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Methods for the validation of biotechnological, thermal and chemical processes for the treatment of animal by-products, sewage sludge and biowastes in order to determine the hygienic safety of the resulting fertilizers or comparable products by exposition of test organisms or test viruses – Part 2 : Validation with test viruses

Methoden zur Validierung von biotechnologischen, thermischen und chemischen Behandlungsprozessen für tierische Nebenprodukte, Klärschlamm und Bioabfall zur Gewährleistung der hygienischen Unbedenklichkeit der hergestellten Dünger oder vergleichbarer Produkte mittels Exposition von Prüforganismen oder Prüfviren – Teil 2 : Validierung mit Testviren

Méthodes pour validation des procès biotechnologiques, thermiques et chimiques pour traitement des sous-produits animaux, boues et des biodéchets pour atteindre la securitee hygienique des engrais ou produits comparable par exposition des organisme de test ou virus de test- Partie 2 : Validation avec des virus t

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Foreword

This document has been prepared in the framework of the project Horizontal.

This document is a working document.

This standard is divided into five parts:

— *part 1 describes a validation procedure with vegetative test bacteria*

— *part 2 describes a validation procedure with test-viruses*

— *part 3 describes a validation procedure with parasites eggs*

— *part 4 describes a validation procedure with bacterial test spores*

— *part 5 describes a validation procedure with prions*

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Introduction

This document is developed in the framework of the project "Horizontal". It is the result of a desk study "*Prevention-Process control and process Validation"*. After discussion with all parties concerned in CEN the standard has been developed further as a modular horizontal method. The capability of a process to inactivate pathogens causing raw-material dependant risks cannot be judged on by analysis of presence or absence of indicators (Bacterial, viral, fungal or parasitic) in the final product. Absence of all or one of the mentioned pathogens or indicators in the final product may be caused by several reasons. They may not be present in the raw material, they may be present in the raw material but in a low count (less than 5 log), the existing methods for reisolation of the target organism are insufficient in the investigated matrix due to ineffective enrichment procedures (e.g. bacteria) or the quantitative isolation of the indicator is technically not possible due to effects of the complicated matrix (e.g. viruses).

Even if the possibility of validating a process by input-output analysis of a certain indicator is generally given and will be covered in WIxxxx., input- output analysis under practical conditions is depending on the microbiological properties of the input materials processed and therefore limited in feasibility under practical conditions. Therefore other strategies must be followed in such a case, e.g. process validation with one or more representative test–organism. Either if the thermophilic process itself or if a thermal treatment shall provide an inactivation of pathogens belonging to the indicated level of thermo- and chemoresistance, representative test-organisms must be exposed in a similar matrix as treated in a suitable test containment system (test-body) in a defined validation experiment. The relevant process parameters must be recorded during the exposure in order to define the technical conditions to be kept for save inactivation according to the results of the survival experiments and to define the critical control points in the application of a HACCP-concept.

WARNING — "Waste and sludge samples can contain hazardous and inflammable substances. They can contain pathogens and be liable to biological action. Consequently, it is recommended that these samples should be handled with special care. The gases which can be produced by microbiological activity are potentially inflammable and will pressurise sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided wherever possible. National regulations should be followed with respect to microbiological hazards associated with this method".

The texts of the chapters are normative; annexes are normative or informative, as stated in the top lines of the annexes.

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1 Scope

This part of the European Standard describes a procedure with test viruses for the validation of biotechnological, thermal and chemical processes for treatment of animal by-products, sewage sludge and biowastes deemed to provide hygienic safety of the resulting fertilizers or comparable products. The method includes the description of one suitable test containment system (test bodies as mentioned in Regulation EC No.208/2006) suitable for the exposure in different treatment processes as well as the methods necessary for the preparation of the inoculums of test viruses and for the sample processing after exposure.

The method is suitable for determination of the efficiency of biotechnological, thermal and chemical treatment process (CEN/TC 308 – doc. 525 (REVISION of Directive 86/278/EEC – 3rd Draft (1)) and Regulation (EC) No 208/2006 (2)) for the elimination of pathogens in untreated substrates. The treatment processes are validated through to a defined 3 Log_{10} reduction with thermo resistant viruses such as *Bovine Parvovirus*.

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this Working document only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies.

REGULATION (EC) No 208/2006: amending Annexes VI and VIII to Regulation (EC) No 1774/2002 of the European Parliament and of the Council as regards processing standards for biogas and composting plants and requirements for manure.

3 Definitions

For the purposes of this European Standard, the following terms and definitions apply.

3.1
Parvo virus
Belong to *Parvoviridae* family, linear, non-segmented single stranded DNA viruses with an average genome size of 5 kbp. Some of the smallest viruses found in nature.

3.2
infectivity
ability of a virus to express in cells its genetic information and/or multiply in them to infectious progeny

3.3
inactivation of viruses
reduction of infectivity of a virus during biochemical, thermal and chemical processes

3.4
reference virus suspension
virus suspension of a defined virus strain which is not passaged more than 10 times, is maintained in national culture collection centres and kept in small volumes (less than 1 ml) at a temperature of −196 °C over liquid nitrogen

NOTE: Stock virus suspensions are prepared from reference virus suspensions.

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3.5
stock virus suspension
virus suspension of a defined strain that is multiplied on a large scale to obtain a virus suspension of the same characteristics as the reference virus suspension and kept in a small volume at a temperature of below −70 °C or preferably at −196 °C over liquid nitrogen

3.6
test virus suspension
virus suspension that is used for the validation of biochemical, thermal and chemical processes

3.7
virus titre
amount of infectious virus per unit volume present in a cell culture

3.8
viral cytopathic effect (CPE)
morphological alteration of cells and/or their destruction as a consequence of virus multiplication

3.9
tissue culture infecting dose ($TCID_{50}$)
virus dose that gives rise to cytopathic change (CPE) in 50 % of the inoculated cell cultures

3.10
spiking
Artificial contamination of a matrix to be exposed to the process with a defined amount of a bacterial suspension of the test strain

3.11
matrix

A matrix is a representative sample of the material deemed to be processed, that is spiked with the test strain of interest in order to evaluate the influence of its chemical, physical and biological properties to the inactivation of the exposed bacteria.

3.12
biotechnological process
A process mainly characterized by the activity of organisms (e.g. microorganisms) under defined conditions for an intended purpose (e.g. composting)

3.13
thermal process
A process mainly characterized by adding energy in the form of heat under defined conditions for an intended purpose (e.g. pasteurisation)

3.14
chemical processes
A process mainly characterized by adding a certain amount of chemicals (e.g. lime) to a matrix under defined conditions for an intended purpose (e.g. disinfection)

4 Symbols and Abbreviations

TCS 3: test containment system type 3

BEL: primary bovine embryo lung cells

BPV: bovine parvovirus

DMEM: dulbecco´s minimum essential medium

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FCS: fetal calf serum

TVS: trypsin-versene solution

PLB: phosphate loading buffer

CPE: cytopathic effects

$TCID_{50}$: tissue culture infecting dose

5 Principle of the validation procedure

The procedure for the validation of various processes using suitable test containment system (TCS 3)

requires following stages:

1. Preparation of the cell line

2. Preparation of the test virus suspension

3. Preparation of test containment system type 3 (TCS 3)

4. Exposure of the TCS 3 to the process at defined places in a suitable manner for the intended exposure time

5. Collecting of TCS 3 and determination of residual virus count from the exposed TCS 3

6. Determination of the inactivation rate.

6 Reagents, diluents and culture media

To ensure reproducible results, prepare culture media and diluents using either constituents of uniform quality and chemicals of recognised analytical grade, or a dehydrated diluent or complete medium prepared following the manufacturer's instructions. Prepare them with fit for purpose demineralised or distilled water free from substances capable of inhibiting growth under the test conditions. [ISO 8199:2005].

6.1 Dulbecco´s minimum essential medium (DMEM) supplemented with antibiotic suspension and fetal calf serum

Prepared according the manufacturer or :

Mix the following substances in 1 000 ml water in a 2 000 ml flat bottom flask:

-	NaCl	64000 mg	-	L-Methionin	30 mg
-	KCl	400 mg	-	L-Phenylalanin	66 mg
-	$CaCl_2$	200 mg	-	L-Threonin	95 mg
-	$MgSO_4 \cdot 7H_2O$	200 mg	-	L-Tryptophan	16 mg
-	NaH_2PO_4	124 mg	-	L-Tyrosin	72 mg
-	D-Glucose	1000 mg	-	L-Valin	94 mg

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-	$Fe(NO_3)_3 \cdot 9H_2O$	0.1 mg	-	Glycin	30 mg
-	Na-Pyruvat	110 mg	-	L-Serin	42 mg
-	Phenorot	15 mg	-	Cholinchlorid	4 mg
-	$NaHCO_3$	37000 mg	-	Folazid	4 mg
-	L-Arginin.HCl	84 mg	-	Myo-Inositol	7,2 mg
-	L-Cystin	48 mg	-	Nicotinamid	4 mg
-	L-Glutamin	580	-	D-Ca-Pantothenat	4 mg
-	L-Histidin.HCl $\cdot H_2O$	42	-	Pyridoxal $\cdot$ HCl	4 mg
-	L-Isoleucin	105 mg	-	Ribiflavin	0.4 mg
-	L-Leucin	105 mg	-	Thiamin $\cdot$ HCl	4 mg
-	L-lysin $\cdot$ HCl	146 mg			

Filter 500 ml of prepared DMEM through a sterile 0.22 µm (pre-filtration) and 0.21 µm pore size membrane filter into glass flask using positive pressure.

Antibiotic suspension

Penicillin G

Streptomycin sulphate

Gentamycin sulphate

Amphotericin

Aseptically, dissolve each vial contents in sterile distilled water according the manufacturer. Store at 4 °C, protected by light (e.g. aluminium foil).

Add 1 ml of Penicillin G, Streptomycin sulphate, Gentamycin sulphate each, 2 ml of Amphotericin and 50 ml of fetal calf serum into 500 ml prepared DMEM. Divided in 20 mL portions into polystyrene flask (7.13). For the titration of BPV (8.2.1) divide 1.8 ml of DMEM into glass tubes.

6.2 Trypsin-Versene solution

Mix the following substances in 1 000 ml water in a 2 000 ml flat bottom flask:

- NaCl 80 g
- KCL 2 g
- KH_2PO_4 2 g
- $Na_2HPO_4 \cdot 12\ H_2O$ 23.10 g
- $MgSO_4 \cdot 7H_2O$ 1 g

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- $CaCl_2 \cdot 2\ H_2O$ 1.32 g
- Trypsin 1:250 12.50 g
- Versene 12.50 g
- Streptomycin sulphate 0.50 g
- Penicillin G 0.50 g

Adjust the pH to 7.0 ± 0,2 à 25°C. Filter 500 ml through a membrane filter (0.22 µm pore size) using positive pressure into glass flask.

6.3 Phosphate loading buffer

Mix the following substances in 1 000 ml water in a 2 000 ml flat bottom flask:

- NaCl 8 g
- KCL 0.20 g
- H_2PO_4 0.12 g
- $Na_2HPO_4 \cdot 12\ H_2O$ 0.91 g

Adjust the pH to 7.0 ± 0,2 à 25°C. Fill into glass and sterilized by autoclave.

6.4 Beef extract

Mix the following substances in 100 ml water in a 200 ml glass flask:

- Dry meat extract 4 g
- NaCl 2.9 g

Adjust the pH to 8.5 ± 0,2 à 25°C. Filter through a membrane filter (0.22 µm pore size) using positive pressure.

7 Apparatus

With the exception of equipment supplied sterile, the glassware shall be sterilised in accordance with the instructions given in ISO 8199:2005.

Usual microbiological laboratory equipment and in particular:

7.1 Apparatus for sterilization

a) for moist heat sterilization, an autoclave capable of being maintained at (121 ±3) °C for a minimum

holding time of 15 min;

b) for dry heat sterilization, a hot air oven capable of being maintained at (180 ±5)°C for a minimum holding time of 30 min, at (170 ±5) °C for a minimum holding time of 1 h or at (160 ±5) °C for a minimum holding time of 2 h;

c) for media sterilization, use suitable membrane filtration apparatus with filters of diameter 47 mm to 50 mm and membranes with 0,22 and - 0,1 µm pore size.

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7.2 Water bath, capable of being controlled at (4 ± 1) °C and (10 ± 1) °C and at additional temperatures ± 1 °C

7.3 CO_2 incubator (95 % air, 5 % CO_2), capable of being controlled at either (36 ± 1) °C or (37 ± 1) °C. An incubator at (37 ± 1) °C may be used if an incubator at (36 ± 1) °C is not available.

7.4 Wide-mouth glass flasks or beakers

7.5 Culture tubes

7.4 pH-meter, having an accuracy of calibration of 0,1 pH units at 25 °C.

7.5 Inverted microscope, for reading cell cultures microscopically.

7.6 Stopwatch.

7.7 Electromechanical agitator, e.g. Vortex® mixer

7.8 96-well microtitre plates, for cell culture use.

7.9 Containers: sterile test tubes, bottles or flasks of suitable capacity.

7.10 Magnetic stirrer, for keeping cells in suspension before seeding.

7.11 Petri dishes, (**plates**) of size 30 mm to 100 mm.

7.12 Graduated sterile pipettes, of nominal capacities 10 ml and 1 ml and 0,1 ml. Calibrated automatic pipettes with disposable sterile tips may be used.

7.13 Polystyrene flask for cell culture use.

7.14 Cellulose nitrate filter membrane, (0,2 μm - 0,1 μm).

7.15 Adjustable micropipettes up to 200 μL capacity.

7.16 Volumetric flasks.

7.17 Mechanical shaker.

7.18 Centrifuge.

7.19 Refrigerator capable of being controlled at 2 °C to 8 °C.

7.20 Tweezers

7.21 Freezer, for storage at −20 °C and −70 °C.

7.22 Biological safety cabinet, class II.

7.23 polycarbonate membrane with 0,01 μm large pores

7.24 sterile Virosorb membrane filter (pore size 0.2 µm, thickness 70 µm, diameter 25 mm)

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8 Procedure

8.1 Preparation of cell line

Primary bovine embryo lung cells (BEL) are used for the propagation and later for the detection of bovine parvovirus (BPV) in the TCS 3. Preparation of cell culture is carried out under sterile conditions. BEL cells are cultivated in sterile polystyrene flask (7.13) with 20 ml DMEM with 5 % fetal calf serum (FCS) and antibiotic suspension (6.1).

8.1.1 Subculturing of the BEL cells

Principle:

- remove DMEM from the cell culture flask using a pipette and discard
- wash cell monolayer with 5 ml TVS (6.2) (37 ± 1 °C), by spreading TVS over the monolayer with a sterile glass pipette and cautiously swaying the flask.
- remove TVS from the cell culture flask using a pipette and discard
- repeat washing procedure with 5 ml TVS (6.2) (37 ± 1 °C)
- remove 4 ml TVS from the cell culture flask using a pipette and discard
- incubate for about 3-5 min at 37 ± 1 °C or until the cells were no longer attached to the bottom of the flask
- resuspend the cells in DMEM with 5% FCS, diluted 1:3 to 1:5 and seeded into new flasks or into 96 well plates for virus titration. For 96 well plates transfer 0,1 ml of resuspended cells into each well

8.2 Preparation of the test virus suspension

The obligatory test virus is: *Bovine Parvovirus*

The stock BPV suspension must be passaged to produce a test virus suspension. The virus is propagated in a BEL cell line (8.1.1) in order to obtain the test virus suspension.

Principle:

- remove the cell culture medium from one day old BEL monolayers
- add 1 ml BPV suspension to cover the cells
- incubate at 37 °C for one hour
- add 20 ml DMEM with FCS (6.1) to the flask and incubate at 37 °C 5 % CO_2 for 2 to 3 days and monitor daily for cytopathic effects (CPE)
- The virus is harvested when approximately 80% of the cell monolayer showed CPE
- centrifuge the resulting suspension at 175 g at 25 °C for seven minutes for the removal of detritus
- use this stock virus suspension for preparation of TCS 3 or store at –80 °C until further use

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8.2.1 Virus quantification

- Take an aliquot of 200 µl out of the stock BPV suspension (8.2)
- Prepare a serial tenfold dilution up to 10^{-8}: starting by pipetting 200 µl of prepared stock BPV suspension (8.1) into 1.8 ml DMEM (6.1).
- add 100 µl of each dilution to a one day old BEL monolayer in each well of a 96 well plate.
- inoculate four wells with each dilution step
- Incubate at 37 °C 5 % CO_2 for 7 days and monitor daily for cytopathic effects (CPE) under an inverse microscope
- calculate the virus titre in mean tissue culture infectious doses ($TCID_{50}$) according to Spearman and Kärber (from Hierholzer and Killington, 1996).

8.3 Preparation of test containment system type 2

Principle:

- dilute virus from stock virus suspension (8.2) 1:10 in phosphate loading buffer (6.3)
- filter 1 ml this virus suspension through a sterile Virosorb membrane filter (pore size 0.2 µm, thickness 70 µm, diameter 25 mm) using sterile syringe, allowing the virus to adsorb to the membrane
- wrap the membranes in a polycarbonate membrane with 0,01 µm large pores
- place the envelope with membranes (3 x 3 cm) in Rashel sacks (5 x 5 cm) or TCS 1 and keep in the refrigerator (approx. 4 °C) until use, but not longer than 18h

An example for preparation of TCS 3 is given in Annex (C 2).

Virus load on the Virosorb membrane is determined at least on one of the prepared membranes before exposure in TCS 3. At least one TCS 3 is kept in the laboratory at a temperature of 4 °C to 8 °C and processed together with the exposed TCS serving as positive control.

Principle:

- place the membrane using sterile tweezers into glass flask containing 1 ml beef broth (6.4)
- treat in an ultrasound bath for 5 min
- transfer the content to disposable centrifuge tube, centrifuge at low speed?
- determine the virus concentration as described in 8.2.1

8.4 Exposure of the TCS 3 to the process

The exposure of the TCS 3 to the treatment process is carried out depending on the situation in the treatment process. The TCS 3 must be exposed for a maximum of time according to the shortest amount of time that substrate can be expected to be exposed to the treatment process (e.g. pasteurisation at 70 °C for 60 min.).

TCS must be introduced at various locations within the process, being representative for the process and such can be regarded as critical control points. In processes where no even distribution of temperature or concentration of chemicals can be expected the sites of exposure shall represent the locations of lowest

temperature or concentration, of the highest temperature or concentration and of a medium temperature or concentration. In general a minimum of 10 TCS must be introduced at such representative locations in the process. In processes in which an even distribution of temperature or chemicals can be expected (e.g. pasteurisation units) due to measurements previously carried out at least 4 TCS shall be exposed. At the places where the TCS are exposed the relevant process parameters (e.g. temperature or pH-value) shall be measured and recorded in parallel, those data are required for future steady supervision of the process at critical control points in routine operation.

8.5 Collecting of TCS 3 and determination of residual virus count

At the end of the treatment process, the TCS 3 must be removed, washed with 2 ml PBS, placed into sterile Petri plates and returned to the laboratory. In order to prevent drying of the TCS 3 during transport, the Petri dishes shall be kept humid by placing the TCS 3 on a layer of wet paper towel in the Petri dish.

For detection and enumeration of the remaining virus, the Virosorb membrane from TSC 3 must be treated as described in 8.2 and 8.3.

8.6 Determination of the inactivation rate.

The validation of biotechnological, thermal and chemical processes must demonstrate that the process achieves the following overall reduction:

- reduction of infectivity titre of thermo resistant viruses such as *parvovirus* by at least 3 log10

If the reduction of parvovirus after exposure does not comply with the above requirement, the process will be regarded as insufficient for hygienisation of substrate.

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Annex C
(informative)

C.1 Calculation of the viral infectivity titre

Example of $TCID_{50}$ determination by the Spearman-Kaerber method

The Spearman-Kaerber method is most simple and gives results most compatible with other calculations. Prerequisite of the evaluation is the use of several dilutions which cover infection of all cell culture units to those in which no virus multiply. For calculation of the negative logarithm of $TCID_{50}$ endpoint titre:

negative lg of $TCID_{50}$ = negative lg of the lowest dilution step – {[© (% positive/dilution)/100] □ 0,5} lg of dilution factor

Table C.1 — Example of titration results

Dilution (-lg)	**Result** [a]	**% positive**
4	444 444	**100**
5	444 344	**100**
6	443 003	**66.7**
7	400 020	**33.3**
8	000 000	**0**
Sum of % positive cultures		300
a 1 to 4 = virus present, degree of CPE in six cell culture units (tubes, microtitre plates) 0 = no virus present		

Negative lg $TCID_{50}$ = – 4 – (300/100 – 0,5) 1 = –6,5

$TCID_{50}$ titre = $10^{-6,5}$

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C.2 Test containment system type 2

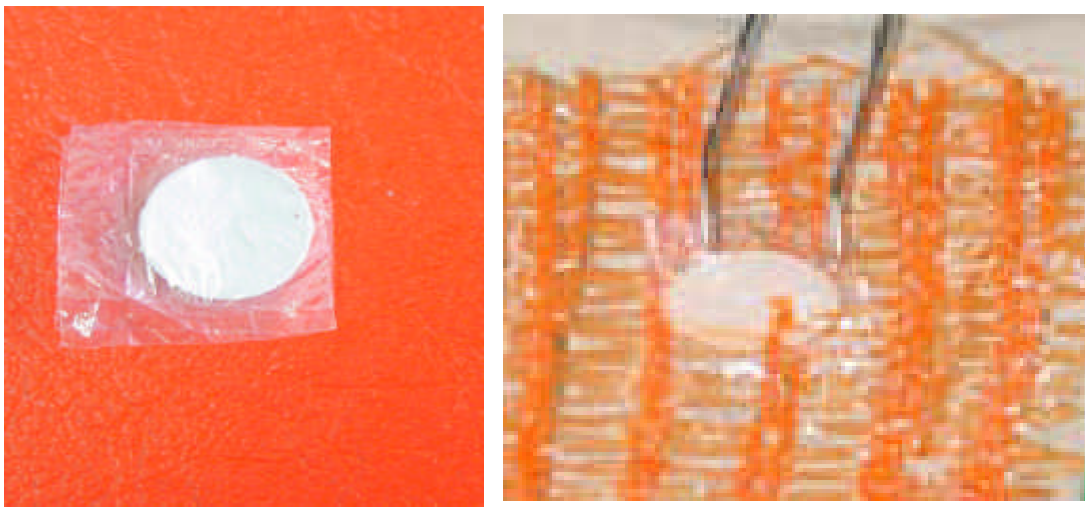

Fig. 1: Envelope (3 x 3 cm) with membranes with BPV

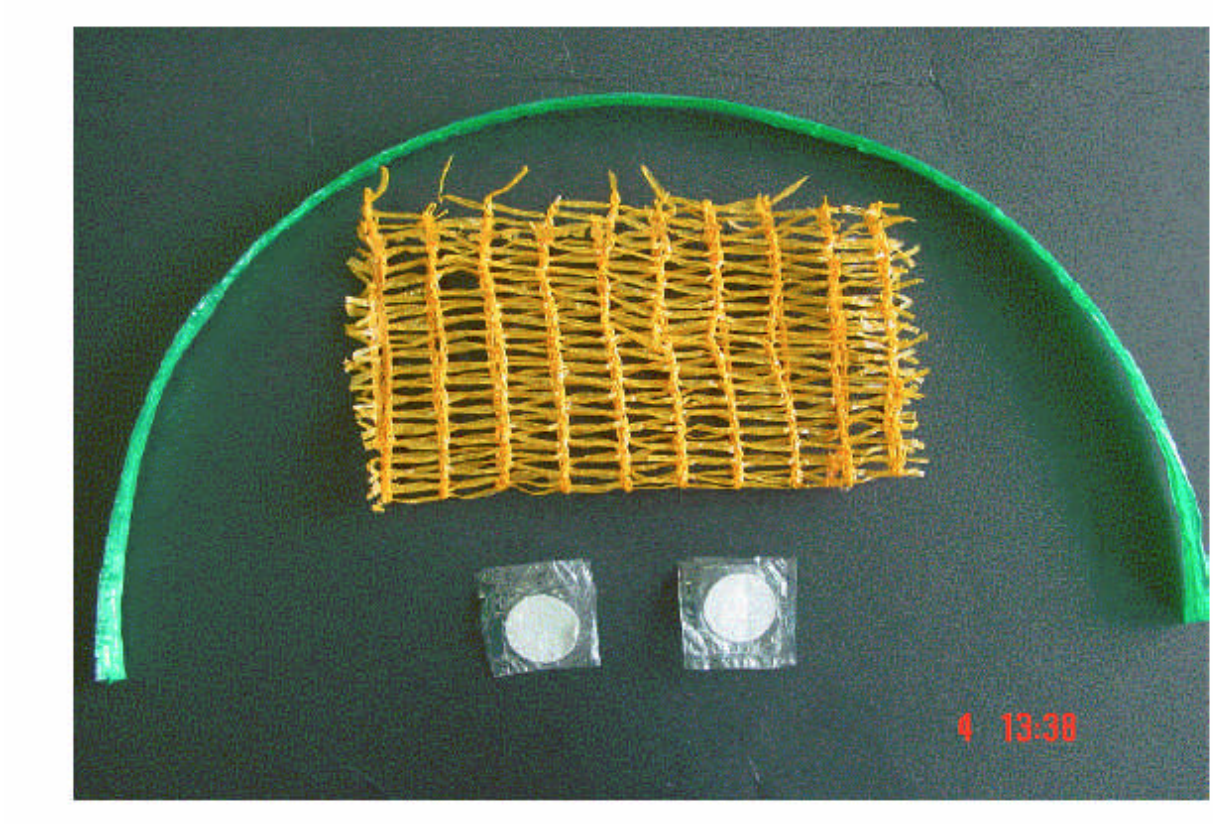

Fig. 2: Envelope (3 x 3 cm) with membranes with BPV and onion sacks

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