28	22	26	11,76	31	35,6
12,04	22	26,9	11,55	21	25	12,04	30	34,6
12,32	21	25,9	11,81	20	24	12,32	30	34,6
12,60	21	25,9	12,08	20	24	12,60	29	33,6
						12,88	28	32,6
						13,16	26	30,6
						13,44	25	29,6
						13,72	23	27,6
						14,00	22	26,6
						14,28	22	26,6
						14,56	21	25,6
						14,84	21	25,6

Summary of the results

E3K8 shows a slight overshoot to about 143°C

E3K9: time to 134°C 2.2 seconds
time above 120°C 6.5 seconds
time above 133°C 5.5 seconds
time above 137°C 4.8 seconds
time taken to reach 137°C 3 seconds
The amount of time above 137°C was 0.8 secs over the desired period of 4 secs.

E3K24: time to 134°C 1.9 seconds
time above 120°C 5.3 seconds
time above 133°C 4.4 seconds
time above 137°C 3.0 seconds
time taken to reach 137°C 3 seconds

Although the period of time above 137°C was 1 second shorter than the desired 4 second period, this test was the best approximation of the industrial 4 second UHT sterilisation.

Sterilised sample E3K24 was used for measurement of infectivity by mouse bioassay.

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 to 11. 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

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

Die Gültigkeit der Eichung erlischt vorzeitig, wenn eine der in § 13 Absatz 1 der Eichordnung beschriebenen Veränderungen eingetreten ist.

Eichscheine ohne Unterschrift und Dienstsiegel haben keine Gültigkeit. Dieser Eichschein darf nur vollständig und unverändert weiterverbreitet werden. Auszüge oder Änderungen bedürfen der Genehmigung der Eichbehörde. Im Zweifelsfalle gilt der deutsche Text des Eichscheines.

Ort und Datum — Ilmenau, 1999-01-14

Dienstsiegel

Unterschrift

Amtsrat

Unterpörlitzer Str. 2 · 98693 Ilmenau · Telefon 03677/650-300 · Telefax 03677/650-400

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.

Samples were taken at most points of the manufacturing process, and some material was lost in the equipment during processing. For these reasons, the obtained amount of gelatine was less than maximally possible.
Calculations performed using the volumes obtained were compensated for this using a calculated correction factor.

Amount of starting material.

Total amount of 301V infected mouse brain on the bone starting material	10.14 g
	====
Crushed bone	1501.0 g
Backbone	501.7 g
301V infective mouse brain	10.14 g
Total amount of spiked bone starting material	2013 g
	====

Correction factor for extracted gelatine

I.	Amount of wet degreased bone and tissue	*1345* g
	Sample taken of wet degreased bone and tissue	28 g
	Amount used in further processing	*1317* g
II.	Weighed amount of sorted dried bone chips	463 g
	Sample taken of sorted dried bone before weighting	25 g
	Original amount of sorted dried bone	*488* g
	Amount of sorted dried bone chips	463 g
	Bone weight equivalent of sample ossein	20 g
	Bone weight equivalent extracted	443 g

Calculated correction factor = (1345/1317) x (488/443) = 1.125

Correction factor for extracted gelatine 1.1

Correction factor for sterilised gelatine.

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

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

Extracts:

	Initial volume	Samples	Remaining
Extract 1	520 ml	52 ml	468 ml
Extract 2	520 ml	55 ml	475 ml
Extract 3	530 ml	55 ml	485 ml

Filtrates:

All samples were taken from the filtrate of Extract 1

Filter bag	40 ml
Diatomaceous earth	40 ml
Schenk filter sheet	40 ml
Total from filtrate 1	120 ml

Apart from removal of samples, some gelatine was also lost during filtration because of absorption by the filter media and because gelatine solution was left 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 in the calculation below.

Effluent ion-exchange:

The effluent was considered as one pool of material

Obtained effluent	1500 ml
Sample	40 ml
Effluent to concentration	1460 ml

Amount of concentrated gelatine: 0.60 x18.8 + 0.65 x 21 = 24.93 Brix

Correction factor =

= 1.125 x ((((4.2-0.52-1.2)x8.2+(5.2-0.55)x4.2+(5.3-0.55)x2.4)x1460/1500)/24.93)x
x1470/1428x1300/1260x1260/1220x1225/1185x1500/1460

= 2.63

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 10.14 g

B. Extracted gelatine

Total volume of extracted gelatine	**1470 ml**
Correction factor for extracted gelatine	**1.1**

C. Sterilised gelatine

Total volume concentrated gelatine	**125 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 disposing of all equipment immediately after use. The risk of cross contamination in this way was small as in most cases starting material and end product did not co-exist. In the cases where both were present, these fractions were kept physically separate such that no cross-contamination could occur. 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 1). 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).

Alkaline 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).

Alkaline 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 (titration 1)	301V -infected mouse brain (titration 2)	Alkaline process - crude gelatine extract	Alkaline process - sterilised concentrated gelatine
10^0	N/A	N/A	18	
10^{-1}		2	18	18
10^{-2}		2	18	18
10^{-3}		2	18	18
10^{-4}	12	6	18	18
10^{-5}	12	6		
10^{-6}	18	6		
10^{-7}	18	12		
10^{-8}	18	12		
10^{-9}	18	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 1). Titrated 10/5/00 and 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.

Alkaline process - crude gelatine extract. Titrated 3/11/99.

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^{-4}. The warmed undiluted (10^{0} group) was also inoculated into mice.

Alkaline 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 1
Titration 372J-1L
Final 420 days post injection.

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

301V mouse brain pool 1
Titration 372S1T/1
Final 358 days post injection.

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

Titration data of gelatine samples.

Sample of extracted gelatine
Titration 372J-1C
Final 666 days post injection.

-log dillution	Number of mice	Mice positive on 301V	Average incubation period (days)
0	18	10	283
1	14	1	378
2	17	0	-
3	16	0	-
4	17	0	-

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

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