

**Validation of the clearance of TSE agent by  
the initial steps of the alkaline gelatine  
manufacturing process**

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Authors:

D.M. Taylor  
R.A. Somerville  
P.J. Steele  
A.H. Grobden

Sponsors:

GME Sector Group  
CEFIC  
Avenue E. Van Nieuwenhuysse 4, bte 2  
B-1160 Bruxelles (Belgium)

European Commission  
Research Directorate-General  
Directorate B.1 – Life Sciences  
Health Food and Environment  
Rue de la Loi 200  
B-1049 Bruxelles (Belgium)

Laboratory:

Institute for Animal Health  
Neuropathogenesis Unit  
Ogston Building  
West Mains Road  
Edinburgh (UK)  
EH9 3JF

**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, 4 July 2003



R.A. Somerville



P.J. Steele



A.H. Grobber

INSTITUTE FOR ANIMAL HEALTH  
BBSRC NEUROPATHOGENESIS UNIT  
OGSTON BUILDING, WEST MAINS ROAD  
EDINBURGH EH9 3JF  
TEL: 0131 667 5204 FAX: 0131 668 3872

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**PERSONNEL INVOLVED.**

Study Directors	D.M. Taylor, PhD MBE R.A. Somerville, PhD
Study Coordinator	A.H. Grobбен, BSc
Project Leaders	D.M. Taylor, PhD MBE R.A. Somerville, PhD A.H. Grobбен, BSc (for GME)
Experimental procedures	A.H. Grobбен, BSc P.J. Steele, BSc
Animal Services Manager	I. McConnell, BSc MIAT
Pathology Laboratory Manager	A. Suttie

## SUMMARY REPORT.

The initial steps of the industrial manufacturing process for the production of limed bone gelatine, in which bones are converted into a gelatine extract, were downscaled to an accurate laboratory scale model. Using this downscaled model process, gelatine extract was made from bones experimentally contaminated with hamster brain infected with the 263K strain of hamster-passaged scrapie agent. Samples of input, intermediate, and output material were taken throughout the experimental process. To determine the capacity of the process to remove/inactivate 263K infectivity, a sample of the infectious brain and crude gelatine extract were assayed for the amount of infectivity present by intracerebral inoculation in experimental hamsters. The measured infectivity of the infectious brain was  $10^{8.0}$  ID<sub>50</sub>/g. The crude extract  $10^{1.1}$  ID<sub>50</sub>/g. the calculated clearance factor for the process steps up to extraction was:  $10^{4.6}$  ID<sub>50</sub>.

### Introduction.

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

This report contains the results of the inactivation and removal of TSE infectivity by the initial steps of the alkaline process (usually called limed bone process), a description of the experiments done, and all other data associated with this study. The experiment is also intended to support and confirm the result of the same experiment using the 301V strain of mouse passaged Bovine Spongiform Encephalopathy agent.

### Industrial manufacturing process steps.

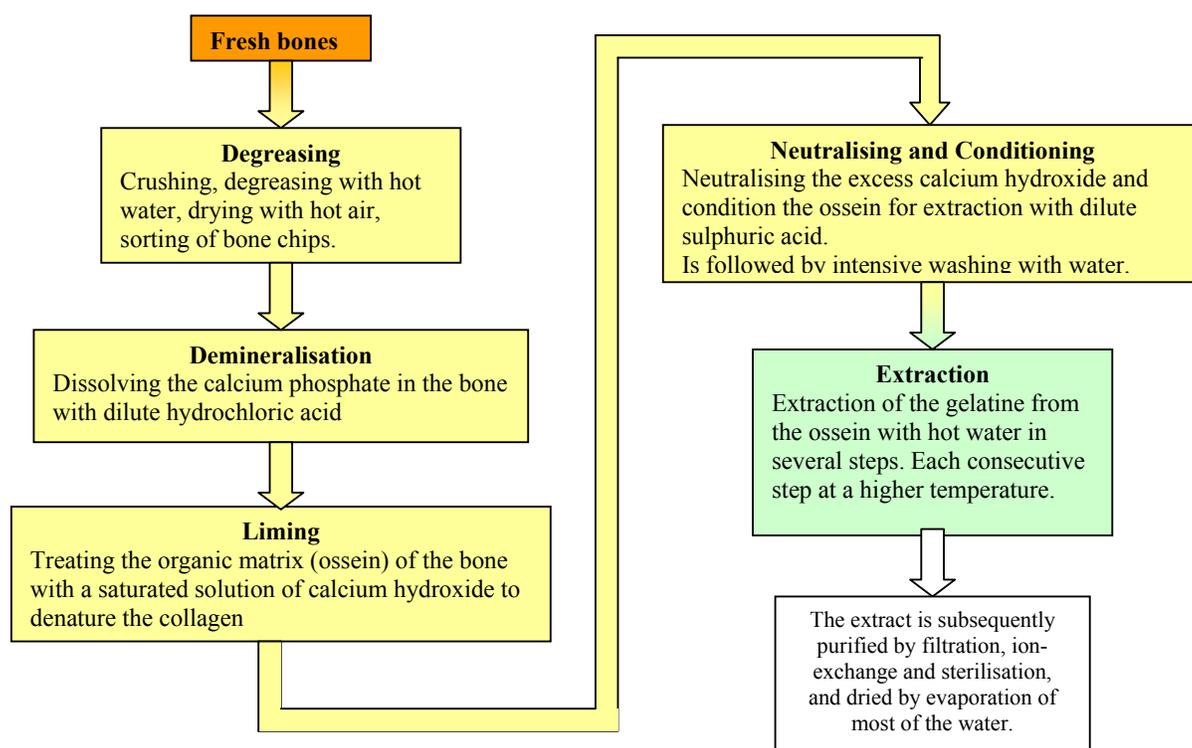
The limed bone manufacturing process is extensively described in Appendix 1 to this report. Here follows a brief description and diagram.

The initial stage of 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, the treatment of dried degreased bone chips with dilute hydrochloric acid to dissolve the calcium phosphate content. The resulting organic matrix, called ossein, is washed with water.
- Liming, where the ossein is treated for at least 20 days with an oversaturated solution of calcium hydroxide, 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.

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



## **Development of the study.**

### *Demands on the study*

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

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

These demands were met by:

- Using an accurately downscaled model of the industrial process, developed to maintain the same manufacturing conditions as those of the industrial process.
- Using industrial crushed bone as starting material. Backbone, which was added to facilitate spiking with infectious material, was treated such that it did not differ from industrial crushed bone.
- Adding infectious material to represent both direct infection and cross contamination of bone material.
- The use of 263K infected hamster brain, a hamster adapted scrapie strain which achieves high titres of infectivity, and is known to be relatively resistant to inactivation.
- Detection of 263K infectivity by hamster bioassay.

### *Downscaled model process*

The study was performed using an accurately downscaled model of the industrial process using the same conditions as in the industrial process. 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 influence inactivation.

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

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

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

### **Materials and methods.**

#### *Agent strain.*

TSE infected brain material was used as the infectious load. Specifically, the hamster-passaged scrapie strain, 263K was used as this strain achieves high levels of infectivity in the brain of infected rodents and is known to be relatively resistant to inactivation. The 263K infected hamster brain material was prepared by IAH-E. To determine the amount of infectivity present, the brain material was titrated by intracerebral inoculation into hamsters.

#### *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 263K spike.*

The raw materials consisted of 2017 g of fresh crushed bone and backbone, which contained 9.98 g 263K infected hamster brain. The process was executed from degreasing up to extraction. The ossein produced by demineralisation was limed for 20 days. 1550 ml of crude gelatine was extracted from the limed ossein. A sample of the crude gelatine extract was titrated by hamster bioassay to determine infectivity titre.

*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 hamsters (50µl/hamster). 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 519 days (spike material) and 631 days (output samples) post-injection. Animals injected with output samples were kept for more than 600 days because treatment of the agent potentially modifies the dose-response curve. The brains of all animals injected were removed and fixed in formol-saline. Sections were subsequently cut and stained with haematoxylin and eosin. These were examined microscopically for the spongiform lesions that are pathognomonic for 263K infection in hamsters. 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 263K spike material and of the output gelatine produced

**Table 1. Infective titres of 263K infected hamster brain and gelatine made by downscaled process.**

Sample name	Study and sample number from protocol	Titre/result (ID <sub>50</sub> )	Observation time
263K brain titration		10 <sup>7.98</sup>	519 days
Limed bone process 263K; extracted gelatine	Study 2 - Sample 3	10 <sup>1.13</sup>	631 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.

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

The data for calculation of clearance factors are in table 2. The calculated clearance factors are in table 3.

**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 <sub>50</sub> /g	Amount of gelatine (g)	Corr. factor	Titre of gelatine ID <sub>50</sub> /g
Alkaline process 263K - extracted gel.	Study 2 - sample 3	9.98	10 <sup>8.0</sup>	1550	1.1	10 <sup>1.1</sup>

**Table 3. Clearance factors.**

Sample name	Total clearance factor ID <sub>50</sub>
Alkaline process 263K - extracted gelatine	10 <sup>4.6</sup>

## Discussion

The titration values recorded show a significant reduction of 263K infectivity of 10<sup>4.6</sup> by the downscaled alkaline manufacturing process steps degreasing, demineralisation, liming, neutralising and extraction.

In a separate parallel experiment the TSE removal/inactivation capacity of the initial stage of the alkaline bone gelatine manufacturing process was tested using mouse brain infected with the 301V strain of mouse adapted BSE agent as spike material. The results of that experiment were reported in the report *Validation of the clearance of TSE agent by the alkaline gelatine manufacturing process* of 20 August 2002. The reduction factor found in that experiment was 10<sup>3.7</sup> ID<sub>50</sub>. Though the clearance factor found in that experiment is not precisely equal to the one reported here, the order is approximately the same. Current data from inactivation studies

suggest that BSE derived strains such as 301V are more thermostable than those derived from scrapie. The larger clearance observed here for the hamster adapted scrapie strain is consistent with these data.

Requirements for this validation studies were mentioned on page 6 and 7.

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.

Hamster brain and spinal cord from the backbone were added to the industrial crushed bone starting material. Of the total raw material weight, 0.5% consisted of infectious hamster brain and approximately 0.5% calf spinal cord. This amount of cerebrospinal tissue is at least 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.

The infectious hamster brain was introduced in a way that resembled incorporation of both directly infected and cross contaminated raw material into the manufacturing process. Based on current data regarding BSE infectivity in cows, the level of infectivity applied in this experiment is much higher than would be encountered in current industrial practice, but is used to facilitate the measurement of definite clearance values.

263K 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, 263K, 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 initial steps of a downscaled model of the limed bone gelatine manufacturing process substantially decreased 263K infectivity, with a factor  $10^{4.6}$ . The process steps tested were degreasing, demineralisation, liming and final washing.
2. The result supports those found in parallel experiments using BSE-derived material reported in *Validation of the clearance of TSE agent by the alkaline gelatine manufacturing process* of 20 August 2002.
3. The gelatine manufacturing process was successfully scaled down; gelatine was prepared from industrial starting material.
4. The study complies with the requirements of a validation study.

## Appendix 1. INDUSTRIAL MANUFACTURING PROCESS STEPS

### Short description of the limed bone process steps

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 subsequently purified by filtration and ion-exchange, followed by concentration by vacuum evaporation, sterilisation of the concentrated solution and drying of the gelled gelatine concentrate in a stream of warm air.

### 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 °C in some installations to 90 °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.

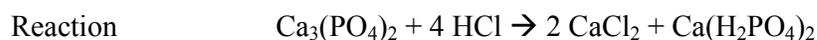
Modern installations are made of stainless steel whereas older installations are made of normal steel.

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 %

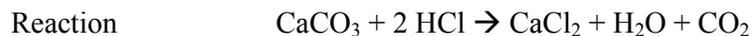
#### *Demineralisation (also called acidulation)*

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.



Mol. weight 310.3 36.5 111 234.2

Equivalent weight 310.3 146



Mol. weight 100 36.5 111 18 44

Equivalent weight 100 73

For the demineralisation of one tonne of bone chips, containing 8% water and 63% hydroxyapatite dry matter one needs:

$$0.90 \times 0.63 \times 1000 \times (0.89 / 310.3 \times 146 + 0.11 \times 100 \times 73) = 283 \text{ kg HCl}$$

Hence, approximately 7,000 litres 4% hydrochloric acid is needed for complete conversion of one tonne 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.

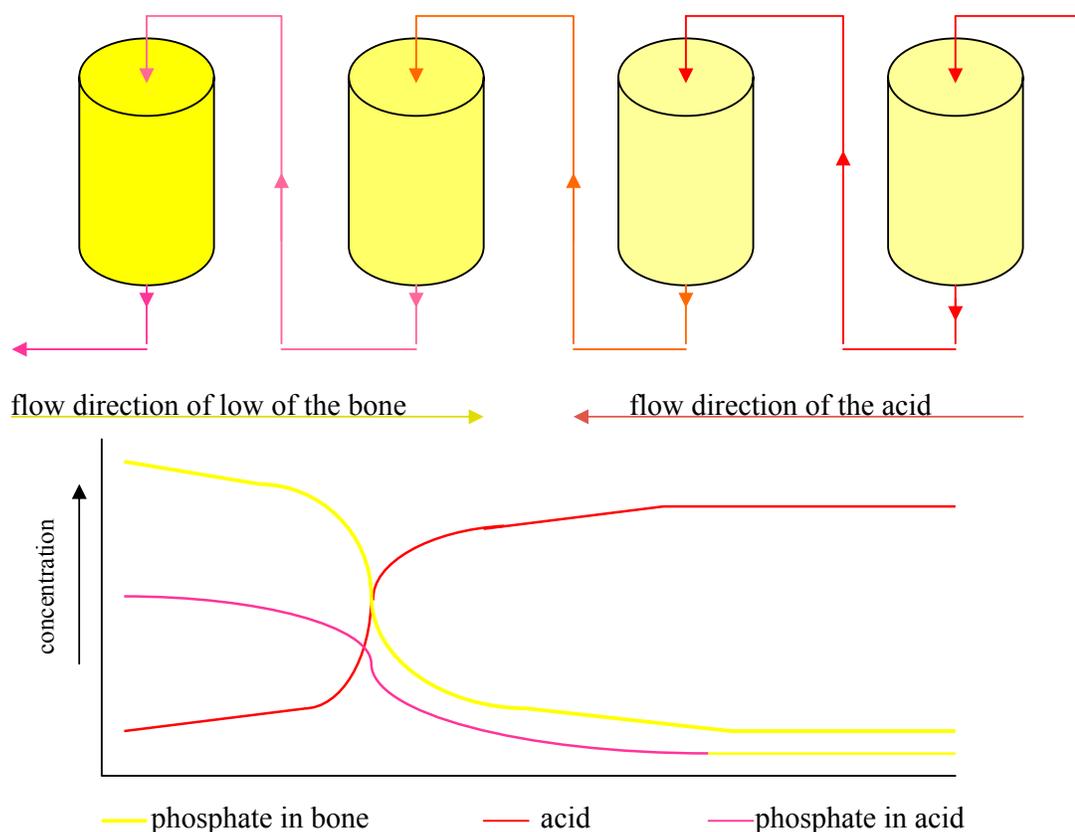


fig. 1 cascade process

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 tonnes 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 tonnes 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 tonnes 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.*

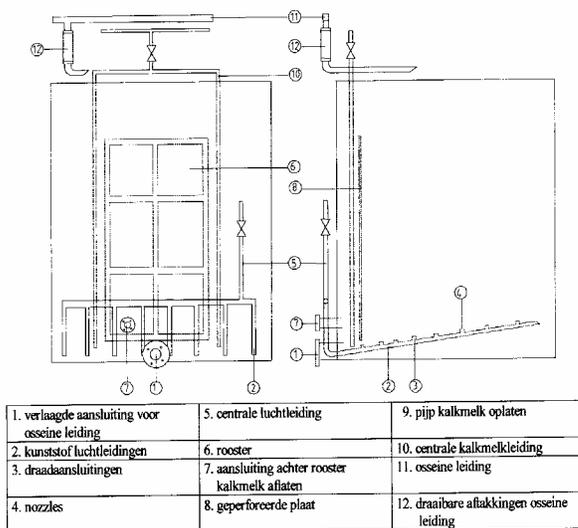
During liming the ossein is immersed in a supersaturated calcium hydroxide solution with a pH  $\geq$  12.5. Being supersaturated the pH remains 12.5, because any consumed dissolved lime is replaced by excess precipitated lime. The ossein is kept immersed in this solution for a period of at least 20 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.



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,  $\text{pH} \approx 12.5$ , 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.*

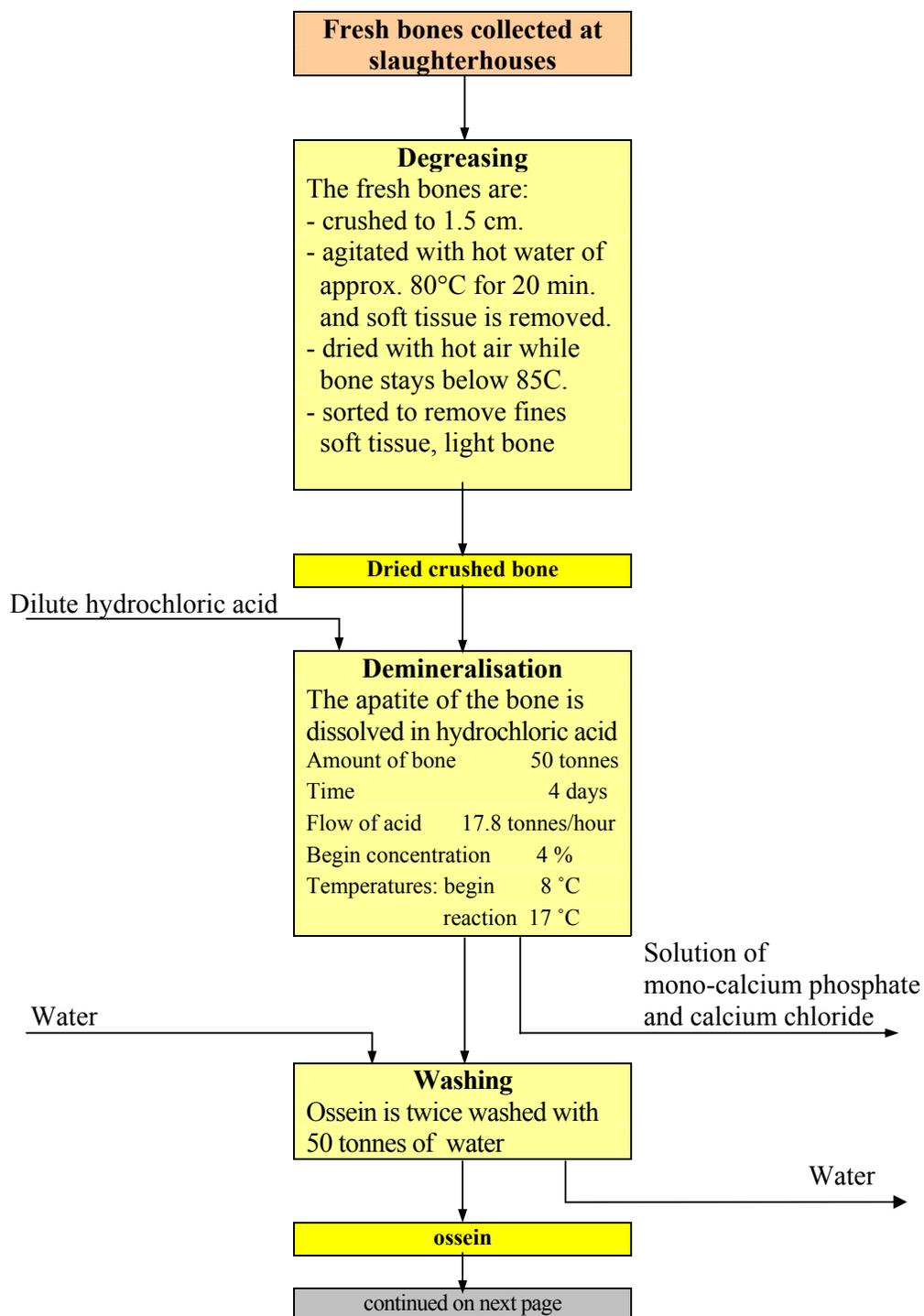
Gelatine is extracted from the denatured collagen in the ossein with warm water and is done in a number of steps, each being an individual extraction. The water temperature is increased with each consecutive extraction. The first is carried out usually at 50 to 60 °C, the last one at 100 °C. Individual consecutive extractions can however be done at the same temperature. The gelatine concentration of the extract is normally between 3 and 8 %.

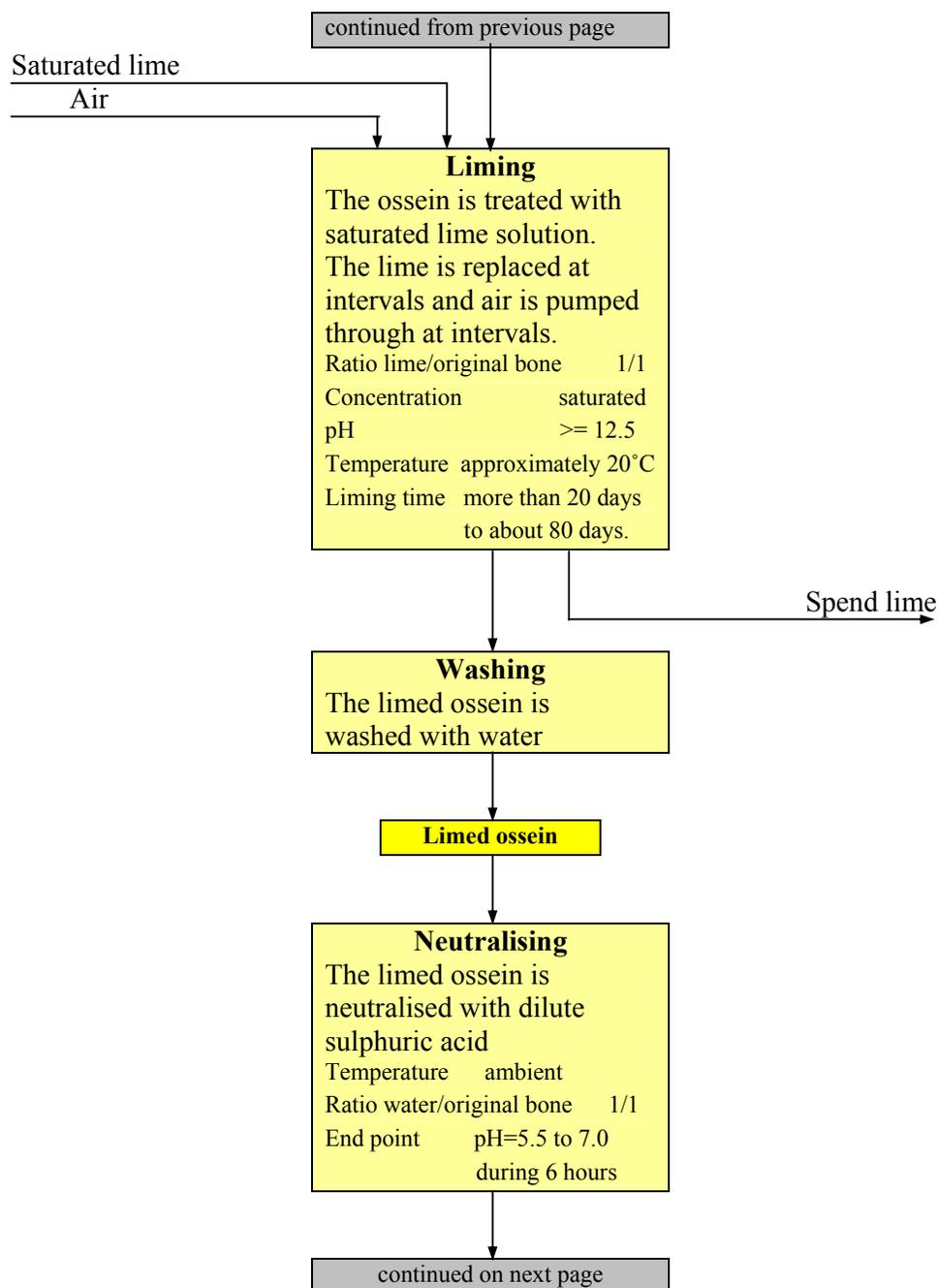
By the extraction process, H-bridge bonds and Van der Waals forces maintaining the collagen helix are broken, allowing individual collagen molecules to dissolve. The process is not ideal and, especially at higher temperatures, peptide bonds in the collagen molecules are broken. Further, not all covalent 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.

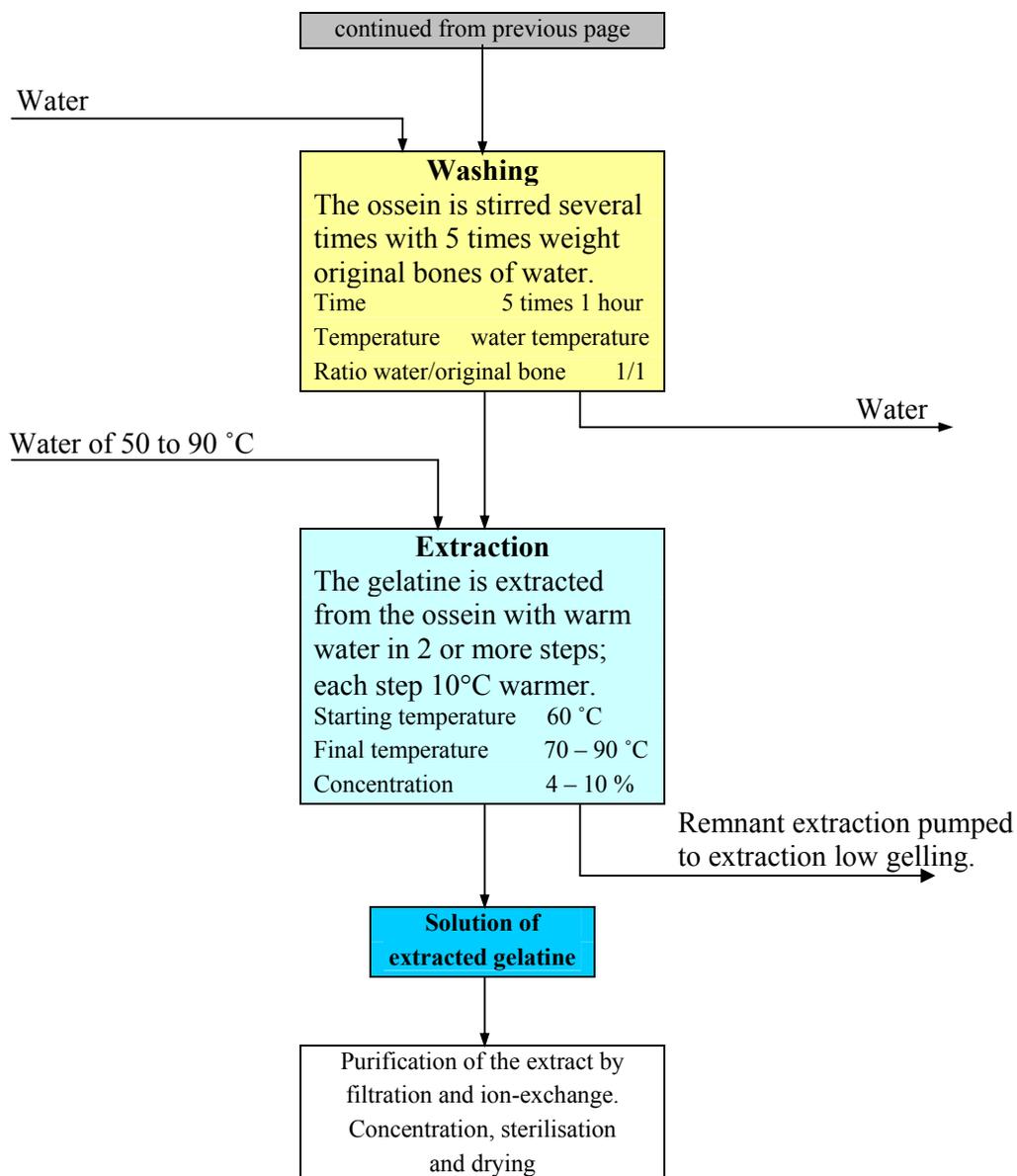
Industrially, extraction is executed as follows. In the extraction pre-heated water is added to the ossein, or cold water is added which is then heated in the extractors. The extractors are either stirred or the water is circulated over the ossein bed. This is continued until a gelatine concentration 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 tonnes 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 extraction 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.

**Flow sheet of the limed bone gelatine manufacturing process steps.**





### 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 tonnes bone chips.  
Dimensions of reactor 50 tonnes: height 7 m, diameter 3.5 m  
4 reactors in cascade  
Duration of process 4 days  
Flow of acid 17.8 tonne/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 tonnes of water.
- Liming:** Liming time: 20 to 80 days  
Refreshing lime and blowing air according table 1  
Washing 2 times with about 50 tonnes 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 tonnes 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.

## Appendix 2. DOWNSCALING PROCEDURE.

Industrial gelatine is manufactured from batches of between 100 tonnes and 250 tonnes 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**

<b>Process step</b>	<b>Minimum process conditions of industrial process</b>	<b>Process conditions of downscaled process</b>
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	Neutralising under stirring with sulphuric acid. Washing in several steps with 5 times original weight of bones of water.	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.
Extraction	Stepwise with sufficient water, at 60, 70, 80, ... °C. Concentration at draining 3-9%	Stepwise with 800 ml water at 60°C and every next time 10°C more. Concentration at decanting 3 to 9%

### 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 batchwise degreasing 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  $\frac{3}{4}$  of the way through the process time stepwise with an amount of fresh water equal to the weight of the crushed bone being degreased. In the final  $\frac{1}{4}$  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.

All together the batchwise degreasing applied in the laboratory process still represents more than a worst case situation, for in the industrial continuous process infectivity washed from the bone will leave the process with the effluent continuously.

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<sub>2</sub>, and 40.5g Ca(H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub> per litre water and a temperature of 18 to 22°C for 24 hours.
2. A solution containing 25g HCl, 23g CaCl<sub>2</sub>, and 17.5g Ca(H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub> 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.

#### 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 root 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 may not always be 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 263K infected hamster 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 hamster 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. The bones without the skull and the backbone of one whole cow weighs approximately 20 to 29 kg, the backbone 4 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 maximal 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 (historical) 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 °C	approx. 31 %	approx. 48 %
Ash 1100 °C	approx. 30.5 %	approx. 47.5 %

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

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

The calf from which the backbone was obtained was bought and slaughtered specifically for use in these experiments. All the relevant associated papers are shown on the following pages together with the health certificate of the bone material used.

 **VEEHANDEL A. ROMMENS**  
Schoutestraat 9 - 4735 TH Zegge  
Telefoon 0165 - 55 74 33  
Fax 0165 - 54 55 94  
B.p.g. 0165 - 53 49 84  
Robobank Rek. nr. 14.43.01.741

Datum: 2-11-1998  
Nota voor: C.v. Elteren  
Kagerstraat 11  
Oud-gastel

AANKOOP: VERKOOP: 1 Rund fl. 1150,-  
P. Rommens nummer: 199 084 528

Levering heeft plaatsgevonden binnen half uur van art. 27, 2e lid O.B. 1968  
Landbouw, Veehandel wetgeving.

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V.o.b. West Brabant  
Postbus 199  
4870 AD Etten Leur  
Tel. 078-503120  
1210582 Gebr. Luijk

Postbus 454, 6800 AL Arnhem  
Verwerkingsdatum 17-04-98

GER DATUM	DRAAGTIJD	SEXE	LEVENSNUMMER
12/03/98	293	vr1	1990 8452 g

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VADER: Roel T1 457834445  
MOEDER: Bianca 2 857016676  
BIJZONDERHEDEN: 168 50%HF 37%RY 12%JER C

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199 084 528  
8452

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Met genoemde rundveebedrijf is evenals het/de betreffende runderen ten tijde van afgifte van deze verklaring gekwalificeerd als:

<p><b>Dierziekte</b></p> <p>Brucellose Tuberculose Leucose Leptospirose IBR Paratuberculose BVD Salmonellose* BSE</p>	<p><b>Kwalificatie</b></p> <p>Officieel vrij Officieel vrij Officieel vrij Vrij Niet vrij Onbekend Vrij</p>
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\*eventueel aanvullende informatie veehouder/praktiserend dierenarts

Deze verklaring is geldig tot maximaal zeven dagen na datum afgifte.  
Datum afgifte: 16-10-1998

De directeur,

### SLAGERIJ VAN ELTEREN V.O.F.

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4761 RX OUD-GASTEL  
TEL 0186 51 25 42  
AUTOTELE 06 53 17 50 60  
FAX 0186 51 18 22

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Koning...

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(Veterinair Certificaat)

Ministerie van Landbouw, Natuurbeheer en Visserij	Ministry of Agriculture, Nature Management and Fisheries	Ministère de l'Agriculture, de la Pêche et de la Faune	Ministerio de Agricultura, Pesca y Caza
Rijksdienst voor de Keuring van Vee en Vlees	National Inspection Service for Livestock and Meat	Service National d'Inspection du Bétail et de la Viande	Servicio Nacional de Inspección de ganado y carne
Gezondheidscertificaat	Health Certificate	Certificat Sanitaire	Certificado Sanitario

I. Certificering voor: **BOVE CHIPS (DRIED BONE PRODUCTS)**

Naam product, afkomst / Name of the product, animal species / Name des Produits, des Types / Nom du produit, espèce animale / Nombre des Produits, espèce animale

Identificering / Marks / Identification / Identification / Marca

Aantal, gewicht / Number, weight / Zahl, Gewicht / Nombre, poids / Quantes, peso

Soort verpakking / Nature of packaging / Art de l'emballage / Sorte d'emballage / Clase de embalaje

Naam en adres producent, herkomstigheid / Processing plant, Farm of origin / Estruga, Herkunftsbetrieb / Productor, establecimiento de origen / Exporteur / Exporthandel / Abnehmer / Expéditeur / Exportador

Naam en adres ontvanger / Name and address of consignee / Name and Address des Empfänger / Nom et adresse du destinataire / Destinataro Land / Country / Land / País / País

Modo van vervoer / Means of Transport / Transporte mittel / Moyen de transport / Clase de transporte

II. Ondergetekende verklaart / The undersigned certifies / Je soussigné, certifie / El que suscribe, certifica

1. That the material from which the degreased whipped bone is prepared are bovine and pig bones, obtained from healthy animals that were slaughtered by an official veterinarian and were declared fit for human consumption.

2. The bovine bones are either obtained from animals from herds that never had a case of BSE reported or were imported from countries where no case of BSE has been officially declared. No bovine bones are obtained from the United Kingdom, Ireland, France, Oman, Switzerland or Portugal.

3. The product does not contain, and is not derived from, specified risk material as defined in Commission Decision 97/381/EC or mechanically recovered meat obtained from the vertebral column of bovine, ovine or caprine animals.

4. The crushed bones are defatted by treating them for 15 minutes with an aqueous solution of pH 4 at 90° C and are subsequently dried with air at 450° C.

5. The crushed bones are not intended to be diverted for direct use in human or animal food.

6. Traceable records of raw material for each batch of degreased chipped bone are maintained and are available for inspection by the authorities.

Handwritten signature and official stamp

## Appendix 5. DETAILED DESCRIPTION OF EXPERIMENTAL PROCEDURES

### Preparation of the starting material

#### *Crushed bone starting material and calf backbone*

Two bags of crushed bone and a piece of intact backbone were taken from the freezer and were thawed in the refrigerator. The backbone was weighed on Ohaus Explorer scales (4100±0.1g cat no EOD120). The difference between the weight of the backbone and 2000 g was calculated and this amount of crushed bone was aliquoted into a tray.

#### *Preparation of the 263K hamster 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 fitted to the stirrer with adhesive tape which also served as a seal. The stirrer was equipped with 30 ml Schütt homogeniser with a PTFE piston (LS cat no 1931 05145/55). Approximately 6 g of 263K infected hamster 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. 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 a jar which was weighed. The jar, the homogeniser tubes and the spatulae were set aside. The glove bag was removed from the cabinet together with the pistons of the homogenisers and disposed of. The stirrer and stand were also removed from the cabinet and put in polythene bags until further use.



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

Left:  
Preparing  
homogenate



Right:  
Weighing  
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 the jar. 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



Injecting spinal cord



Syringe and backbone

after which the syringe was weighed again. The tray with the spiked backbone was put in a polythene bag and put in the refrigerator.

The tray with crushed bone was taken from the refrigerator and placed in the biological safety cabinet. The spatulae used previously with adherent brain tissue were weighed before the brain tissue was smeared on the crushed bone. The spatulae were weighed again and disposed of.

The brain tissue left in the jar was also smeared on a few pieces of the crushed bone by wiping the inside of the jar with pieces of crushed bone. The jar 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 4 g of brain macerate was taken from the stock jar and smeared on the crushed bone. The tray with crushed bone was placed in a polythene bag and put in the refrigerator. The stock jar with brain macerate was put back in the laboratory freezer.

All materials and equipment remaining in the biological safety cabinet was disposed of and the cabinet decontaminated using a sodium hypo 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		1820 g
Weight of empty tray with polythene bag		383 g
<b>Crushed bone</b>		<b>1437 g</b>
Bagged tray with backbone		593 g
Empty tray with bag		23 g
<b>Back bone</b>		<b>570 g</b>
Stock jar before taking out	107.25 g	
Stock jar after taking out to homogeniser tube	100.23 g	
Taken out of stock jar		7.02 g
Beaker with tube with brain	151.05 g	
Beaker with clean tube	144.95 g	
Brain in tube		6.10 g
Beaker with used spatula I	107.19 g	
Beaker with clean spatula I	106.30 g	
Brain on spatula I		0.89 g
Brain in tube and on spatula I		6.99 g
Evaporation of water		0.03 g
Syringe with brain homogenate	22.78 g	
Syringe after injection in spinal cord	19.67 g	
Injected in spinal cord		3.11
Beaker with spatula I	107.19 g	
Beaker with spatula I after smearing crushed bone	106.33 g	
Smear on crushed bone		0.86 g

Beaker with tube and spatula II	160.38	
Beaker with tube and spatula II after smearing on crushed bone	106.33	
	<hr/>	1.28 g
Smeared on crushed bone		
Jar with left brain homogenate	24.55 g	
Jar after smearing on crushed bone	23.83 g	
	<hr/>	0.72 g
Infectious brain smeared on crushed bone		
Stock jar with brain	100.19 g	
Stock jar with brain after smearing on crushed bone	96.21 g	
	<hr/>	3.98 g
<b>Total amount of 263K infected hamster brain with the bone</b>		<b>9.98 g</b>
		<hr/> <hr/>
Jar with 263K hamster brain macerate before spiking	107.25 g	
Jar with 263K hamster brain macerate after spiking	96.21 g	
	<hr/>	
263K hamster brain macerate taken from jar	11.04 g	
Total spike with bone	9.98 g	
	<hr/>	1.06 g
Total losses		
Brain left on spatula I	0.89 g	
Jar after filling syringe	24.55 g	
Jar empty	23.69 g	
	<hr/>	0.86 g
Homogenate left in jar		
Jar with homogenate from tube	28.24 g	
Jar after filling syringe	24.55 g	
	<hr/>	3.69 g
Brain taken from jar		

Syringe with homogenate	22.78 g	
Empty syringe	19.71 g	
	3.07 g	
Brain left on spatula II		0.62 g
Brain macerate put in tube	6.10 g	
Brain homogenate put in jar	4.55 g	
	1.55 g	
Syringe after injection	19.67 g	
Syringe empty	19.71 g	
	-0.04 g	
Difference		-0.04 g
Total left in and on equipment		3.88 g
On bone from spatula I	0.86 g	
On bone from tube and spatula II	1.28 g	
On bone from jar	0.72 g	
	2.86 g	
Smeared from equipment on bone		2.86 g
Difference		1.02 g
Losses not accounted for		0.04 g

(The losses are partly due to brain tissue left on piston homogeniser tube and other equipment, and partly due to evaporation of water from macerate and homogenate. In this latter case this means that the actual amount of infectivity is actually somewhat higher than the measured amount)

Reference: Note book 31-12-98 to 24-3-99 page 25 to 30. Date 25-1-99.

### *Sawing of the backbone*

A glove bag was set up in the biological safety cabinet. One plastic and one aluminium tray, two pairs of tweezers (Bochem 18/8 steel LS cat no 3305 01000), two pairs of forceps (arterienklemme stainless steel LS cat no 3351 11038) and two scalpels (scalpel handle LS cat no 3351 18061, blade LS cat no 3351 18064) and a vice were put in the bag. A hole was made in the side of the bag through which a Stryker TPS Micro Sagittal saw (ref 5100-34) with a Stryker Long Wide Aggressive Blade 34.5mm x 16.5mm (ref 2296-3-504) was put

inside the glove bag. The saw handle and the cord of the saw were covered with a protective sleeve. The sleeve was taped to the hole to close this hermetically. The glove bag was intended to protect against the saw-dust created by sawing of the backbone.

The tray with the backbone was taken from the refrigerator and put in the glove bag. The backbone was placed into the vice and sawn to 1.5 cm pieces as described below. The soft tissue on the backbone was cut away with scalpels and the backbone material was handled using the tweezers and forceps. Care was taken when separating the pieces that the spinal cord was cut to size and remained connected to the backbone. Care was also taken that the dorsal root ganglia remained between the vertebrae. The pieces of backbone were put into the tray which had originally contained the whole backbone.



Glove bag with backbone and sawing equipment

#### *Description of sawing of the backbone:*

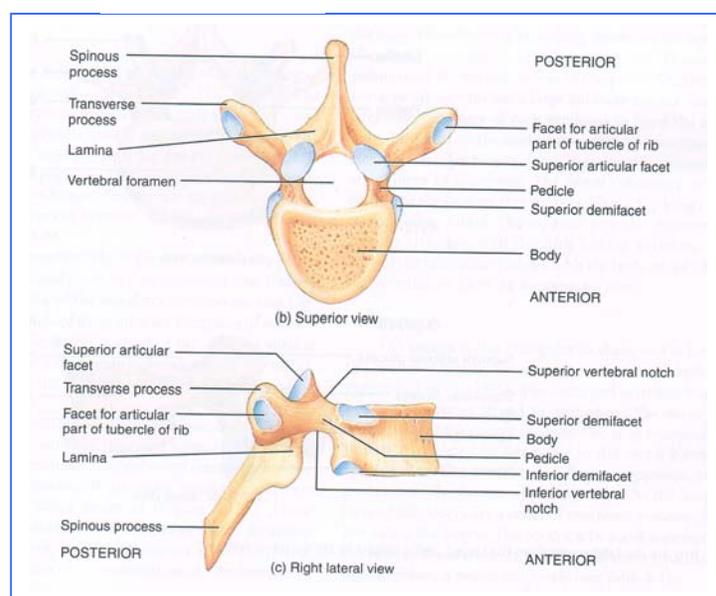


Sawing the backbone

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

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 hypo-chlorite as before.

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

Reference: Note book 31-12-98 to 24-3-99 page 31. Date 27-1-99.

## **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<sub>2</sub>SO<sub>4</sub>, was heated on a hotplate (Schott-Geräte CERAN hotplate 1800W type 930 00) to 90°C. One of the 600 g portions of crushed bone was added to the water and stirred for 20 minutes at approximately 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 263K infected hamster 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



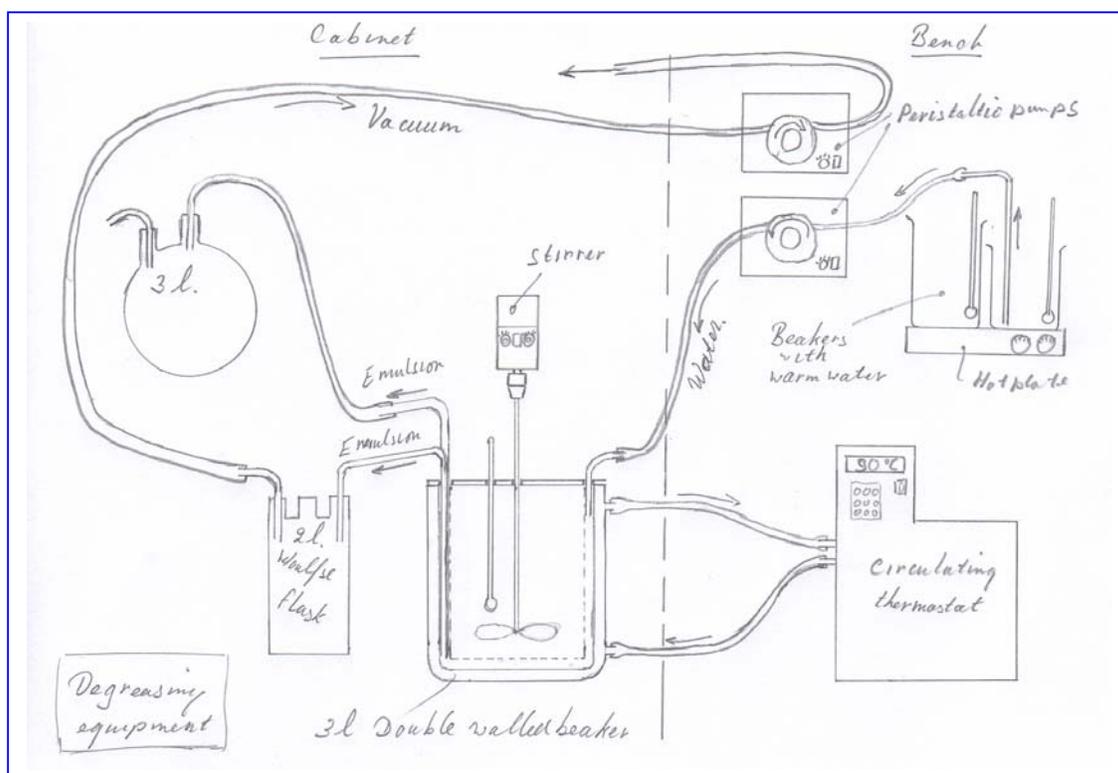
Degreasing equipment in safety cabinet

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.

<b>1:</b>	
Tray+bag +bone	994.8 g
Tray+bag	24.0 g
	<hr/>
Bone	970,8 g
<b>2:</b>	
Tray+bag +bone	1000.2 g
Tray+bag	24.0 g
	<hr/>
Bone	976,2 g

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

**List of equipment:**

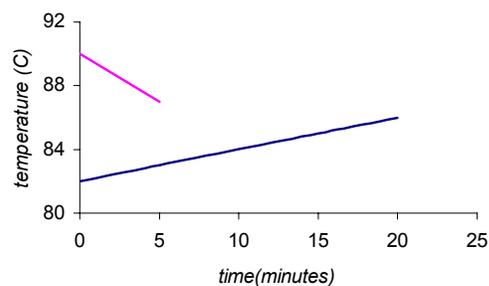
tempering beaker 3 litre custom made  
 basket custom made  
 ring custom made  
 lid custom made  
 U-tube custom made  
 Large siphon custom made  
 Small siphon custom made  
 Small tube custom made  
 Thermometer  $-10 \dots 110^\circ$  cat no 67370413  
 Woulfe flask 3 neck 2000 ml cat no 832502179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



The basket was wrapped in a polythene bag and filled with crushed bone portion 1. The stirrer was placed in the basket and the lid fitted on top. The basket was fixed to the ring and the whole assembly was fitted on to the stand. The polythene bag was removed and the basket assembly lowered into the double-walled beaker immersing it in the warm degreasing liquid. The propeller stirrer was fixed to the stirrer, the water inlet siphon connected and the emulsion outlet siphon put in the emulsion. The stirrer was started and 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 18 minutes 1 litre of emulsion was pumped out of the beaker and 1 litre of clean water pumped in. Samples of the degreasing emulsion were taken after 5 minutes and 15 minutes.

#### Temperature during degreasing.

Time (min)	temperature degreasing emulsion	temperature fresh water
0	82°C	90°C
5	83°C	87°C
15	85°C	-
20	86°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 85 to 86°C. After 3 minutes a sample was taken from the emulsion.

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.

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



Degreased bone

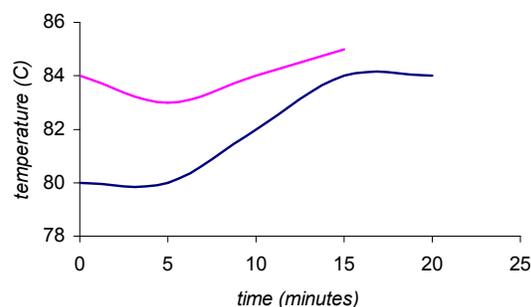
3 litre double walled beaker and was warmed to 80°C. Two beakers with 1 and with 2 litres of water were heated on the hotplate. The basket was filled with the second half of the spiked crushed bone and sawn backbone, the propeller stirrer was put in the basket, the lid put on it and the basket fitted 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 18 minutes.  
2 samples of the degreasing emulsion were taken, one after 5 minutes and one after 15 minutes. One sample was taken from the short rinse with clean water after 3 minutes.

#### Temperature during degreasing.

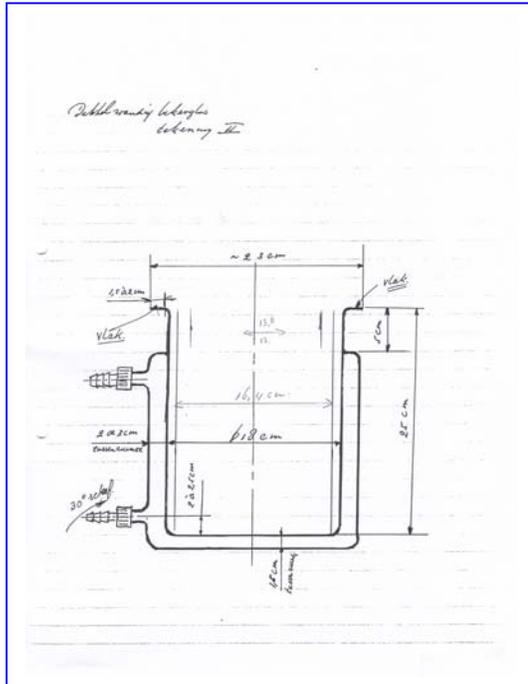
Time (min)	temperature degreasing emulsion	temperature fresh water
0	80°C	84°C
5	80°C	83°C
10	82°C	84°C
15	84°C	85°C
20	84°C	



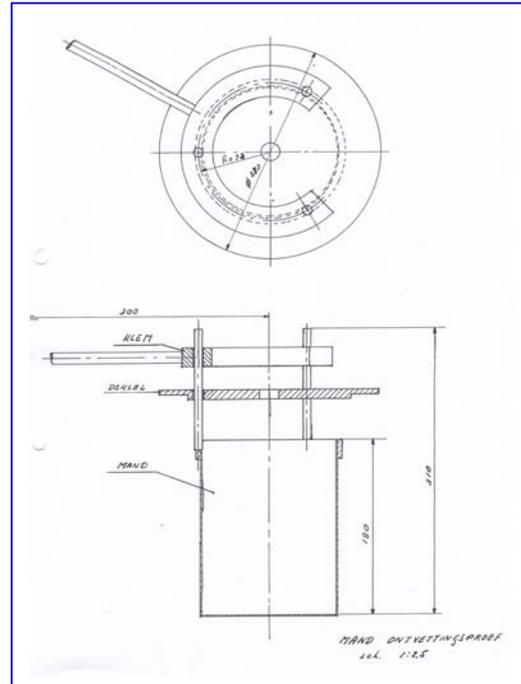
#### Temperature during 3 minute rinse.

Time (min)	temperature emulsion
0	84°C
3	-

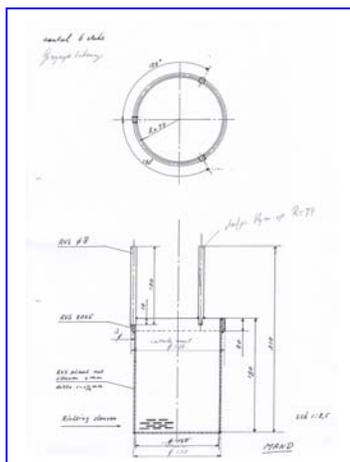
Custom made equipment



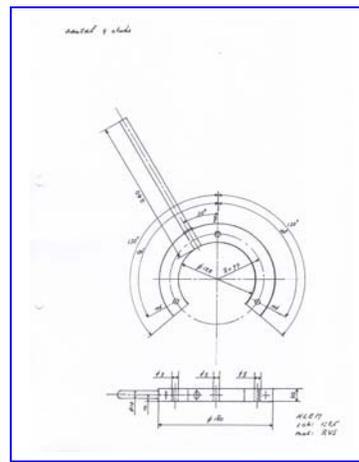
3 litre double walled beaker



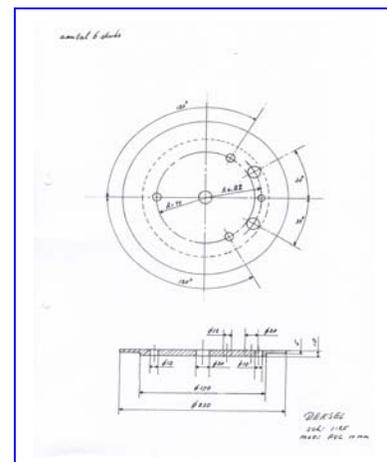
basket assembly



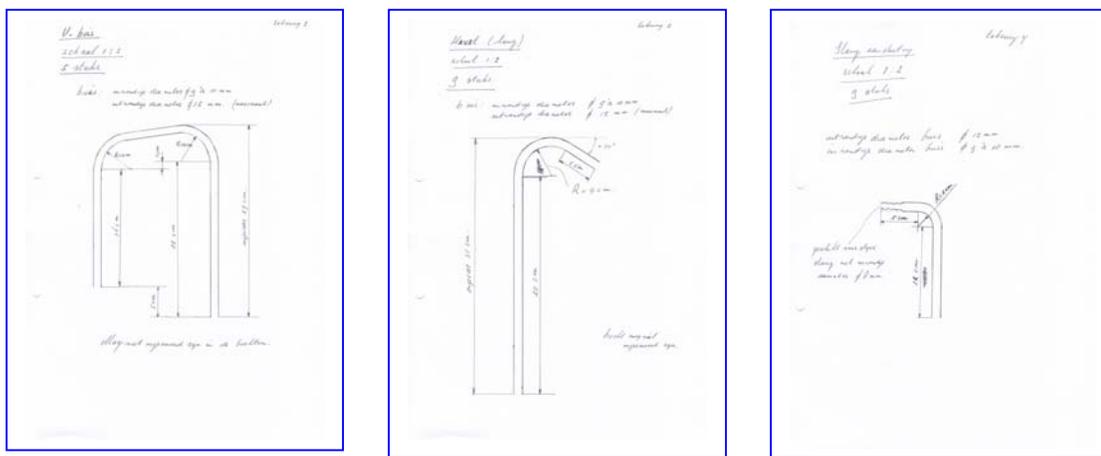
basket



ring



lid



siphons

Tray with bag and degreased bone	1490 g
Tray with bag	383 g
	<hr/>
Bone chips and meat	1107 g

A sample of 42.7 g was taken of the degreased bone.

The tray with degreased bone was put in a polythene bag and set aside.

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

#### *Separating bone and soft tissue*

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	669 g
Empty tray and bag	24 g
	<hr/>
Sorted wet bone	645 g

*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 112°C and the maximum temperature between the bone was 79°C.

Tumbler jar with dried bone	837 g
Tumbler jar empty	354 g
Dried bone	483 g
Loss of water	162 g



Drying of the bone

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

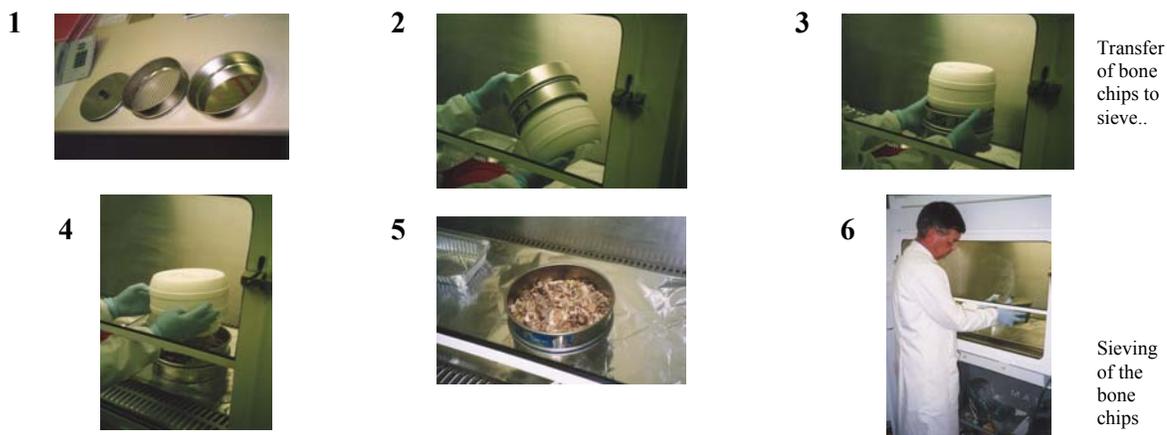
*Tumbling, sieving and sorting of the dried bone.*

The tumbler jar (Curtec Keg wide mouth HDPE 3.5 litre BDH cat no 215/0380/12) was closed and sealed with a piece of steel wire. The jar was put on a roller bank and rotated for 5 hours.

Tumbling of the dried bone 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.

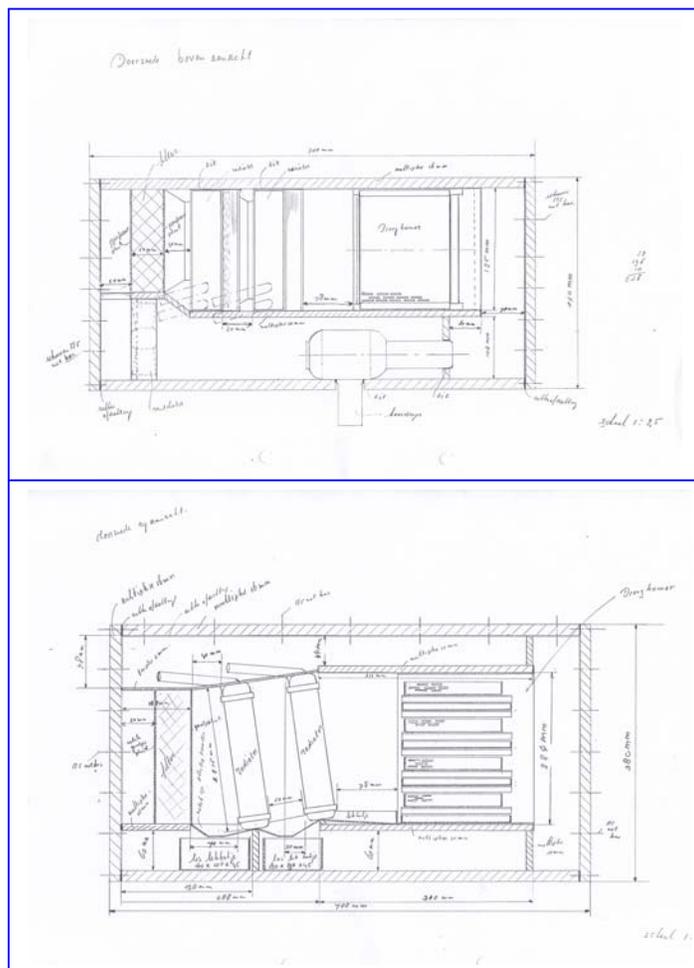


The sieve was taped shut and the bone chips were sieved for 10 minutes. (Sieve 4.00 mm, 200x50 mm DIN ISO 3310/1 C rvs LS cat no 01310310066, Sieve lid 200mm rvs LS cat no 01051070252, Sieve bottom 200x50 mm rvs LS cat no 0105010022). The bone was then transferred to a tray and the sieve assembly disposed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



### *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.



Drier, side view and top view

Tray with bag and bone chips

473 g

Empty tray and bag

20 g

**Sorted degreased dried bone chips****453 g**

A 20.9 g sample was taken of the bone chips.

Sorted degreased dried bone chips

453 g

Sample

20.9 g

**Bone chips for demineralisation****432 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 32-40. Date 28-1-99 to 31-1-99.

### **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.

4 litre solution: 260 g bone ash  
377 ml 36% hydrochloric acid  
3625 ml water

Solution 2: Composition: 2.5% hydrochloric acid, the remainder mono-calcium phosphate and calcium chloride.

6 litre solution: 169 g bone ash  
566 ml 36% hydrochloric acid  
4675 ml water (750 g ice added later)

Solution 3: Composition: 4% hydrochloric acid.

10 litre solution: 943 ml 36% hydrochloric acid  
6570 ml water (2400g ice added later)

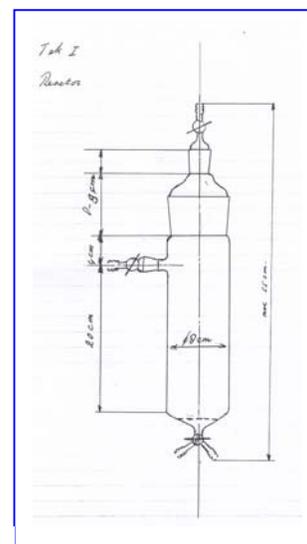
(Bone ash: Industrial quality bone ash Smits-Vuren BV, Vuren, The Netherlands, Hydrochloric acid: Merck (BDH) Normapur AR min 36% d. 1.19 analytical reagent cat.no 20252.324)

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

Reactor flask demineralisation



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 147 ml/hour. After 3 hours the solution had filled the bone



Reactor  
flask with  
bone chips

bed. After 24 hours solution 1 was replaced by solution 2, which was replaced after a further 28 hrs by solution 3. Solution 3 was pumped through for 51 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

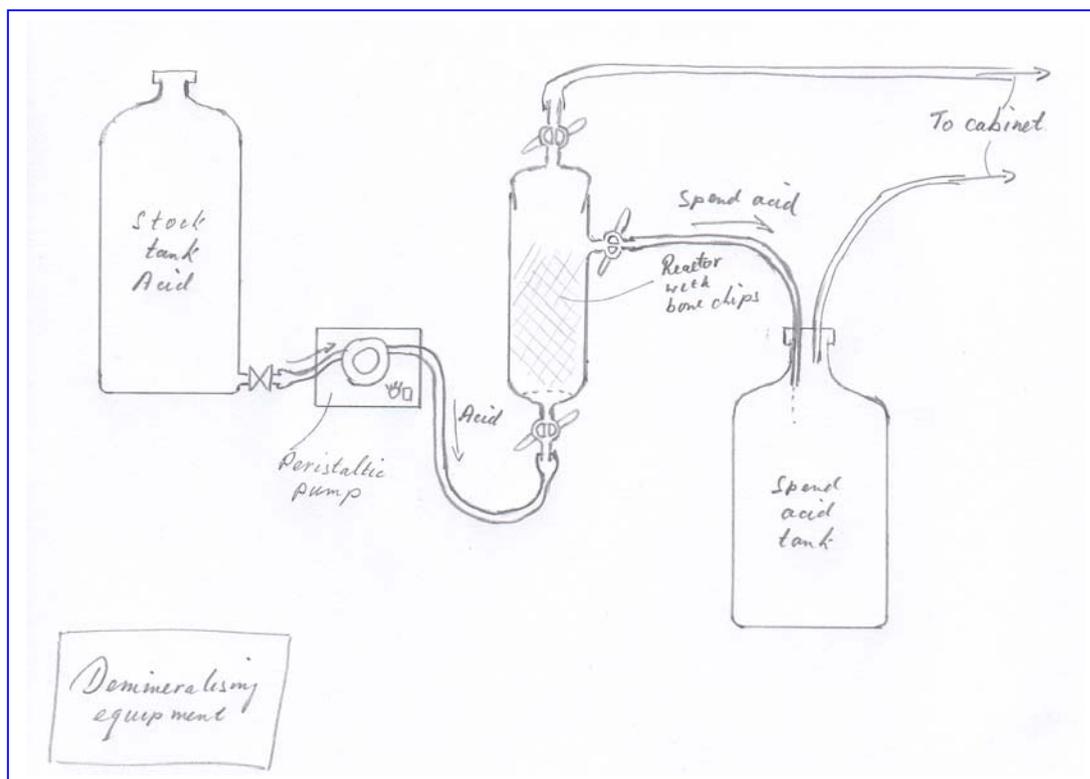
new iced solution. The waste solution was also removed and replaced by an empty container. A sample of the spent acid was taken and the remainder disposed off. The valves were re-opened and the pump switched on.

Solution 1:	Started 4-2-99 at 9.30 h.	Stopped 5-2-99 at 9.30 h.
Solution 2:	Started 5-2-99 at 9.30 h.	Stopped 6-2-99 at 13.30 h.
Solution 3:	Started 6-2-99 at 13.30 h.	Stopped 8-2-99 at 16.30 h.

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



Demineralisation equipment



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

### *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. Through the second valve enough water was added 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 carefully decanted from the beaker, the stirrer



Washing of ossein after demineralisation

blade removed and disposed of. The washed ossein was transferred to the cleaned reactor and allowed to drain for 15 minutes. Samples were taken of all drained washing waters.

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.



Transfer of ossein to beaker



Stirring of ossein with water

Reference: Notebook 31-12-98 to 24-3-99 page 40-42 and page 48-49. Date 3-2-99 to 8-2-99.

### **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 third day and

replaced with the same volume and concentration of fresh lime. Air was pumped through the reactor for 1 hour on the first second and third day. Draining and replacing of the lime, and pumping of air was repeated several times during the course of the liming period (see table below).

Samples were taken of the drained lime and stored in a freezer.

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 glass beaker. The reactor flask and all equipment which had been potentially in contact with test materials were disposed of.

date	replacing lime	Pumping air through for 1 hour
8-2-99	first lime added	
9-2-99	X	X
10-2-99		X
11-2-99	X	X
13-2-99		X
17-2-99	X	
19-2-99		X
23-2-99		X
24-2-99	X	
27-2-99		X
28-2-99	final draining of lime	



Transferring limed ossein to beaker for washing

### *Washing*

Washing was done in the safety cabinet in a 2 litre glass beaker 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 first washing the pH was 12.2 after the second washing 12.3 and 12.1 after the third washing. Before each measurement the pH meter was adjusted using pH4 and pH7 buffer solutions.

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

References: Notebook 31-12-98 until 24-3-99 page 49-50 and 73-74. 8-2-99 until 28-2-99



Washing equipment



Final washing after liming

### 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 2 M sulphuric acid were added to the ossein while the pH was observed. Addition of acid was continued until the pH remained stable at approximately pH5. During neutralisation, the pH meter was calibrated at least twice a day with pH4 and pH7 buffers and further tested against these buffers at intervals, where a deviation of 0.1 was allowed.



Neutralisation

Neutralisation data are in the table below.

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 and a sample of it was taken.

Table of neutralisation data

date	time	pH	Amount of 2M H <sub>2</sub> SO <sub>4</sub> added	pH after addition of H <sub>2</sub> SO <sub>4</sub> .
28-2-99	15.10	12.1	5 ml	2.6
	15.15	4.0		
	15.20	10.0		
	15.24		5 ml	2.1
	15.25	2.4		
	15.40	4.7	2.5 ml	2.4
	15.45	3.2	2.5 ml	2.2
1-3-99	10.25	6.3		
	10.30		2 ml	3.0
	12.00	4.3		
	12.50	4.6		
	13.10	4.7		
	14.00	4.8		
	14.55	4.9	2 ml	2.8
	16.10	4.1		
	16.40	4.3		
	17.10	4.4		
	17.15		1 ml	3.3

date	time	pH	Amount of 2M H <sub>2</sub> SO <sub>4</sub> added	pH after addition of H <sub>2</sub> SO <sub>4</sub> .
2-3-99	10.10	4.6		
	11.40	4.7		
	14.10	4.8		
	16.20	4.9		
	17.15	4.9		
3-3-99	9.15			
	10.20	5.0		
	14.10	5.0		
	15.15	5.0		



Washing of the neutralised ossein

Washing	pH
1	5.1
2	5.1
3	5.1
4	5.2
5	5.1

The pH meter was calibrated before every measurement with pH4 and pH7 buffer solutions.

After the last washing the beaker with washed 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 75 to 78. Date 28-2-99 until 3-3-99.

## 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 650 ml was pumped into the ossein. The water was added at 18.50 hrs and stirring was started. At 19.00 hrs all equipment was then switched off and left to stand overnight. The heating equipment was switched on at 9.30 hrs the next day and, when the



Extraction equipment

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). Extraction was continued until 17.00 hrs, then all equipment was then switched off and left to stand overnight. The next day equipment was switched on at 9.45 hrs. When the index of refraction reached 8.0 the extract was pumped



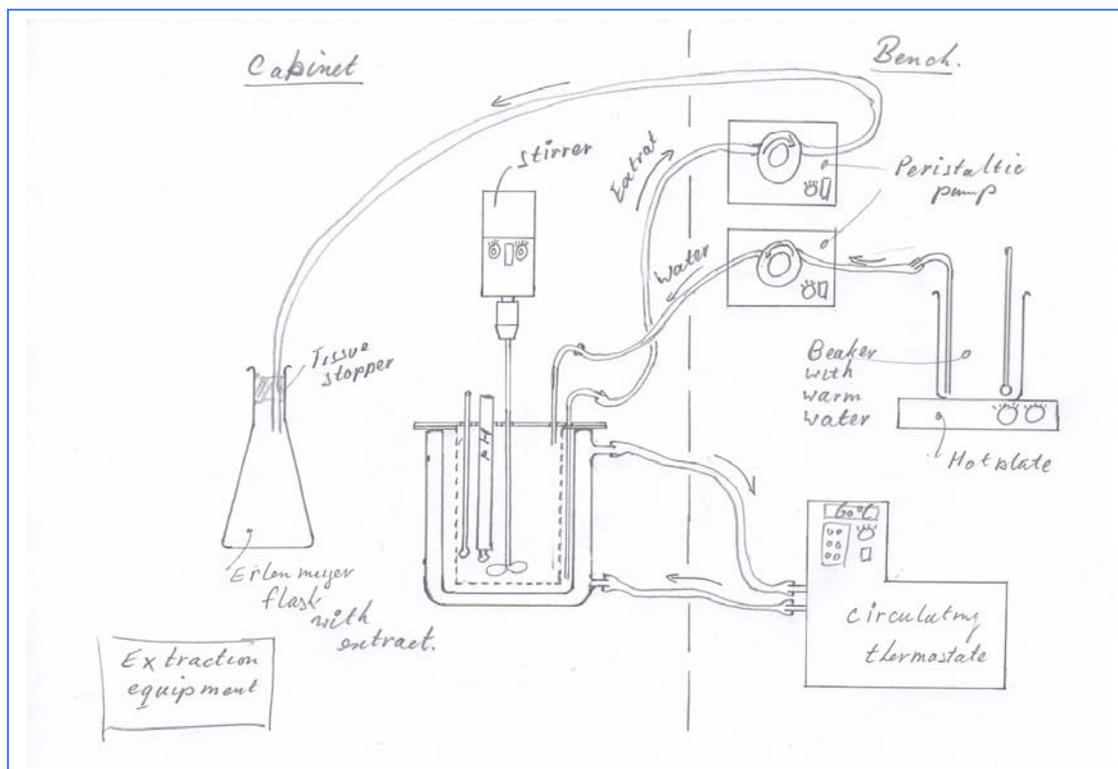
pH



index of refraction



over the 0.1 mm mesh 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 450 ml gelatine extract was obtained. A 40 ml sample was taken.



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  $-10 \dots 110^{\circ}$  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

Day	Time	Temperature	pH	Brix
3-3-99	18.50	switched on		
	19.00	switched off		
4-3-99	9.30	switched on		
	11.00	61°C	5.4	4.4
	12.50	60°C	5.4	5.5
	14.40	61°C	5.4	6.5
	16.00	62°C	5.4	7.0
	17.00	switched off		
5-3-99	9.45	switched on		
	10.45	59°C	5.5	7.8
	11.45	60°C	5.6	8.0



Extraction

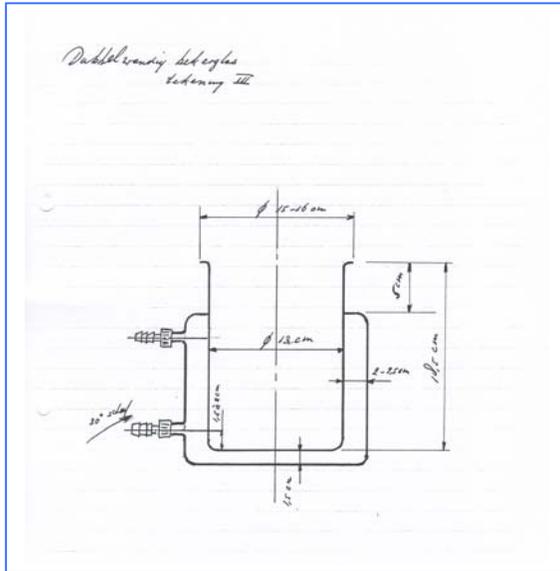
Extraction was continued at the same temperature. 750 ml of water at 60°C was pumped into the beaker and stirring restarted. Extraction was continued until 17.00 hrs. The next day equipment was switched on at 9.30. The pumping thermostat was set at 77°C to maintain an extraction temperature of 70°C. When an index of refraction of 4.0 Brix was reached the gelatine solution was pumped out and left to cool. Approximately 450 ml extract was obtained. A 40 ml sample was taken.

Day	Time	Temperature	pH	Brix
5-3-99	12.55	60°C	5.8	1.2
	14.20	60°C	5.8	1.9
	17.00	60°C	5.8	2.1
6-3-99	17.00	switched off		
	9.30	switched on		
	10.00	66°C	5.6	2.5
	11.50	71°C	5.7	2.9
	14.15	40°C	5.7	3.6
	15.10	70°C	5.7	4.0
	15.20	-	-	4.0

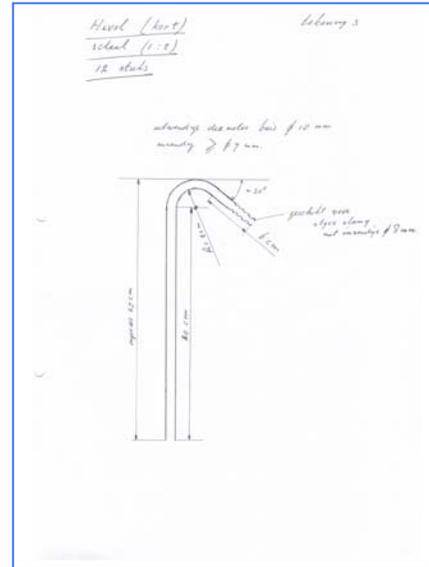


Measuring extraction temperature

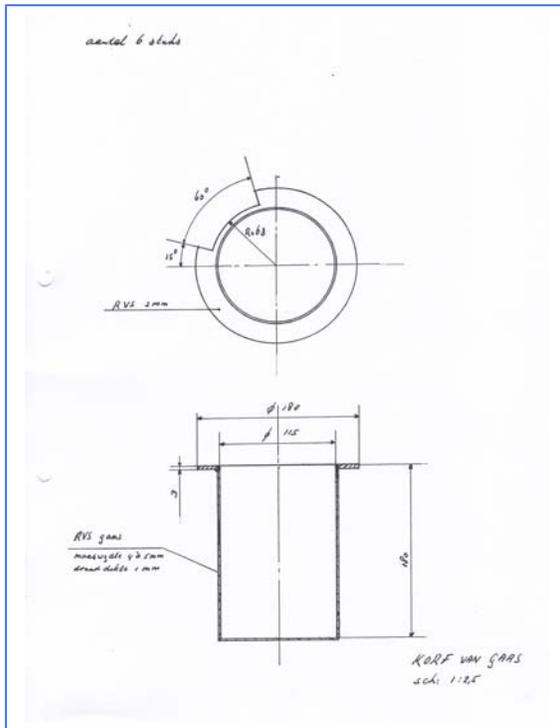
The circulating thermostat was set at 87°C, to maintain an extraction temperature of 80°C. 700 ml of water of 88°C was pumped in and stirring restarted. A maximum index of refraction of 2.0 Brix was measured. The gelatine solution was pumped out of the double walled beaker in the same fashion as before. Approximately 650 ml extract was obtained of which a 40 ml sample was taken. There was approximately 100 ml ossein residue left in the double walled beaker.



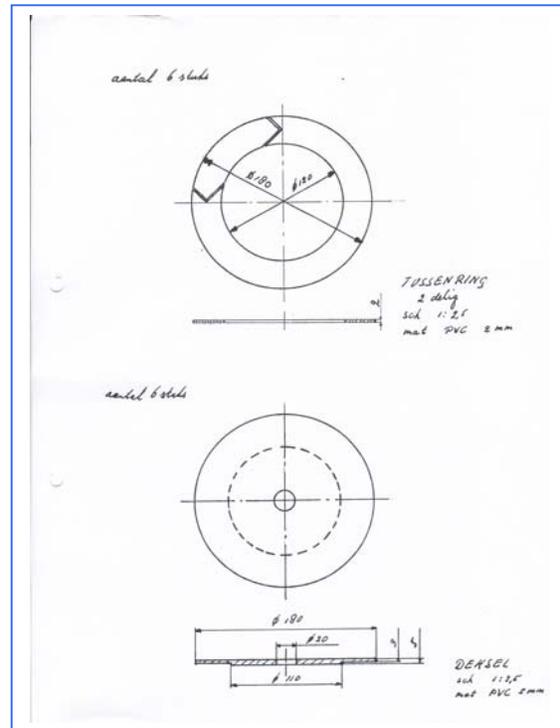
2 litre double walled beaker



siphon



Basket



Lid and ring

Day	Time	Temperature	pH	Brix
6-3-99	15.55	78°C	5.8	0.8
	17.45	81°C	5.9	1.6
	18.45	78°C	5.9	2.0



Draining the  
extract by  
pumping out

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 79 until 83. Date 3-3-99 until 6-3-99.

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 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.

Laboratory thermometers for general temperature measurements were disposed of after every test. These thermometers were not calibrated on every use.

Results of checking general use thermometers against a verified thermometer

<b>Verified:</b>	<b>26.5</b>	<b>30</b>	<b>62</b>	<b>70</b>	<b>78</b>	<b>81.2</b>	<b>92.5</b>
<b>100°C:</b>							
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
<b>Max 150°C:</b>							
1		31	63	71		81.2	92
2		30	62	70		82	93

#### *pH meters*

Unless otherwise indicated, the pH meters were calibrated before and after each measurement with standard buffer solutions of pH4 and pH 7 or pH7 and pH10.

#### **General observation.**

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

## Certificate verified thermometer:

**2 zum Eichschein**  
of verification certificate

144 09

**zliche Angaben zum Gegenstand der Eichung**  
of correct measurement object of verification

bereich: 0 °C bis 250 °C

Stellungswert: 1 °C  
Anz.:  
marke bei: 0 °C  
mit:

**verfahren**  
of verification

Die Eichung erfolgte im geeichten Flüssigkeitsbad durch Vergleich mit den Flüssigkeits-Glaskthermometer-Normalen  
The verification was carried out in a liquid glass standard thermometer

194D1566, 136D1566, 136G1566, 136C1566, 136D1566.

Wie in PTB-Prüfregel 14.01-07, 'Flüssigkeits-Glaskthermometer' beschrieben.  
As described in PTB testing instructions 14.01-07 'Liquid-Glass-Thermometer'.

**bedingungen**  
of verification

Wie in PTB-Prüfregel 14.01-07, 'Flüssigkeits-Glaskthermometer' beschrieben.  
As described in PTB testing instructions 14.01-07 'Liquid-Glass-Thermometer'.

Thermometeranzeige Thermometer reading °C	Anzeige-korrektur Correction for thermometer reading °C	Messunsicherheit Uncertainty of measurement °C
0,0	0,2	0,2
100,0	0,0	0,2
200,0	0,4	0,2
250,0	0,0	0,3

**unsicherheit**  
of measurement

Die Messunsicherheit setzt sich aus den Unsicherheiten des Eichverfahrens und denen des Gegenstandes der Eichung und der Eichung zusammen. Ein Anteil für die Langzeitstabilität des Gegenstandes der Eichung ist nicht enthalten, jedoch ist die erweiterte Messunsicherheit, die sich aus der Standardmessunsicherheit durch Multiplikation mit dem Expansionsfaktor  $k = 2$  ergibt. Die Werte gemäß DIN, Lauffaden zur Angabe der Unsicherheit beim Messen, sind ablesbar. Der Wert Messgröße liegt im Regelfall mit einer Wahrscheinlichkeit von annähernd 95% im angegebenen Wertebereich.  
The uncertainty of measurement is composed of the uncertainty of the verification method and the uncertainties of the object to be verified. The uncertainty of measurement stated in the expanded uncertainty which is  $k = 2$  is from the standard uncertainty of measurement by multiplication by the expansion factor  $k = 2$ . It was determined in accordance with, Guide to the use of standards in measurement. Thereby, with a probability of approx. 95%, the value of the measured lies within the interval assigned.

**einheit**  
The temperature corresponding to the thermometer reading is obtained according to the relative  
Temperatur = Thermometeranzeige + Anzeige-korrektur.  
Temperature = Thermometer reading + correction for thermometer reading.

Die angegebenen Anzeige-korrekturwerte gelten für:  
a) den Außendruck 1 · 10<sup>5</sup> Pa, ambient pressure 1 bar Pa,  
b) den Außendruck 1 · 10<sup>5</sup> Pa, ambient pressure 1 bar Pa,  
c) unter der Voraussetzung, dass das Thermometer einschließlich der Fadennuppe, aber ausschließlich der Expansionsverlängerung abgelesen wird.  
or reading of the thermometer is total measured.

Wenn das Thermometer nicht ganz einbauecht benutzt wird, ist die Thermometeranzeige durch Abbringen einer Fadennuppe zu berichtigen.  
If the thermometer is not used bauecht, an expansion column corrects it necessary.

Die Werte sind auf die ITS-90 bezogen.  
The results are in accordance with the ITS-90.

**des Eichscheines**  
of verification certificate

**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

DE BEI DEN MESSUNGEN VERWENDETE NORMALE SIND AN DIE NATIONALEN NORMALE DER BUNDESREPUBLIK DEUTSCHLAND BEI DER PHYSIKALISCH-TECHNISCHEN BUNDESANSTALT PTB ANGESCHLOSSEN.  
THE STANDARDS USED FOR THE MEASUREMENTS ARE TRACEABLE TO THE NATIONAL STANDARDS OF THE FEDERAL REPUBLIC OF GERMANY AT THE PHYSIKALISCH-TECHNISCHES BUNDESANSTALT (PTB).

**Eichschein** 144 09

Verification certificate

Gegenstand der Eichung Object of verification	Flüssigkeits-Glaskthermometer
Identifikation Identification	Laborthermometer
Auftraggeber/Hersteller Client/Manufacturer	

Anzahl der Seiten des Eichscheines: 2  
Number of pages of the verification certificate

Ort und Datum der Eichung: Ilmenau, 1999-01-13  
Place and date of verification

Das Messgerät entspricht den Eichvorschriften (EO 14.1 und PTB-A 14.1).  
The measuring instrument complies with legal regulations (EO 14.1 and PTB-A 14.1).

Gültigkeit der Eichung: 10 Jahre  
Validity of verification

Die Gültigkeit der Eichung erlischt vorzeitig, wenn eine der in § 13 Absatz 1 der Eichordnung beschriebenen Veränderungen eingetreten ist.  
The validity of the verification has ceased to exist if one of the changes listed in § 13 section 1 of the Eichordnung are occurred.

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.  
Verification certificates without signature and official stamp are not valid. This verification certificate may only be reproduced in complete and unchanged form. Extracts or amendments require the approval of the verification office. In case of doubt, the German text shall prevail.

Ort und Datum: Ilmenau, 1999-01-14  
Place and date

Dienstsiegel: Unterschrift:   
Official stamp Signature

Adametz

Unterpörlitzer Str. 2, 98693 Ilmenau, Telefon 03677/850-300, Telefax 03677/850-400

**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 using a calculated correction factor.

*Amount of starting material.*

Total amount of 263K infected hamster brain on the bone starting material	9.98 g =====
Crushed bone	1437 g
Backbone	570 g
263K infective hamster brain	9.98 g
Total amount of spiked bone starting material	2017 g =====

*Correction factor for extracted gelatine*

I.	Amount of wet degreased bone and tissue	1107 g
	Sample taken of wet degreased bone and tissue	43 g
	Amount used in further processing	1064 g
II.	Weighed amount of sorted dried bone chips	453 g
	Sample taken of sorted dried bone before weighting	21 g
	Original amount of sorted dried bone	432 g

Calculated correction factor =  $(1107/1064) \times (458/432) = 1.103$

**Correction factor for extracted gelatine            1.1**

***Summary of data for the calculation of the clearance factors*****A. Total amount of 263K infected hamster brain on the bone starting material 9.98 g****B. Extracted gelatine 1550 ml****C. Correction factor for extracted gelatine 1.1**

**Appendix 7: PREVENTION OF CROSS-CONTAMINATION.**

The whole series of experiments was done in a laboratory room free of any background contamination. During the entire series of experiments special care was given to prevent background contamination. Handling of infectivity was exclusively done in a biological safety cabinet, which was decontaminated 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 of 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 hypo-chlorite 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 intracerebral inoculation of 50 µl into the LVG strain of Syrian hamsters. 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 263K infected hamster brain.** Undiluted macerated tissue ( $10^0$  log dilution) serially diluted to produce a series of log 10 dilutions. Each hamster was injected with 50µl 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 hamster was injected with 50µl of the appropriate dilution (see table).

To be prepared as follows:

	263K infected mouse brain	Crude gelatine extract
$10^0$	N/A	12
$10^{-1}$		12
$10^{-2}$		12
$10^{-3}$		12
$10^{-4}$	8	12
$10^{-5}$	8	
$10^{-6}$	12	
$10^{-7}$	12	
$10^{-8}$	12	
$10^{-9}$	12	

**Bioassay procedure**

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

**Titration method**

The biological safety cabinet was switched on and the working surface covered with 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 263K infected hamster brain.****Titrated 30/8/00.**

A 10% brain homogenate was prepared from the same undiluted macerated 263K-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 5/12/00.**

The undiluted gelatine extract was liquified by warming in a water bath at 50°C. Dilutions were then made of this solution as for the 263K-infected hamster brain, up to and including  $10^{-4}$ . The warmed undiluted ( $10^0$  group) was also inoculated.

**Inoculation of experimental animals**

Prior to inoculation, the experimental protocol for the bioassay was completed and the requisite number of cages of hamsters 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 hamster data card identifying the experimental group each animal belonged to. These were kept separate to the cage to allow blind clinical assessment throughout the experiment.

The safety cabinet was then set up for inoculation of each group of hamsters. 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 hamsters was anaesthetised and one at a time placed into the cabinet. Each hamster was then inoculated intracerebrally with 50µl of the inoculum. After inoculation, each hamster was removed to a clean cage to recover. The next cage of hamsters

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.****263K hamster brain pool**

Titration 372J-1R

Final 519 days post injection.

<b>-log dilution</b>	<b>Number of hamsters</b>	<b>Hamsters positive 263K</b>	<b>Average incubation period (days)</b>
-	-	-	-
-	-	-	-
-	-	-	-
4	8	8	101
5	8	8	112
6	11	10	168
7	11	3	164
8	11	0	-
9	12	0	-

**Titration data of gelatine sample.****Sample of extracted gelatine**

Titration 372J-1Y

Final 631 days post injection.

<b>-log dilution</b>	<b>Number of hamsters</b>	<b>Hamsters positive 263K</b>	<b>Average incubation period (days)</b>
0	9	2	221
1	9	1	-
2	9	0	-
3	11	0	-
4	11	0	-