The development and evaluation of reference materials for food microbiology

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Paul in 't Veld

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificius
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op maandag 7 december 1998
des namiddags te 4 uur in de aula.

The research described in this thesis was conducted at the National Institute of Public Health and the Environment (RIVM, Bilthoven, The Netherlands) at the Laboratory for Water and Food Microbiology (LWL) and, since 1996, the Microbiological Laboratory for Health Protection (MGB). The work was financially supported by the European Commission's Communities Bureau of Reference (BCR) and later the Standards, Measurement and Testing Programme (SM&T).

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

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Development and evaluation of reference materials for food microbiology
– in 't Veld P.H. – 1998

Thesis Landbouwuniversiteit Wageningen. – With ref.-
With summaries in English and Dutch
Subject headings: reference materials/quality assurance/food microbiology

ISBN 90-5485-952-0

1. Het aantonen van lage aantallen middels hitte beschadigde *Listeria monocytogenes* cellen is niet goed mogelijk met de huidige ISO 11290-1 methode voor de detectie van *Listeria monocytogenes*, als gevolg van de te korte incubatietijd (24 uur) van de eerste ophopingstap in het "half Fraser" medium.
   (dit proefschrift).

2. Referentiematerialen worden, internationaal gezien, nog weinig gebruikt voor de kwaliteitsborging van microbiologische analyses. Dit heeft meerdere oorzaken, zoals bijvoorbeeld onbekendheid met de beschikbaarheid van de materialen en de gebruikskosten zonder dat er een direct voordeel duidelijk is.
   (dit proefschrift).

3. De frequentie van het analyseren van referentiematerialen bij routinematig gebruik wordt te makkelijk gesteld op "bij elke serie analyses". Daarentegen wordt de frequentie voor het gebruik van gecertificeerde referentiematerialen te makkelijk op nul gesteld.
   (dit proefschrift).

4. De opmerking in de ISO 11290 betreffende de detectie en enumeratie van *Listeria monocytogenes* dat er ook haemolyse negatieve *Listeria monocytogenes* bestaan, is van generlei belang voor het vinden van pathogene *Listeria monocytogenes*.

5. Het huidige systeem voor het maken van internationale standaarden door de ISO of de CEN houdt een zodanig lange procedure in dat op het moment van uitgifte van dergelijke standaarden deze vaak al niet meer *up to date* zijn.

6. De resultaten gevonden met de, door Beecher en Wong beschreven, HBL plaat methode om het *Bacillus cereus* HBL enterotoxine complex aan te tonen, komen niet overeen met die verkregen met PCR methoden (gericht op het aantonen van de enterotoxine genen) en de Oxoid Reverse Passive Latex Agglutination test (gevoelig voor het L_2 deel van het HBL-complex).
   (Beecher, D.J. and Wong, A.C.L. (1994) Appl. Environm. Microbiol., 60 (5), 1646 - 1651).

7. De algemene eisen voor accreditatie die aan laboratoria gesteld worden zijn in internationale normen vastgelegd om een gelijk (kwaliteits)niveau van geaccrediteerde laboratoria te realiseren. Echter, op het niveau van de beoordeling door een vakdeskundige blijkt dat, internationaal gezien, gelijkwaardigheid van de interpretatie van de eisen nog ver te zoeken is.

8. De kwalitatieve methoden voor het aantonen van pathogenen in levensmiddelen zouden moeten worden vervangen door kwantitatieve bepalingsmethoden om hiermee een betere bijdrage te kunnen leveren aan de risicoanalyse.

9. De vraag of de oorzaak van het slecht begrijpen van een publicatie, zoals bijvoorbeeld een proefschrift, gezocht moet worden in een gebrekkige formulering dan wel in de leesvaardigheid van de lezer is niet altijd naar ieders tevredenheid te beantwoorden.

10. Het probleem voor een simpele en snelle oplosprocedure voor de referentiematerialen, bedoeld voor de evaluatie van tellingsmethoden, blijkt niet simpel en snel op te lossen.

11. De tijdsbesparing als gevolg van het gebruik van computers voor het maken en corrigeren van rapporten en verslagen wordt teniet gedaan door de aandacht voor de presentatie.

12. Wie niet in staat is verbanden te leggen, doet er beter aan eens een E.H.B.O.-cursus te volgen.

13. Het te vroeg op de markt brengen van nieuwe producten kan na gebleken problemen leiden tot extreme reacties bij zowel producent als consument die niet in relatie staan tot de proporties van het probleem, zoals dit bijvoorbeeld werd gezien bij de introductie van de A-klasse van Mercedes-Benz.

14. De mate van dominant gedrag van honden die, maatschappelijk gezien, wordt getolereerd is omgekeerd evenredig met de grootte van de hond.

15. Uitgaande van het gegeven dat muziek tenslotte geluid is, verkeren sommige musici ten onrechte in de veronderstelling dat geluid ook altijd muziek is.

Stellingen behorende bij het proefschrift 'The development and evaluation of reference materials for food microbiology'.

Paul in 't Veld, Wageningen, 7 december 1998.

Voor mijn ouders.

Paul in 't Veld (1998) *Development and evaluation of reference materials for food microbiology.* PhD-thesis, Wageningen Agricultural University, The Netherlands (184 pp., summaries in English and Dutch).

This thesis describes the evaluation and certification of reference materials (RMs) for quality assurance of the microbiological examination of food, the use of RMs and certified RMs (CRMs) in food microbiology laboratories and the setting up of regular production and distribution of such materials. The microbiological RMs were produced by spray drying a bacterial culture suspended in milk. The highly contaminated milk powder (HCMP) obtained was mixed with sterile milk powder and filled into gelatin capsules. There are two types of these RMs, one for the evaluation of enumeration methods and the other for detection methods. The RMs for *Bacillus cereus* (containing *ca* 10^4 colony forming particles (cfp) per capsule) and *Listeria monocytogenes* (containing *ca* 5 cfp per capsule) were evaluated with respect to the general requirements for RMs (stability, homogeneity and representativity). Both RMs fulfilled these requirements. The use of the *L. monocytogenes* RM in laboratories was further evaluated in three collaborative studies, testing the RM both with and without competitive micro-organisms (the latter as capsules containing a mixture of micro-organisms or as real food samples). Most laboratories were able to isolate *L. monocytogenes* when no competitors were present. However, the addition of competitive micro-organisms reduced the number of positive isolations, while in the presence of food there was an even greater reduction in the number of positives. Several RMs were certified by the European Commission and became the first microbiological CRMs available world-wide. The *Salmonella* CRM was certified for the fraction of capsules in which no *Salmonella* could be detected by the ISO isolation method (2.7 %). The *B. cereus* CRM was certified using both MEYP (53.4 cfp per plate) and PEMBA (55.0 cfp per plate) agars. Based on the variance components user tables were constructed that presented the 95 % confidence limits for the number of capsules likely to be examined in practice. Fluid bed spray granulation was evaluated as an alternative method to spray drying for the preparation of HCMPs. Several strains were dried in milk by this method and the resulting material tested for homogeneity and stability when stored at -20 °C and 22 °C. The stability of the HCMPs stored at -20 °C was comparable to that of the spray dried material but at 22 °C was less stable. RMs are mainly intended for the quality control (QC) of routine examination. Control charts can be produced for enumeration methods even when the RMs used are not fully stable. CRMs are used to determine the accuracy of a method or laboratory. A power analysis indicates the minimal difference between the certified value and the true laboratory mean that can be detected using a CRM. Market research was undertaken to obtain information on the need for RMs and to compare this need to the characteristics of the RMs developed. Commercial production of RMs was set up at the Foundation for the Advancement of Public Health and the Environment (SVM). RMs and CRMs are produced in the same way, but for CRMs there is an additional certification study. The sale of RMs is increasing gradually and interviews conducted after the initial market research indicated that the QC of routine analysis is increasing in importance. The RMs developed were proved to be competitive (in performance and price) with alternative RMs available from other sources. It is concluded, therefore, that stable, homogeneous and representative microbiological RMs and CRMs can be produced and that they play an important role in quality assurance of the microbiological examination of food.

ABBREVIATIONS

AOAC	Association of Official Analytical Chemist.
ATCC	American Type Culture Collection.
a_w	water activity.
BCR	Communities Bureau of Reference (see also SM&T).
BGA	Brilliant Green Agar.
BHI	Brain Heart Infusion broth.
BHIA	Brain Heart Infusion Agar.
BMT	Management & Marketing Consultants.
BPLSA	Brilliant green Phenol red Lactose Sucrose Agar.
BPw	Buffered Peptone water.
ca	circa.
CB	Collaborative Study.
CEN	European standardisation organisation
cfp	colony forming particle (same as cfu).
cfu	colony forming unit (same as cfp).
CMP	Contaminated Milk Powder.
CRM(s)	Certified Reference Material(s).
EC	European Commission
EU	European Union.
(½) FB	(half) Fraser Broth.
FDA	Food and Drug Administration.
KFA	Kenner Fecal Streptococcus Agar.
HCMP	Highly Contaminated Milk Powder.
i.d.	initial decrease
IDF	International Dairy Federation.
IRMM	Institute for Reference Materials and Measurements.
ISO	International Organisation for Standardisation.
LEB	Listeria Enrichment Broth.
LL	Lower Limit.
LPM	Lithium chloride-Phenylethanol-Moxalactam agar.
LSA	Lauryl Sulphate Agar.
m-EA	membrane-Enterococcus Agar according to Slantez and Bartley.
MEYP	Mannitol Egg-Yolk Polymyxin agar.
MK	Muller Kauffmann broth.
mmp	mixed milk powder.
mp	milk product no. 17 Nestlé.
NCTC	National Collection of Type Cultures.
NIST	National Institute of Standards & Technology.
NSM	non-standard method.
PEMBA	Polymyxin pyruvate Egg-yolk Mannitol Bromothymol blue Agar.
PHLS	Public Health Laboratory Service (UK).
PS	Peptone Saline solution.

QA	Quality Assurance.
QC	Quality control.
RIVM	National Institute of Public Health and the Environment.
RM(s)	Reference material(s).
RV	Rappaport-Vassiliadis magnesium chloride malachite green broth.
RVS	RV prepared with Soya peptone.
s.d.	standard deviation.
SM	standard method.
smp	skim milk powder.
SBA	Sheep Blood Agar.
SM&T	Standards Measurements and Testing Programme (former BCR).
SOP	Standard Operating Procedure.
SVM	Foundation for the advancement of Public Health and Environmental Protection.
T7A	Lactose 2,3,5-Triphenyl Tetrazolium Chloride agar with Tergitol-7.
TSA	Tryptone Soya Agar.
TSAYE	Tryptone Soya Agar supplemented with Yeast Extract.
TSB	Tryptone Soya Broth.
TSBY(E)	Tryptone Soya Broth supplemented with Yeast Extract.
UL	Upper Limit.
USDA	United States Department of Agriculture.
XLD	Xylose Lysine Desoxycholate agar.

SYMBOLS

α	significance level.
$1-\beta$	power of the test.
β	regression coefficient.
β_1	intercept.
β_2	slope of first line segment.
$\beta_2 + \beta_3$	slope of second line segment.
χ^2	Chi-square test statistic.
ε	error component.
$\hat{\mu}$ or μ_{cert}	certified value.
μ_{lab}	laboratory mean.
$\hat{\sigma}^2_{cap}$	variance component for capsules.
$\hat{\sigma}^2_{L}$	variance component for laboratories.
$\hat{\sigma}^2_{sub}$	variance component for replicates.
$\hat{\sigma}^2_{x}$	variance for a combination of capsules and replicates.
C_{cfp}	number concentration of colony forming particles of the test strain in a broth culture or in contaminated milk.
$F_{I-1,N}$	F test statistic with I-1 and N degrees of freedom.

I	(total) number of capsules examined.
J	number of replicate samples examined per capsule.
L	number of laboratories used for the determination of the (certified) value.
N	total number of capsules tested (minus the number of homogeneity tests performed).
$N_{S,cfp}$	number of colony forming particles of *Salmonella* in one capsule.
p_{lab}	fraction of negative capsules (capsules not containing the target organism) observed by a laboratory.
p_{neg}	certified fraction of negative capsules.
p_y	chance of finding "y" positive isolations.
r	repeatability.
R	reproducibility.
$\overline{R}$	average moving range.
t	storage time.
t_s	time point at which a structural break occurred.
$t_{1-\alpha/2,L-1}$	Student t-test value at (1 - α/2) fractile and L-1 degrees of freedom.
T_1	Cochran's dispersion test statistic, to determine the variation in z between samples from one reconstituted capsule.
T_2	Cochran's dispersion test statistic, to determine the variation in z between samples from different reconstituted capsules of one batch.
T_{hom}	T_2 applied to four sets of long term stability test data to estimate homogeneity.
X	$\log_{10}$ count (number of cfp) per capsule.
x_i	$\log_{10}$ count of the I^{th} capsule.
y	number of positive isolations.
z_{ij}	count of sample j of capsule I.
z_{i+}	sum of counts of all replicate samples examined of capsule I.
z_{++}	sum of counts of all replicate samples examined for all capsules.
z_i	count of capsule I.
z_+	sum of counts of all capsules.

THE MEASUREMENTS PROCESS IN MICROBIOLOGY

There are many kinds of measurement processes, varying from relatively simple ones such as measuring the temperature of air, to complex ones, such as measuring the concentration of dioxins in milk. Measurement processes in microbiology differ from those in other disciplines in that the analyte is a living organism.

Several techniques exist to determine the presence or concentration of a certain type or group of micro-organism(s) in a sample. Detection (or qualitative) methods are used to determine the presence or absence of a specific micro-organism in a defined quantity of sample (in food microbiology usually 25 gram). The principle of this technique is that the sample is homogenised in an enrichment broth and after incubation and subculture to a solid medium the suspect target organism is isolated. Identification of the colonies isolated as the target organism confirms the presence of that organism. Results are expressed as presence or absence of the organism sought in a defined quantity (gram or ml) of sample.

The, so called, Most Probable Number (MPN) technique is an extension of this basic detection procedure to allow quantification of low levels of target organisms. It is based on detection of the organism in various quantities or dilutions of the sample. The number of positive results obtained for the various dilutions of the sample are used to estimate, from appropriate tables, the concentration of the target organism in the sample. Results are normally expressed in the same manner as for quantitative techniques i.e. per gram or ml of sample, but are less precise than quantitative methods. The MPN technique is not used in food microbiology as often as the detection or enumeration techniques, it is usually for specific applications. Therefore it is not specifically dealt with in this thesis.

Enumeration (or quantitative) methods are used to determine the concentration of total or specific micro-organisms in a sample. The principle of these methods is that the sample is first homogenised in a dilution fluid (for food microbiology usually 10 gram of food in 90 ml of peptone saline solution). This suspension is then decimally diluted and defined quantities of various dilutions are plated (usually in duplicate) onto solid media which may be general media for the total flora or selective media for specific target organisms. After incubation of the plates the number of colonies (total or suspected target organisms) are counted and, if necessary, confirmed by means of biochemical tests. Counts are expressed as the number of colony forming particles (or units) per gram or ml sample.

ACCURACY OF MICROBIOLOGICAL MEASUREMENTS

In general a measurement technique should produce accurate results, aiming to approximate the true value. The principles and definitions in relation to accuracy are described in the ISO standard 5725-1 (Anon., 1994a). The accuracy of a measurement result depends on two factors which are related as follows: accuracy = trueness + precision. **Accuracy** is defined as the closeness of agreement between a test result and an accepted reference value. **Trueness** is defined as the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. An accepted reference value is used, when the true value is not known. The accepted reference value (Anon., 1994a) is a

value that serves as an agreed-upon reference for comparison, and is derived as one of the following:

a) a theoretical or established value, based on scientific principles.
b) an assigned or certified value, based on experimental work by a national or international organisation.
c) a consensus or certified value, based on collaborative experimental work under the auspices of a scientific or engineering group.
d) when a), b) and c) or not available, the expectation of the measured quantity, i.e. the mean of a specific population of measurements.

Precision is defined as the closeness of agreement between independent test results obtained under stipulated conditions (Anon., 1994a). Precision data should be obtained under repeatability and reproducibility conditions to assure independence of the test results. **Repeatability** is defined as the precision of independent test results obtained using the same method on identical test items in the same laboratory by the same operator using the same equipment within a short interval of time. **Reproducibility** is defined as the precision of test results obtained with the same method on identical items in different laboratories by different operators using different equipment. So, in other words, trueness is related to the systematic errors and precision to the random errors of the measurement process.

Normally repeatability is expressed as the repeatability limit representing the maximum value for the absolute difference between two test results obtained under repeatability conditions. This value is calculated as 2.8 times the standard deviation (s.d.) for repeatability. Similarly the reproducibility limit is calculated as 2.8 times the s.d. for reproducibility. The Association of Analytical Chemists (AOAC) expresses the precision of their methods in an alternative manner. They specify the s.d. for repeatability and reproducibility and the relative s.d. for repeatability and reproducibility ($RSD_{r/R}$) based on transformed counts. The $RSD_{r/R}$ is the s.d. for r or R divided by the mean count (Youden and Steiner, 1975; Park *et al.*, 1996).

How do the principles of accuracy, trueness and precision apply to microbiological measurements? The definitions given above are used for **quantitative** data that conform to a normal distribution. In theory the distribution of micro-organisms in a sample is not described by a normal distribution but by a Poisson distribution (Niemelä, 1983; Heisterkamp *et al.*, 1993). For quantitative microbiological methods log transformation of the counts is appropriate to approximate a normal distribution of the log transformed counts. Using log transformation it is possible to calculate the precision of methods. Precision limits can be expressed as a difference in log units between two log values or as a ratio between values on the normal scale.

For **qualitative** methods the situation is more difficult. The ISO 5725 (Anon., 1994a) is only applicable to quantitative methods. Recently the first attempts were made to define precision for qualitative methods within the framework of a project financed by the European Commission to determine the precision of various ISO methods. Hopefully within a few years techniques will become available to allow expression of precision for qualitative methods.

The **true** value in microbiology is not known. Results depend on the method used and even non-selective methods do not recover all organisms present in a sample. Therefore, the overall mean of a set of results obtained in different laboratories is used as the accepted

reference value for quantitative methods. The validation procedure for rapid (quantitative as well as qualitative) methods set up by Microval uses the term relative trueness when the mean value of the rapid method is compared to the reference method used in the validation study (Anon., 1997). An example of the use of the overall mean obtained in an interlaboratory study as a true value is in the certification of reference materials as described in this thesis.

MICROBIOLOGICAL REFERENCE MATERIALS

Reference materials (RMs) are defined in the ISO guide 30 (Anon., 1992) as a material or substance one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials. The property values of a material are established by subjecting that material to a collaborative study in which at least two independent laboratories determine the property value using their own measurement procedure. The value obtained in the collaborative study is the consensus true value (trueness in the measurement process). The property value of the RMs is expressed as the mean value including its uncertainty. When more than one measurement procedure determines the property of the RM, the values obtained must be within the uncertainty assigned to each measurement procedure. When the differences between measurement procedures are too large the value can be expressed on a method dependent basis.

From the above description a number of general requirements for RMs can be derived (Griepink, 1989; Anon., 1989) including:

- Homogeneity.
  The homogeneity of the RM, which is the variation in the property value between portions of the RM, must be within specified limits. In the ideal situation the variation in the property value between portions of the RM is negligible compared to the variation in the measurement method.
- Stability.
  The property value must remain stable within specified limits over a specified period of time during storage and transport.
- Representativity.
  The RM should be representative for its intended use in respect to, for example, the type of matrix and level of contamination.

In addition to RMs there are also certified reference materials (CRMs). A CRM is a reference material that has been certified for its properties by means of a technically valid procedure and should be accompanied by, or traceable to, a certificate or other documentation which is issued by a certifying body (Anon., 1989). ISO/REMCO (Anon., 1994b) reported the existence of a total of 46 certifying bodies world-wide that currently produce CRMs. The two major certifying bodies are the Community Bureau of Reference (BCR) of the European Commission, currently called the Standards, Measurement and Testing programme (SM&T) and the National Institute of Standards & Technology (NIST) in the United States of America.

Many RMs have been produced and certified world-wide, especially in the fields of physics and chemistry, but, until recently, none in microbiology. There are various reasons for

the lack of microbiological RMs and CRMs. These are mainly related to the fact that this type of RM contains living organisms and thus to the fact that stability over a long period of time and homogeneity of the number of organisms is difficult to achieve. It is also well recognised that differences between the results of different microbiological measurement procedures exist. This means that the values stating the properties of an RM are expressed according to a specific measurement procedure, which may limit their use.

In 1985, Beckers *et al.* (1985) reported for the first time the availability of a microbiological reference material for the evaluation of the method for detecting *Salmonella* in food. This RM evolved from air drying salmonellae in milk (Van Schothorst and van Leusden, 1972). Since 1987 the development of these RMs received additional impetus when the BCR/SM&T supported the development and evaluation of this type of material. Currently, a number of the RMs developed are available for use by laboratories from the Foundation for the Advancement of Public Health and the Environment (SVM).

In the last few years other microbiological RMs have become available. These alternative RMs (Oxoid's Quanti-Cult$^{PLUS\ ™}$ marketed by Unipath Ltd, U.K. and the RMs produced by the National Food Administration in Sweden) are both based on the stabilisation of micro-organisms by freeze drying (Peterz and Steneryd, 1993). The Quanti-Cult$^{PLUS\ ™}$ systems contain a single type of organism per RM and cover a range of organisms not specifically intended for food microbiology. The two RMs prepared by the National Food Administration in Sweden contain a mixture of various organisms (pathogens and non-pathogens) commonly found in foods.

PRODUCTION OF MICROBIOLOGICAL REFERENCE MATERIALS

Production process

The spray drying process described by Beckers *et al.* (1985) has been used as a basis for the preparation of the microbiological RMs developed by the RIVM/BCR. A bacterial suspension in milk is spray dried to produce a contaminated milk powder, referred to as "highly contaminated milk powder" (HCMP) as this powder contains more organisms per gram than the final reference material. After spray drying the HCMP is stored for some time to stabilise the concentration of micro-organisms.

In order to produce an RM the micro-organism has to survive the spray drying process. Micro-organisms capable of surviving HTST pasteurisation (15 s at 72 °C) can also survive the spray drying process (Stadhouders *et al.*, 1982). Among these so called thermoresistant bacteria are spores of *Bacillus* and *Clostridium* species, *Enterococcus* and *Micrococcus* species. Survival of bacteria in the spray drying process is related to the outlet temperature of the dryer (LiCari and Potter, 1970; Miller *et al.*, 1972; Chopin *et al.*, 1977; Thompson *et al.*, 1978). Evaporation of water from the milk droplet after atomisation in the hot (inlet) air will cool the milk droplet and reduce the air temperature. The temperature of the milk droplet does not exceed the outlet air temperature. Most of the drying occurs at even lower temperatures (Verhey, 1973). The reduction in water content of the milk during the drying process increases the heat resistance of the bacteria present. Dega *et al.* (1972) observed an increase in the heat resistance of *Salmonella* and *Escherichia coli* when milk was

concentrated. LiCari and Potter (1970) observed D values for various *Salmonella* serotypes 2 to 5 times higher during spray drying compared to their D values in liquid milk. Reduction of non thermophilic bacteria in a spray dryer is in the range of 1 - 5 $\log_{10}$ units. For examples, reductions of 1 - 1.5 $\log_{10}$ units have been observed for *Listeria monocytogenes* (Doyle *et al.*, 1985), 2 - 5 $\log_{10}$ units for various *Salmonella* serotypes (LiCari and Potter, 1970), 1 - 6 $\log_{10}$ units for various *Salmonella* serotypes and 3 - 5 $\log_{10}$ units for *Escherichia coli* (Miller *et al.*, 1972) in relation to the outlet temperature of the spray dryer.

The HCMP produced by spray drying is used as stock powder for the preparation of the final RM. For this purpose the HCMP is mixed with sterile (γ-irradiated) milk powder. Mixing is performed in steps using a mortar and pestle for quantities up to *ca* 200 g mixed powder followed by the use of a mixing apparatus for larger quantities. For each step an equal quantity of contaminated milk powder is mixed with sterile milk powder. The mixing procedure is the critical step in the production process to obtain homogeneous RMs. The stock powder can be used for many years (an HCMP prepared in 1978 is still being used today) and the level of contamination of the final batch of RM can be adjusted by changing the mixing ratio. The mixed powder is packed into gelatin capsules in quantities of *ca* 0.3 g of powder.

Two types of RMs can be identified based on their use. The first type is a quantitative RM (also called a high level RM) and is intended for evaluation of enumeration methods. The level of contamination in a quantitative RM is between 10^3 and 10^4 colony forming particles (cfp) per capsule. The second type, a qualitative RM (also called a low level RM), is intended for the evaluation of presence/absence or detection methods. The level of contamination is *ca* 5 cfp/capsule.

Control of production

During production the level of contamination and homogeneity of the material is checked quantitatively. The results obtained are compared to predefined limits and when a deviation from (one of) these limits is observed the level of contamination and/or homogeneity is adjusted by (re)mixing of the batch or the entire batch is rejected for further use. For this check the RMs need to be reconstituted in a standard manner before counting of organisms. The **quantitative** RMs are reconstituted in a test tube containing 10 ml peptone saline solution. During incubation at 38.5 °C for 30 minutes, the solution is agitated several times at regular intervals to promote dissolution of the gelatin and dispersion of the milk powder. After reconstitution 0,1 ml of the suspension is used for inoculation of plates according to standardised methods (In 't Veld *et al.*, 1995).

The **qualitative** RMs are reconstituted in a Petri dish containing five ml peptone saline solution. The dishes are incubated at 38.5 °C for 45 minutes with shaking. After reconstitution a non-selective agar is added and the dishes are incubated for four h to resuscitate injured organisms. After resuscitation a selective agar is added on top of the non-selective agar and incubated further.

The reconstitution procedure for qualitative RMs when used in presence/absence testing is simple. The RM capsule is added intact to the (pre)enrichment broth. Depending on the incubation temperature to be used, pre-warming of the broth, before addition of the capsule,

may be required. For incubation temperatures below 35 °C it is recommended that the broth is pre-warmed to 37 °C, before addition of the capsule and then the broth is incubated at the desired temperature. The in-use reconstitution procedure for quantitative RMs is identical to the procedure used to check the level of contamination and homogeneity at production (described above).

The **homogeneity** of the RM is checked by means of two tests. Both tests are based on Cochran's dispersion test statistic (Cochran, 1954) and uses the count values for the number of cfp on the plates. The first test, also called the T_1-test (Heisterkamp *et al.*, 1993), determines the variation between replicate samples from the same reconstituted capsule. T_1 is approximately χ^2 distributed with $I \bullet (J - 1)$[1] degrees of freedom assuming that the variation between counts from the same reconstituted capsule is a Poisson distribution. The second test, called the T_2 test, determines the variation between the sums of the replicate sample counts per reconstituted capsule. The test statistic T_2 is also approximately χ^2 distributed with $(I - 1)$ degrees of freedom. The formulae for calculating the T_1 and T_2 test statistics are presented in chapter 1. When the variation between the counts conforms to a Poisson distribution the value of $T_2/(I - 1)$ equals one. Both T_1 and T_2 tests are used to determine the homogeneity of the quantitative reference materials. For the qualitative RM only the T_2 test is used as for this type of RM the entire capsule is used for the enumeration method.

The procedure for enumeration of organisms in qualitative RMs is specifically designed to check the level of contamination and homogeneity of a batch of RMs during production. This cannot be achieved by just testing for presence or absence of the organism in a capsule. It is possible to estimate the mean level of contamination (assuming a Poisson distribution) on the basis of the fraction of negative capsules found by testing for the presence or absence in a capsule. However, no information on the variation in the number of cfp between capsules can be obtained by such tests.

The homogeneity tests verify whether or not the distribution between the counts on the plates conform to a Poisson distribution. For other purposes (such as expression of a mean value or analysis of results from a collaborative study) a $\log_{10}$ transformation of the counts (obtained with the quantitative RMs) is used in order to obtain normally distributed data. As a result of the $\log_{10}$ transformation mean values on the normal scale represent the geometric mean. In contrast, the mean values calculated for the qualitative RMs represents the arithmetic mean because at the low level of organisms $\log_{10}$ transformation is not suitable for obtaining normally distributed data.

OBJECTIVES OF THE WORK

The main objective was to evaluate the possibility of producing microbiological reference materials that could fulfil the general criteria for reference materials stipulated by Griepink (1989) and Anon. (1989). Secondly, the possibility of certification of the microbiological RMs was evaluated. As an off-shoot of the work protocols for carrying out microbiological collaborative studies and statistical techniques for control of production and evaluation of collaborative studies were developed. A third objective was to set up production facilities for

[1] I = number of capsules examined, J = number of replicates examined per capsule.

microbiological RMs to ensure continuation of production once the possibility of producing microbiological RMs and the need for these materials in laboratories were established.

OUTLINE OF THE THESIS

The first chapter describes the production and characterisation of a quantitative RM (used for the evaluation of enumeration procedures) and a qualitative RM (used for the evaluation of detection procedures). For the qualitative RM the *Listeria monocytogenes* material is described and for the quantitative RM the *Bacillus cereus* material. The RMs are characterised by their stability, homogeneity and representativity. The implications of changes in the characteristics of the bacteria due to the drying process are discussed.

After an RM has been produced and tested for its stability and homogeneity, it is evaluated by other laboratories in a collaborative study. The second chapter, therefore, describes the evaluation of the *Listeria monocytogenes* RM in three European collaborative studies. The RM was evaluated both in the presence and in the absence of competitive micro-organisms. Competitive organisms were inoculated into appropriate media together with the RM either as a mixture of dried strains or as a naturally contaminated food sample.

After an RM has been successfully evaluated for stability and homogeneity and in a collaborative study, certification is the next step. Chapter 3 presents the certification study presented to the Community Bureau of Reference (BCR) for the qualitative *Salmonella typhimurium* RM and chapter 4 for the quantitative *Bacillus cereus* RM. The certification procedure, the results of the study and the certified values are presented.

The general procedure for the production of RMs and CRMs is based on the preparation of a HCMP by spray drying a bacterial culture in milk. Spray drying uses high temperatures for drying the milk, which affects the survival of the organism. An alternative procedure for the production of an HCMP, without the application of high drying temperatures, is described in chapter 5. This method is evaluated for the production of HCMPs for various organisms, most of which are either difficult or impossible to dry by means of spray drying. The HCMPs obtained were evaluated for stability and homogeneity.

Once the materials have been developed they can be used in practice. For this it is necessary to determine who the candidate users are and how they can and will use the RMs and CRMs. The various possibilities for use of RMs and CRMs are described in chapter 6. The possibilities and limitations for use are divided into routine and incidental use and a further division is made between the quantitative and qualitative RMs and CRMs. For identification of the customers and how they will use the materials a market research was conducted in collaboration with a marketing consultant bureau. The customers' needs were identified and compared to the properties of the materials (see chapter 7). The possibilities for improvement of the design quality of the materials are presented. As a result of the market research the production of RMs was established at the Foundation for the Advancement of Public Health and the Environment (SVM). Chapter 7 also describes the setting up and the reproduction quality of the RMs and CRMs.

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Production of reference materials for *Bacillus cereus* and *Listeria monocytogenes* and some of their characteristics.

ABSTRACT

Reference materials (RMs) for *Bacillus cereus* and for *Listeria monocytogenes* were developed based on spray drying of artificially contaminated milk. The spray dried powders obtained were used to prepare batches of RMs at the desired level of contamination by mixing the contaminated milk powder with sterile milk powder. Quantitative RMs containing *B. cereus* at a level of *ca* 10^4 colony forming particles (cfp) per capsule and qualitative RMs containing *Listeria monocytogenes* at a level of *ca* 5 cfp per capsule were prepared. The batches prepared were used to characterise the behaviour of the RMs in relation to the general requirements for reference materials (stability, homogeneity and representativity). For determining the representativity of the *B. cereus* RM the effects of osmotic shock and heat injury and of heat shock, storage time and lysozyme on germination were tested. For the *L. monocytogenes* RM the parameters examined included the effects of osmotic shock, heat injury, pre-warming of enrichment broth and incubation time on recovery. Both RMs fulfilled the general requirements for reference materials. Differences found in stability and the effect of heat injury and osmotic shock between the two RMs are related to the use of spores for the *B. cereus* RM. Based on the stability of the RMs at higher temperatures it is concluded that the *B. cereus* RM can be shipped to other laboratories without special precautions, but for the *L. monocytogenes* RM cooling during transport is necessary.

Parts of this chapter have been published:

- In 't Veld, P.H., Soentoro, P.S.S., Delfgou-van Asch, E.H.M. and Notermans, S.H.W. (1991) Influence of reconstitution on isolation and enumeration of *Listeria monocytogenes* from milk powder used for reference samples. *J. Food Prot.* **54 (2),** 124 - 126.
- In 't Veld, P.H., Soentoro, P.S.S. and Notermans, S.H.W. (1993) Properties of *Bacillus cereus* spores in reference materials prepared from artificially contaminated spray dried milk. *Int. J. Food Microbiol.* **20,** 23 - 36.

INTRODUCTION

The development of microbiological reference materials (RMs) at the National Institute of Public Health and the Environment has a long history. In 1965 Kampelmacher was already using artificially inoculated minced meat samples in comparative studies (Kampelmacher, 1967) as a standard sample for the evaluation of the performance of the *Salmonella* detection procedure in various laboratories. Later *Salmonella* was dried onto milk powder which resulted in the production of a reference material (gelatin capsules containing spray dried milk artificially contaminated with *Salmonella*) for use in laboratories methods trials (Beckers *et al.*, 1985). The development of other reference materials and their evaluation was initiated in 1986 when the first contract for water microbiology with the former European Communities Bureau of Reference (BCR) was agreed. A contract for the development of reference materials for food microbiology followed in 1987. These reference materials (RMs) are prepared from spray dried artificially contaminated milk. The initial spray dried powder (called highly contaminated milk powder or HCMP) is mixed with sterile milk powder to give the desired level of contamination and the mixed powder is subsequently packed into gelatin capsules.

There are two types of such reference materials. The first, the so-called qualitative RM, is intended for the evaluation of qualitative tests (detection or presence/absence procedures) while the second, the so called quantitative RM, is intended for the evaluation of quantitative tests (enumeration procedures). Reference materials must fulfil a number of general requirements (Griepink, 1989). These requirements are:

- stable within specified limits over a defined period of time.
- homogeneous within defined limits.
- representative for their intended use.

After a micro-organism is spray dried it has to survive in the dry conditions. The stability (rate of decrease in the level of contamination over time) of the micro-organism in these conditions will depend on the storage temperature and water content (or water activity) of the milk powder, the type of micro-organism and the particular strain present. Stability is of importance as the RMs must be usable for a long period of time without change in the number of micro-organisms present. In general the lower the percentage of moisture in the milk powder or the lower the relative humidity under which it is stored the better the survival (Higgingbottom, 1953; Palumbo and Williams, 1990). The same is true for the storage temperature of the milk powder (Palumbo and Williams, 1990), i.e. the lower the storage temperature the better the survival.

Most experiments related to the survival of micro-organisms in milk powder are directed either towards the rate of decrease under the normal storage conditions for milk powder or to conditions that accelerate their destruction. Chopin and co-workers (1978) observed a decrease for *Staphylococcus aureus* of between 0.5 and 2.5 $\log_{10}$ units after seven weeks storage at ambient temperature depending on the strain used and the water activity of the powder. For *E. coli* a reduction of 2 $\log_{10}$ units was observed after four weeks storage at 25 °C (Thompson *et al.*, 1978). A reduction of ≥ 3 $\log_{10}$ units during storage for 4 - 8 weeks at 45 °C or 55 °C was observed for *Salmonella* (LiCari and Potter, 1970). Both LiCari and

Potter (1970) and Chopin and co-workers (1978) observed a decrease in the rate of destruction with increasing storage time.

Homogeneity of an RM is of importance as a significant difference between counts (within or between laboratories) is detected sooner when the variation between the number of micro-organisms is smaller. The homogeneity of a spray dried RM will be influenced by the dispersion of the micro-organisms in the milk used for drying and by the mixing procedure used for diluting the HCMP to achieve the desired level of contamination of the final RM.

The spray drying process and subsequent storage of the contaminated milk powder may affect the behaviour of the micro-organism. For example, the procedure for rehydration of *Salmonella* from dried products can affect their recovery (Van Schothorst *et al.*, 1979). Changes observed in the behaviour of the test organisms must not interfere with the intended use of the RM, otherwise the representativity of the RM is affected.

This chapter will focus on the preparation and characterisation of both quantitative and qualitative RMs. For qualitative tests the RM containing *Listeria monocytogenes* is described and for quantitative tests the RM containing *Bacillus cereus*. The evaluation of representativity will focus on the behaviour of the strains after drying and not on the evaluation of the RMs in collaborative studies, this aspect is described mainly in chapter 2.

MATERIALS AND METHODS

Preparation of highly contaminated milk powder (HCMP)

Bacillus cereus RM

For the development of a reference material for *B. cereus*, a strain (identification number ATCC 9139) from the American Type Culture Collection (Rockville, USA) was used that showed typical reactions on the media used for the enumeration of *B. cereus*. In August 1990 the strain was streaked for purity on sheep blood agar (SBA) and incubated for *ca* 24 h at (37 ± 1) °C. After incubation a single colony was suspended in 10 ml peptone saline solution (PS) by mixing on a vortex mixer. This suspension was used to inoculate 10 plates (0.3 ml per plate) containing the Polymyxin pyruvate Egg yolk Mannitol Bromothymol blue Agar (PEMBA) of Holbrook and Anderson (1980) prepared from original ingredients. The PEMBA plates were used for rapid sporulation of *B. cereus* and were incubated at (37 ± 1) °C. After *ca* 24 h incubation five ml peptone saline solution (PS) were added to each plate and the cells were suspended in the fluid using a sterile glass spreader. The suspension from each plate was pipetted into a single tube and heated for 10 minutes in a waterbath at (80 ± 0.5) °C to inactivate vegetative cells.

The heated suspension was added to three litres of Friesche Vlag Halvamel (sterile milk evaporated to a dry mass concentration of 240 $g.l^{-1}$, dry fat mass concentration 40 $g.l^{-1}$), which was then spray dried using a Niro mobil minor spray dryer operated at an inlet temperature of *ca* 190 °C and an outlet temperature of *ca* 70 °C. No special precautions were taken to prevent contamination of the HCMP by the air used for drying. It was assumed that the level of contamination of the HCMP resulting from contaminated inlet air was negligible compared to the contamination level of the organism inoculated into the milk. The

HCMP thus obtained was sealed in a polyethylene bag (0.2 mm thick) and stored at 5 °C. The water activity of the powder was determined using a Novasina Thermoconstanter TH2 (Defensor, Pfäffikon, Switzerland) set at 25 °C.

The HCMP was filled into gelatin capsules (0.27 g per capsule) to determine the number of colony forming particles (cfp) per gram of the HCMP. Five capsules containing the HCMP were analysed after reconstitution and appropriate dilution of the suspension. For enumeration the capsules were reconstituted as described below:

a. test tubes (diameter 26 mm) were filled with 10 ± 0.2 ml of PS.
b. the tubes were pre-warmed in a waterbath maintained at (38 ± 0.5) °C for 30 minutes.
c. the capsules were added to the test tubes (one per tube) and mixed on a vortex mixer for a few seconds 10, 20 and 30 minutes after the addition of the capsule. The tubes were out of the waterbath for as short a time as possible.
d. after the last mixing the tubes were transferred from the waterbath to iced water.
e. the reconstituted capsule solutions were used within two hours.

0.1 ml volumes of dilutions of the reconstituted capsules were spread on each of two plates of Mannitol Egg Yolk Polymyxin agar (MEYP, Mossel *et al.*, 1967). The plates were incubated for (24 ± 2) h at (30 ± 1) °C before counting.

Listeria monocytogenes RM

For the development of a reference material for *L. monocytogenes* the Scott-A strain was chosen. This strain is a human isolate from a food-borne outbreak involving pasteurised milk (Fleming *et al.*, 1985) and was obtained from Dr. M. Doyle (University of Wisconsin-Madison, USA). Using this strain two HCMPs were produced. The first one (HCMP 2-1) was prepared in January 1988. The strain was streaked for purity on sheep blood agar and incubated for *ca* 48 hours at (37 ± 1) °C. A single colony was suspended in Tryptone Soya broth (Oxoid CM 129) and incubated for *ca* 24 hours at (37 ± 1) °C. This culture was subsequently diluted 100 times in PS and five ml of this dilution used to inoculated each of 16 Roux bottles containing 200 ml Tryptone Soya Agar (Oxoid CM 131). The Roux bottles were incubated for *ca* 24 hours at (37 ± 1) °C. The cells were harvested by carefully shaking the Roux bottles with 10 ml sterilised milk (Friesche Vlag Goudband, milk evaporated to a dry mass concentration of 320 $g.l^{-1}$, dry fat mass concentration of 90 $g.l^{-1}$) and some sterile glass beads. The suspension of organisms in milk from all the Roux bottles was transferred to a *ca* three l sterile glass bottle containing three litres of sterilised milk (mixture of 50 % Friesche Vlag Goudband and 50 % Friesche Vlag Halvamel). This final mixture was homogenised by shaking and spray dried using a Niro mobil minor spray dryer at an inlet temperature of *ca* 200 °C and an outlet temperature of *ca* 70 °C. The resulting HCMP (given the batch number 2-1) was sealed in a polyethylene bag (0.2 mm thick) and stored at 5 °C until required.

In December 1989 a second HCMP was produced. The Scott-A strain of *L. monocytogenes* was streaked for purity on sheep blood agar and incubated for *ca* 48 hours at (37 ± 1) °C. A single colony was suspended in 10 ml Brain Heart Infusion broth (BHI, Difco 0037-01-6) and incubated for *ca* 24 hours at (37 ± 1) °C. This culture was subsequently diluted in PS and one ml of a 10^{-4} dilution was inoculated into 225 ml Tryptone

Soya Broth (Oxoid CM 129) and incubated for *ca* 24 hours at (37 ± 1) °C. After incubation 0.1 ml of the Tryptone Soya Broth culture was inoculated into three l of sterilised milk (Friesche Vlag Halvamel). The milk was homogenised by shaking and spray dried using a Niro mobil minor spray dryer at an inlet temperature of *ca* 200 °C and an outlet temperature of *ca* 70 °C. The resulting HCMP (given the batch number 2-2) was left for stabilisation sealed in a polyethylene bag (0.2 mm thick) and stored at 5 °C until required.

Both HCMPs were filled into gelatin capsules (0.27 g per capsule) to determine the number of colony forming particles (cfp) per gram of the HCMP. For both HCMPs five capsules containing the HCMP were analysed after reconstitution and appropriate dilution of the suspension. The capsules were reconstituted as described for *B. cereus* and 0.1 ml volumes of dilutions of the reconstituted capsules were spread on each of two plates of Oxford agar (Curtis *et al.*, 1989). The plates were incubated for (48 ± 4) h at (30 ± 1) °C before counting.

The water activity of the powders was determined using a Novasina Thermoconstanter TH2 set at 25 °C.

Preparation of the reference materials

Bacillus cereus RM

Based on the observed number of cfp per gram of HCMP a mixing schedule was established. A 0.5 g quantity of the HCMP was mixed in steps with a total of 1.0 kg of γ-irradiated (dose 10 kGy) skim milk powder (smp; DOMO, Beilen, The Netherlands) in a Turbula type 10 b mixing apparatus (W.A. Bachofen A.G., Basel, Switzerland) using a 17 litre stainless steel drum. In the first step, 0.5 g HCMP was mixed with 2.0 g smp. In the following steps approximately equal amounts of mixed powder and smp were further mixed until a final quantity of one kg mixed powder was obtained. Each mixing step was done for one hour at room temperature. The drum used for mixing was sterilised by autoclaving at (121 ± 1) °C for 15 minutes. The final mixed powder was stored at 5 °C. Mixing commenced 24 days after production of the HCMP.

The mixed powder was distributed into size one ochre/white γ-irradiated (dose 10 kGy) gelatin capsules (Elanco Qualicaps, Fegersheim, France) using an aluminium filling apparatus in a laminar air flow cabinet, 17 g powder were distributed into 60 capsules (0.28 g per capsule). The filling apparatus was cleaned with 70 % ethanol and dried for five hours at (90 ± 5) °C. Sets of five capsules were packed together with a bag of desiccant (silica gel) into a screw capped plastic container and stored at -20 °C. Both the bag of desiccant and the container were pre-sterilised by γ-irradiation (dose 10 kGy).

Listeria monocytogenes RM

Table 1 summarises the production and use of the five batches of *L. monocytogenes* RM. Batches L-1 to L-4 were mixed in steps using approximately equal amounts of contaminated milk powder and sterile milk powder. All mixing steps were done using the Turbula mixer at room temperature for four hours each (one hour each for batch L-4). Mixing of batch L-5 was done using a mortar and pestle and the Turbula mixer as described by In 't Veld and co-workers (1996). Sterile milk product (mp; milk product no. 17 from Nestlé,

Amsterdam, The Netherlands) was used instead of smp because of the better reconstitution properties of the mp.

Table 1. Production and use of batches of the *Listeria monocytogenes* RM.

Batch code	Mixing	Used for testing
L-1[a]	0.5 g HCMP 2-1 with 1.6 kg smp[d]	osmotic shock and heat injury
L-2[a]	2.5 g batch L-1 with 1 kg smp	osmotic shock and heat injury
L-3[a]	0.8 g batch L-1 with 1 kg smp	osmotic shock and heat injury
L-4[b]	45 g HCMP 2-2 with 1,5 kg mp[e]	stability at storage temperature
L-5[c]	120 g HCMP 2-2 with 2.2 kg mp	stability test at higher temperatures, effect of pre-warming the enrichment broth and effect of incubation time on recovery

a mixing started *ca* 10 months after production of HCMP 2-1.
b mixing started *ca* 2 years after production of HCMP 2-2.
c mixing started almost 5 years after production of HCMP 2-2.
d smp = skim milk powder.
e mp = milk product no. 17.

All the final mixed powders were distributed as described for the *B. cereus* RM except that the capsules used were orange/white and only 16 g powder were distributed into 60 capsules (0.27 g per capsule). Sets of 10 or 5 capsules were packed as described for the *B. cereus* RM and stored at -20 °C.

Characterisation of the reference materials

Stability

For testing the stability of the RM two tests were carried out. The first determined stability of the RM at the storage temperature of -20 °C over a long period of time (*ca two* years) and the second (challenge test) determined stability at various temperatures over a period of *ca four* weeks.

- *Bacillus cereus* RM

Every four weeks, over a period of *ca* two years, five capsules were enumerated to demonstrate stability at the -20 °C storage temperature. For the challenge test capsules were stored at -20 °C (reference), 22 °C, 30 °C and 37 °C. Each week, over a period of four weeks, the organisms in five capsules per temperature were enumerated. For enumeration the capsules were reconstituted as described for the preparation of the HCMP. 0.1 ml volumes of the reconstituted RMs were spread on each of two plates of sheep blood agar (SBA) and two plates of MEYP (for the stability test at the -20 °C storage temperature only). All plates were incubated for (24 ± 2) h at (30 ± 1) °C before counting. Counts of *B. cereus* in the capsules were expressed as the number of cfp per 0.1 ml of reconstituted capsule. The counts obtained were $\log_e$ transformed and subsequently analysed using linear regression to

determine the regression coefficient and corresponding *t*-value. The *t*-values were used to determine whether or not the regression coefficients differed significantly from zero.

- *Listeria monocytogenes* RM

Batch L-4 was used for testing the stability at the -20 °C storage temperature. Approximately every four weeks, over a period of *ca* two years, the *L. monocytogenes* in 50 capsules were enumerated. The study began in November 1992. To determine the stability at various temperatures capsules from batch L-5 were stored at -20 °C (reference), 5 °C, 22 °C and 30 °C. Once a week, over a period of five weeks, the organisms in 25 capsules from each storage temperature were enumerated. This study began in November 1994. At the start of the study 100 capsules were enumerated.

For enumeration the procedure used was as described below:

a. dissolution of the capsules.
   Each capsule was placed in a separate Petri dish and 5 ml ± 0.5 ml of pre-warmed PS (38.5 °C) was added. All dishes were transferred directly to a basket placed at an angle of *ca* 20 degrees. The basket was then placed at an angle of *ca* 20 degrees in a shaking incubator operating at (38.5 ± 1.0) °C and the dishes incubated for (45 ± 5) minutes while shaking at *ca* 100 rpm.
b. repair of sublethally injured *Listeria.*
   To each Petri dish 5 ml ± 0.5 ml double strength Tryptone Soya Agar (TSA) was added and mixed carefully with the dissolved capsule. The plates were incubated at 30 °C for (4 ± ½) h.
c. selective growth of *Listeria.*
   To each Petri dish prepared in (b) above 10 ml ± 1 ml of double strength Oxford agar was added over the surface of the TSA. The plates were incubated further at 30 °C for (44 ± 2) h.

Counts were expressed as the number of cfp of *L. monocytogenes* in the reconstituted capsule. The counts obtained were analysed (without log transformation of the counts) using generalised linear models (McCullagh and Nelder, 1983) to determine the regression coefficients and corresponding *t*-values. For the model a Poisson distribution between counts was assumed. The *t*-values were used to determine whether the regression coefficients differed significantly from zero.

Homogeneity

The homogeneity of the RM was checked by means of two tests, both of which are based on Cochran's dispersion test statistic (Cochran, 1954). The first test, also called the T_1 test (Heisterkamp *et al.*, 1993), determines the variation between replicate samples from the same reconstituted capsule and is calculated as follows:

$$T_1 = \sum_{i=1}^{I} \sum_{j=1}^{J} \frac{\left(z_{ij} - \frac{z_{i+}}{J} \right)^2}{\frac{z_{i+}}{J}}$$

where: z_{ij} = count of sample j of capsule i.
z_{i+} = sum of counts of all replicate samples examined of capsule i.
J = number of replicate samples examined per capsule.
I = total number of capsules examined.

T_1 is approximately χ^2 -distributed with $I \bullet (J - 1)$ degrees of freedom assuming that the variation between counts from the same reconstituted capsule is Poisson distributed. The second test, called the T_2 test, determines the variation between the sums of the replicate sample counts per reconstituted capsule and is calculated as follows:

$$T_2 = \sum_{i=1}^{I} \frac{\left(z_{i+} - \frac{z_{++}}{I}\right)^2}{\frac{z_{++}}{I}}$$

where: z_{++} = sum of counts of all replicate samples examined for all capsules.

The test statistic T_2 is also approximately χ^2-distributed with $(I - 1)$ degrees of freedom. When the variation between the counts conforms to a Poisson distribution the value of T_2 divided by $(I - 1)$ equals one. Overdispersion is expected and as a criterion for acceptance of a batch of RM a value for $T_2/(I - 1) \leq 2$ is used. Both T_1 and T_2 tests are used to determine the homogeneity of the quantitative reference materials.

For the qualitative RM only a modified T_2 test is used as for this type of RM the entire capsule is used for enumeration. The formula for the T_2 test will then be calculated as follows:

$$T_2 = \sum_{i=1}^{I} \frac{\left(z_i - \frac{z_+}{I}\right)^2}{\frac{z_+}{I}}$$

where: z_i = count of capsule i.
z_+ = sum of counts of all capsules.

The homogeneity of a batch of RM is determined using the results of the stability tests. Each time capsules were enumerated for the stability test, the T_1 and/or T_2 value were calculated. The value for homogeneity of the RM is then calculated as the sum of the T_2 values of the various days divided by the total number of capsules examined minus the number of examinations (= no of test days) of the capsules. This value is also called T_{hom}/N and is used to quantify the homogeneity of a batch of RM. When the variation between the counts conforms to a Poisson distribution the value of T_{hom} does not differ significantly from a χ^2-distribution with N degrees of freedom.

Testing the representativity of the reference material

The representativity of the RM after spray drying and subsequent storage at -20 °C was determined by testing the effect of the various conditions of preparation, composition and storage of the RMs.

Bacillus cereus RM

- Osmotic shock

For this purpose, osmotic shock is defined as the difference in counts of the organism between encapsulated milk powder and free milk powder (In 't Veld et al., 1991a). For the experiments using encapsulated milk powder, 10 capsules were reconstituted according to the method described for the stability test, except that the counts were done singly. For the experiments using free milk powder the contents of 10 capsules including the opened capsules were each added to a tube (Ø 26 mm) containing 10 ml PS of 38 °C. After addition they were immediately mixed for five seconds on a vortex mixer and placed in iced water. 0.1 ml volumes of both suspensions were spread onto TSA and MEYP plates. The plates were incubated at (30 ± 1) °C and the number of colonies counted after *ca* 20 h incubation. The effect of osmotic shock was tested for significance using the *t*-test (Wardlaw, 1985) based on $\log_{10}$ transformed counts for each medium separately. The experiments were performed five months after spray drying (corresponding to four months after preparation of batch 90-2) and was repeated 3½ months later to determine the effect of storage time.

- Heat injury

Heat injury is defined as the difference in counts on a non-selective medium (TSA) and a selective one (MEYP) (In 't Veld et al., 1991a). This difference was tested for significance using the *t*-test (Wardlaw, 1985) based on $\log_{10}$ transformed counts. The results from the experiments described for testing the effect of osmotic shock were used for the *t*-test. This was done separately for encapsulated and free milk powder.

- Heat shock on germination

The effect of heat shock is defined as the difference in counts between heat treated and non heat treated reconstituted capsules. For this the contents of a capsule including the opened capsule were reconstituted as described for testing the effect of osmotic shock, except that saline solution (9.5 $g.l^{-1}$ NaCl; Merck 1540) was used for reconstitution instead of PS. Four portions of *ca* 1.5 ml were each transferred into a small polyethylene bag (0.05 mm thick), sealed and placed in iced water. The remaining suspension in the tubes was kept in iced water and used as the unheated control. The bags were subsequently heat shocked (fully submerged) for 10 minutes in a waterbath at (50 ± 0.5) °C, (60 ± 0.5) °C, (70 ± 0.5) °C and (80 ± 0.5) °C, respectively. After cooling in iced water 0.1 ml volumes of each bag were spread onto TSA and MEYP. The plates were incubated at 30 °C for 20 h. The experiment was performed with 10 capsules. The effect of heat shock was tested for significance using an one way analysis of variance (ANOVA) based on $\log_{10}$ transformed counts.

- Germination in relation to storage time

To test for germination the content of a capsule including the opened capsule was added to a tube (∅ 26 mm) containing 10 ml prewarmed saline solution at (38 ± 0.5) °C. The tubes were placed on a vortex mixer and mixed for *ca* five seconds at the highest speed. The tubes were replaced in the waterbath and incubated for up to one hour. After several time intervals the tubes were transferred to iced water and a sample of *ca* 1.5 ml was removed and placed in a polyethylene bag, sealed and put in iced water. The sealed bags were heated submerged for 10 minutes in a waterbath at 80 °C. After cooling in iced water 0.1 ml from each bag was inoculated onto a TSA plate. The plates were incubated at 30 °C for 20 h. The experiment was performed 1, 5 and 9 months after production of the HCMP to test the effect of storage time of the milk powder. Three capsules were examined for each time/temperature combination. In addition the effect of reconstitution temperature (30 °C instead of 38 °C) and the effect of reconstitution medium (PS and BHI instead of saline solution) was also determined.

- Lysozyme on germination

To determine the effect of lysozyme on germination of spores capsules were reconstituted as described for the stability test. The reconstituted capsules were inoculated onto freshly prepared TSA supplemented with 0, 10, 100 and 200 $\mu g.ml^{-1}$ lysozyme (Sigma L-6876), MEYP base (MEYP without egg yolk and polymyxin) and MEYP base supplemented with 200 $\mu g.ml^{-1}$ lysozyme. The suspension from one reconstituted capsule was used for inoculation of all the media. The tests were carried out as two experiments, 30 capsules examined in the first and 25 in the second. The effect of lysozyme was tested for significance with an one way ANOVA for results obtained using TSA and a *t*-test for MEYP-base both based on $\log_{10}$ transformed counts.

Listeria monocytogenes RM

- Osmotic shock

The effect of osmotic shock was tested using the three batches of RMs prepared from HCMP 2-1. Using batch (L-1) the same procedure was followed as described for the *B.* cereus RM, with the following exceptions: use of Oxford agar (Oxoid CM 856 + SR 140) instead of MEYP agar and incubation of the plates at (37 ± 1) °C for *ca* 48 h. Differences in counts between encapsulated milk powder and free milk powder were tested for significance using the *t*-test (Wardlaw, 1985) based on $\log_{10}$ transformed counts for each medium separately.

With batches (L-2) and (L-3) two enrichment methods were used. In the first, a two step enrichment, a capsule or free milk powder was added to 100 ml Tryptone Soya Broth (TSB, Oxoid CM 129) supplemented with 6 $g.l^{-1}$ yeast extract (Oxoid L 21) and incubated for (18 ± 2) h at (30 ± 1) °C. After incubation 0.1 ml was transferred to 10 ml of LEB medium according to the IDF standard 143 (Anon., 1990) (TSB supplemented with 6 $g.l^{-1}$ yeast extract, 10 $mg.l^{-1}$ acriflavine (Brocacef AC 463), 40 $mg.l^{-1}$ nalidixic acid (Sigma N 8878) and 50 $mg.l^{-1}$ cycloheximide (Sigma C 6255)). The LEB was incubated for (48 ± 4) h at 30 °C. *L. monocytogenes* was isolated after 24 and 48 h incubation by streaking a 10 µl loopful onto Oxford agar which was then incubated for (24 ± 2) h at 30 °C. In the second method, a one

step method, a capsule or free milk powder was added to LEB and incubated for (48 ± 4) h at 30 °C. *L. monocytogenes* was isolated after 24 and 48 h incubation by streaking a 10 µl loop onto Oxford agar.

For each method and each batch of RM 50 capsules and 50 portions of free milk powder were examined. All experiments were carried out *ca* one year after spray drying. For the enrichment experiments the osmotic shock is defined as the difference in percentage positive *Listeria* isolations between encapsulated milk powder and free milk powder. Differences in number of positive and negative isolations between encapsulated milk powder and free milk powder were tested for significance using the following χ^2-test (Wardlaw, 1985) based on a 2 x 2 contingency table:

$$\chi^2 = \frac{N\left(|(a \bullet d) - (b \bullet c)| - 1/2N\right)^2}{(a+b) \bullet (c+d) \bullet (a+c) \bullet (b+d)}$$

where: N = total number of observations.
a = positive isolations obtained with treatment 1.
b = negative isolations obtained with treatment 1.
c = positive isolations obtained with treatment 2.
d = negative isolations obtained with treatment 2.

Encapsulated milk powder and free milk powder are the two treatments in this example. All χ^2-values obtained were tested for significance at one degree of freedom (critical value is 3.8 at α = 0.05). The combination of results obtained with the two batches and two methods were also tested for a significant effect using the Cochran-Mantel-Haenszel statistics (Rothman, 1986) as calculated by the statistical programme SAS (version 6.11).

- Heat injury

Heat injury using batch L-1 is defined as the difference in counts on a non-selective medium (TSA) and a selective one (Oxford agar) and for batches L-2 and L-3 as the difference in the percentage of positive *Listeria* isolations. The difference in counts using batch L-1 was tested for significance using the *t*-test based on $\log_{10}$ transformed counts. For batches L-2 and L-3 the difference in number of positive and negative isolations were tested for significance using the χ^2-test as described for osmotic shock of *L. monocytogenes*. The results from the experiments described for testing the effect of osmotic shock were used.

- Pre-warming of the enrichment broth

The effect of the different pre-warming temperatures, 30 °C and 37 °C, of the enrichment broth on the recovery of *L. monocytogenes* was tested. Three jars containing 225 ml each of LEB and half Fraser Broth (½ FB, consisting of FB base (Oxoid CM 895) supplemented with one vial Fraser supplement (Oxoid SR 156) and ammonium-iron(III)citrate (250 mg) per litre FB base) were incubated overnight at (30 ± 1) °C and (37 ± 1) °C. One capsule was added to each pre-warmed jar and all jars were incubated further at 30 °C. After 20 and 26 hours incubation a 0.1 ml sample of each culture was spread onto Oxford agar and incubated at 37 °C for *ca* 24 h. The number of positive capsules, i.e. these producing at least one typical colony on Oxford agar, was determined. The experiment was repeated six times using LEB and four times using ½ FB. The effect of

pre-warming temperature, incubation time and type of medium was tested for significance using the Cochran-Mantel-Haenszel statistics as described for osmotic shock.

- Incubation time on recovery

The effect of incubation time on the recovery of low numbers of *L. monocytogenes* using RMs, heat treated and vital cells was tested in LEB and ½ FB.

Reference materials:

Using RMs this effect was examined by using 10 capsules per medium type. One capsule was added to each jar containing 225 ml pre-warmed (overnight incubation at 37 °C) enrichment broth and incubated at 30 °C. After 7, 24, 31 and 48 hours incubation a one ml sample was withdrawn from each jar into a sterile tube and immediately placed in iced water. Serial dilutions of each sample were made in PS and 0.1 ml of the appropriate dilution was spread, in duplicate, on Oxford agar. The number of typical colonies was counted after 24 hours incubation at 37 °C.

Heated and unheated *Listeria*:

The recovery of a heated and unheated *Listeria* culture was tested as follows: A tube containing 40 ml BHI was inoculated with *L. monocytogenes* and incubated at 30 °C for 24 hours. 0.1 ml of this 24-hour culture was inoculated into five ml fresh BHI, transferred into a polyethylene bag (0.05 mm thick), sealed and heated in a waterbath at (56 ± 0.5) °C (fully submerged) for five minutes. The remaining inoculated BHI solution in the tubes was used as unheated control. After cooling in iced water the number of uninjured and injured cfp was determined by serial diluting in PS and plating (0.1 ml spread plates) in duplicate on Brain Heart Infusion Agar (BHIA, Difco 0037 + Oxoid L11) and BHIA supplemented with 4% NaCl (Merck 1540). The number of cfp was determined after *ca* 48 hour incubation at 30 °C. The non selective medium (BHIA) supports the growth of both injured and uninjured cells, whereas the selective medium (BHIA + 4% NaCl) only supports the growth of uninjured cells.

Serial dilutions of the unheated and heated culture were made in PS and 0.5 ml of the appropriate dilution were inoculated into 225 ml LEB and 225 ml ½ FB. The inoculation was replicated twice for each enrichment broth. The incubation temperature, sampling intervals and counting procedure were the same as described for testing the RMs.

RESULTS

Bacillus cereus RM

Preparation of the highly contaminated milk powder and reference material

The identification of the strain is described in chapter 5. Due to the spray drying an almost fourfold decrease occurred in the number of viable spores. The contamination level of the milk before spray drying was 7.6×10^7 cfp.g^{-1} dry weight of the milk compared to 2.0×10^7 cfp.g^{-1} dry weight of the milk powder after drying. The water content of the HCMP was 3.7 % (w/w) corresponding to a measured water activity of 0.25. The water activity of the sterile skim milk powder used for mixing was 0.21.

Characterisation of the reference material

- Stability tests

The results of the stability test on MEYP at the -20 °C storage temperature over 101 weeks are presented in Figure 1 as geometric means of the number of cfp per 0.1 ml sample, including the 95 % confidence limits. Statistical analysis of the data showed that for MEYP as well as for SBA no significant increase or decrease could be detected. The results of the stability test at higher temperatures are presented in Figure 2. Statistical analysis showed that at none of the temperatures tested did a significant increase or decrease occurred. The regression coefficients and corresponding *t*-values for both stability tests (at the storage and higher temperatures) are presented in Table 2.

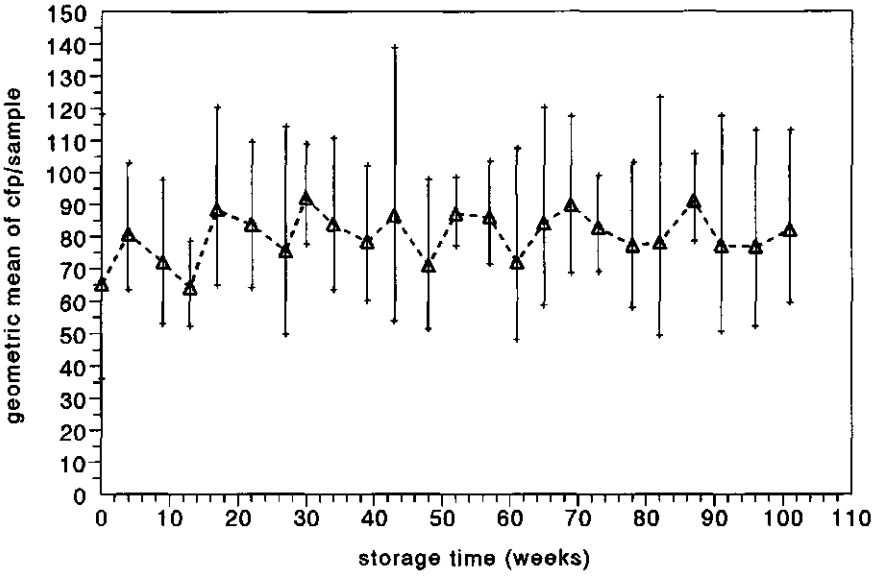

Figure 1. Level of *B. cereus* in the RM as indicated by the geometric mean number of cfp per sample and 95% confidence limits on MEYP in relation to storage time at -20 °C.

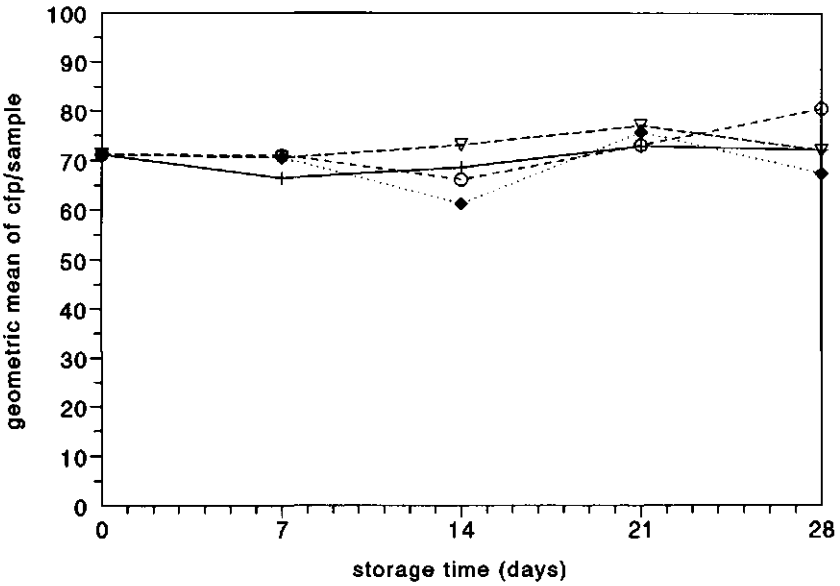

Figure 2. Level of *B. cereus* in the RM as indicated by the geometric mean number of cfp per sample on SBA in relation to storage time at various temperatures (-20 °C +, 22 °C ∇, 30 °C O, 37 °C ♦).

Table 2. Regression coefficients and corresponding *t*-values for *B. cereus* stability tests.

Storage temperature	Counting medium	Regression coefficient[e]	t-value
-20 °C[a]	SBA[c]	0.000034	0.57
	MEYP[d]	0.000130	1.85
-20 °C[b]	SBA	0.001448	0.68
22 °C[b]	SBA	0.003078	1.45
30 °C[b]	SBA	0.002899	1.36
37 °C[b]	SBA	- 0.000094	0.04

a stability test at the -20 °C storage temperature (over a period of 101 weeks).
b stability test at higher temperatures (over a period of 4 weeks).
c SBA = Sheep blood agar.
d MEYP = Mannitol Egg Yolk Polymyxin agar.
e regression coefficient = rate of change in cfp/sample per day based on $\log_e$ transformed counts.

- *Homogeneity tests*

The stability test at -20 °C consisted of 24 sets of observations on five reconstituted capsules, examined in duplicate on MEYP and SBA. For each set of observations on MEYP or SBA, the corresponding T_1 and T_2 values were calculated. In only one case did the T_1-test indicate a significant deviation from the Poisson distribution. This indicates that, on average, the variation in the number of viable spores within one reconstituted capsule was not different from a Poisson distribution. The T_2-test indicated more frequently that the variation between capsules was significantly greater than would be expected for a Poisson distribution, on 16 out of 24 times on MEYP and on 9 out of 24 times on SBA. In six cases, the T_2-test was significant on both media. The homogeneity parameter of the batch (T_{hom}/N) was estimated as 3.0 on MEYP and 2.6 on SBA based on all 24 sets of observations. The values for the homogeneity parameter indicate that the variation of the number of viable spores between capsules was higher than expected for a Poisson distribution (on average by a factor of 2.6 on SBA).

Testing the representativity of the reference material

The strain used for the preparation of the RM was regarded as representative for *Bacillus cereus* as it:

- showed typical growth on MEYP agar.
- showed typical growth and rapid sporulation on PEMBA agar.
- showed typical reactions using the confirmation tests described by ISO 7932 (Anon., 1993a).
- belonged to an official culture collection (ATCC).

After spray drying and subsequent storage the representativity was tested further for the effect of the following conditions:

- Osmotic shock

The results of the experiments are presented in Table 3. No significant difference (critical *t*-value: 2.10 at $\alpha = 0.05$) could be detected between the free and encapsulated milk powder on both TSA (*t*-test values after 5 and 8½ months, 1.47 and 0.69, respectively) and MEYP (*t*-test values after 5 and 8½ months, 0.91 and 0.87, respectively).

- Heat injury

The results of the experiments are presented in Table 3. No significant difference (critical *t*-value: 2.10 at $\alpha = 0.05$) could be detected between the counts on TSA and MEYP for both free (*t*-test values after 5 and 8½ months, 0.19 and 0.80, respectively) and encapsulated milk powder (*t*-test values after 5 and 8½ months, 0.58 and 1.08, respectively).

- Heat shock on germination

The effect of heat shock (10 minutes at 50 °C, 60 °C, 70 °C and 80 °C) on the germination of the spores was examined after storage of the spray dried milk for five months. The results of the experiments are presented in Table 4. No significant effect ($\alpha = 0.05$) of the temperature for the heat treatment on both TSA (F-value 2.4, $p = 0.06$) and MEYP (F-value 0.67, $p = 0.61$) was detected.

Table 3. Geometric mean, mean log_{10} and log_{10} standard deviation of the number of cfp per sample of *B. cereus* counted on MEYP and TSA for encapsulated and free milk powder in relation to the time after spray drying.

Time after drying		Encapsulated milk powder[d]		Free milk powder[e]	
		TSA	MEYP	TSA	MEYP
5 months	geom. mean[a]	98.2	102.4	110.0	111.9
	mean log_{10} [b]	1.99	2.01	2.04	2.05
	log_{10} s.d.[c]	0.078	0.109	0.091	0.075
8½ months	geom. mean[a]	75.9	82.1	81.1	87.4
	mean log_{10} [b]	1.88	1.91	1.91	1.94
	log_{10} s.d.[c]	0.076	0.063	0.105	0.073

a geom. mean = geometric mean 10 values.
b mean log_{10} = mean of log transformed counts.
c log_{10} s.d. = standard deviation of log transformed counts.
d encapsulated milk powder = capsule reconstituted in 10 ml PS at 38 °C for 30 minutes.
e free milk powder = capsule opened and reconstituted in 10 ml PS at 38 °C by mixing for 5 seconds.

Table 4. Geometric mean, mean log_{10}, log_{10} standard deviation of the number of cfp per 0.1 ml volume of the reconstituted RM of *B. cereus* after heat shock counted on MEYP and TSA.

Heat treatment (10 min at)		TSA	MEYP
control	geom. mean[a]	74.1	81.9
	mean log_{10} [b]	1.67	1.91
	log_{10} s.d.[c]	0.085	0.098
50 °C	geom. mean[a]	67.4	78.3
	mean log_{10} [b]	1.83	1.89
	log_{10} s.d.[c]	0.040	0.103
60 °C	geom. mean[a]	85.5	80.9
	mean log_{10} [b]	1.93	1.91
	log_{10} s.d.[c]	0.055	0.065
70 °C	geom. mean[a]	73.5	83.6
	mean log_{10} [b]	1.87	1.92
	log_{10} s.d.[c]	0.065	0.048
80 °C	geom. mean[a]	78.3	75.3
	mean log_{10} [b]	1.69	1.68
	log_{10} s.d.[c]	0.110	0.060

a geom. mean = geometric mean of 10 values.
b mean log_{10} = mean of log transformed counts.
c log_{10} s.d. = standard deviation of log transformed counts.

- Germination temperature

The rate of germination of spores at 38 °C depended on the storage time of the milk powder (see Figure 3). One month after spray drying more than 90 % of the spores germinated within 30 minutes, 8 months later less than 10 % of the spores germinated in the same period. Comparable results were obtained both at 38 °C and at 30 °C (the latter results are not presented). Germination of the spores also depended on the type of medium used for reconstitution of the milk powder. The results of the experiments performed nine months after spray drying are presented in Figure 4. The number of spores present in saline solution directly after reconstitution (0 h) is used as reference (100 % spores).

- Lysozyme on germination

Table 5 presents the effect of the addition of lysozyme (up to 200 $\mu g.ml^{-1}$) to TSA and MEYP base. No significant effect ($\alpha < 0.05$) of the addition of lysozyme to TSA (F-value 0.29, $p = 0.84$) and to the MEYP base (F-value 1.74, $p = 0.09$) could be detected.

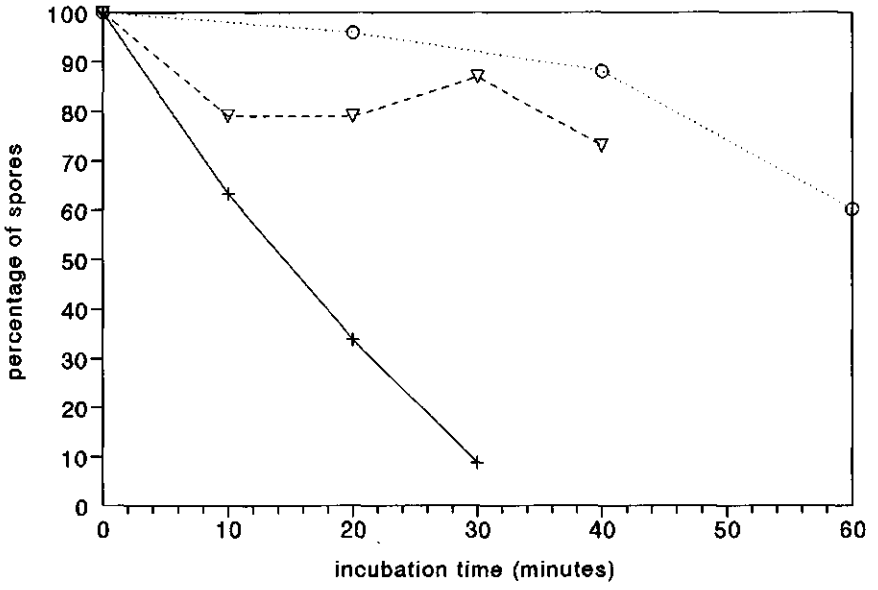

Figure 3. Germination of *B. cereus* in saline solution at 38 °C in relation to reconstitution time and storage time of the HCMP (1 month +, 5 months ∇, 9 months O).

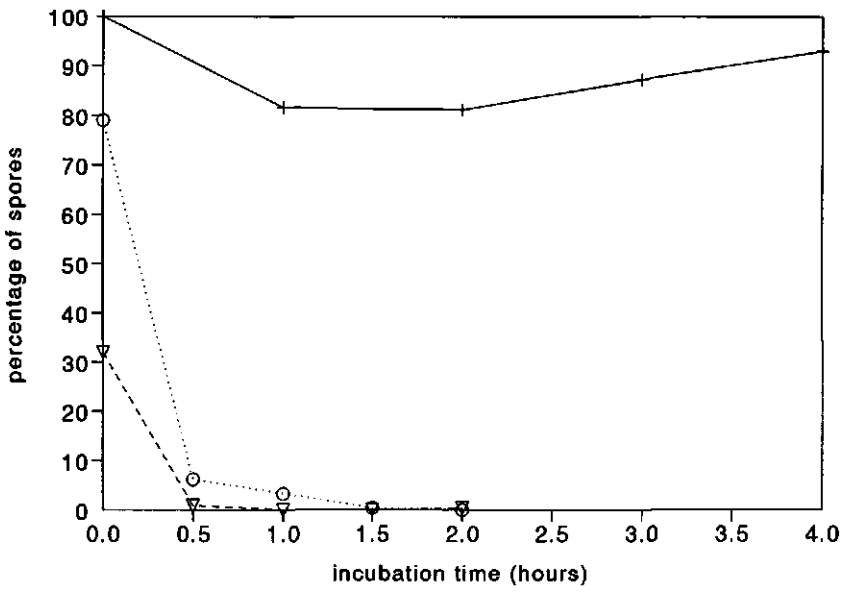

Figure 4. Germination of *B. cereus* at 38 °C in relation to reconstitution time and the type of reconstitution medium (saline +, BHI ∇, peptone saline solution O).

Table 5. Geometric mean, mean log_{10} and log_{10} standard deviation of the number of cfp per sample of *B. cereus* counted on MEYP base and TSA and the effect of lysozyme.

Medium	Lysozyme added ($\mu g.ml^{-1}$)	Geom. mean[d]	Mean log_{10} [e]	Log_{10} s.d.[f]
TSA	**0**[b]	79.5	1.90	0.057
	10[b]	81.7	1.91	0.049
	100[b]	81.4	1.91	0.058
	200[b]	80.7	1.91	0.056
MEYP-base[a]	**0**[c]	74.8	1.87	0.077
	200[c]	81.4	1.91	0.073

a MEYP-base = MEYP without egg yolk and polymyxin added.
b 30 capsules used in experiment.
c 25 capsules used in experiment.
d geom. mean = geometric mean.
e mean log_{10} = mean of log_{10} transformed counts.
f log_{10} s.d. = standard deviation of log_{10} transformed counts.

***Listeria monocytogenes* RM**

Preparation of the highly contaminated milk powder and reference materials

The strain exhibits the typical biochemical reaction pattern for *L. monocytogenes*. Results of these test were presented by In 't Veld and co-workers (1996). The serotype of this strain was 4 b and with phagetyping (according to the procedure of Rocourt and co-workers (1985)) it reacted with phage 2425A only.

For the production of HCMP 2-1 milk was used which had a contamination level of *ca* 3×10^9 cfp.g^{-1} dry weight. The HCMP itself had a contamination level of *ca* 7×10^7 cfp.g^{-1} dry weight (as determined one week after drying). The water activity of the powder was 0.20. For the production of HCMP 2-2 milk was used which had a contamination level of *ca* 4×10^5 cfp.g^{-1} dry weight of the milk, the HCMP itself contained *ca* 2×10^3 cfp.g^{-1} dry weight (as determined five weeks after drying). The water activity of this HCMP was 0.28. A difference in survival of *L. monocytogenes* between both HCMPs was found, which is probably related to the difference in water activity and the storage time of the HCMPs before the level of contamination was determined.

Characterisation of the reference materials

- Stability tests

The results of the stability test at the -20 °C storage temperature over a period of 96 weeks are presented in Figure 5. The results are presented as the arithmetic mean of the number of the cfp per capsule, including the 95 % confidence limits. Statistical analysis showed that the contamination level did not change significantly over the period tested. The results of the stability test at higher temperatures (challenge test) are presented in Figure 6. At 30 °C the results did not meet the linear assumption, so no regression coefficient was determined. At 5 °C and 22 °C a significant decrease in the mean number of cfp per capsule was observed of 0.47 % and 1.62 % per day respectively. The regression coefficients and corresponding *t*-values for both stability tests (at storage and higher temperatures) are presented in Table 6.

- Homogeneity tests

The stability test at -20 °C consisted of 19 sets of observations on 50 reconstituted capsules each. For each set of observations, the corresponding T_2 and $T_2/(I\text{-}1)$ values were calculated. None of the $T_2/(I\text{-}1)$ values, ranging between 0.66 and 1.25, was significantly greater than expected for a Poisson distribution. The homogeneity parameter of the batch (T_{hom}/N) based on all sets of observations was 0.96. This value indicates that the variation in number of cfp between capsules is not different from a Poisson distribution.

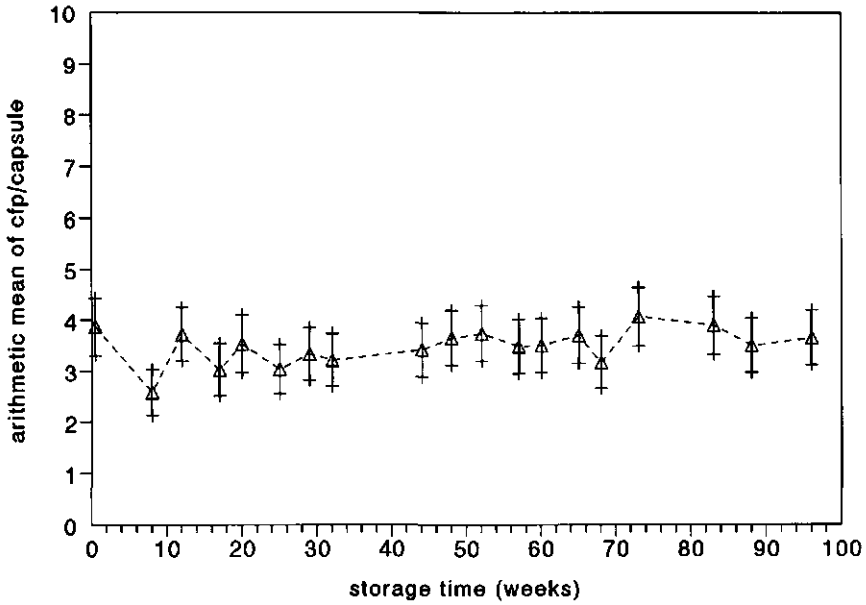

Figure 5. Level of *L. monocytogenes* in the RM as indicated by the arithmetic mean number of cfp per capsule and 95 % confidence limits in relation to storage time at -20 °C.

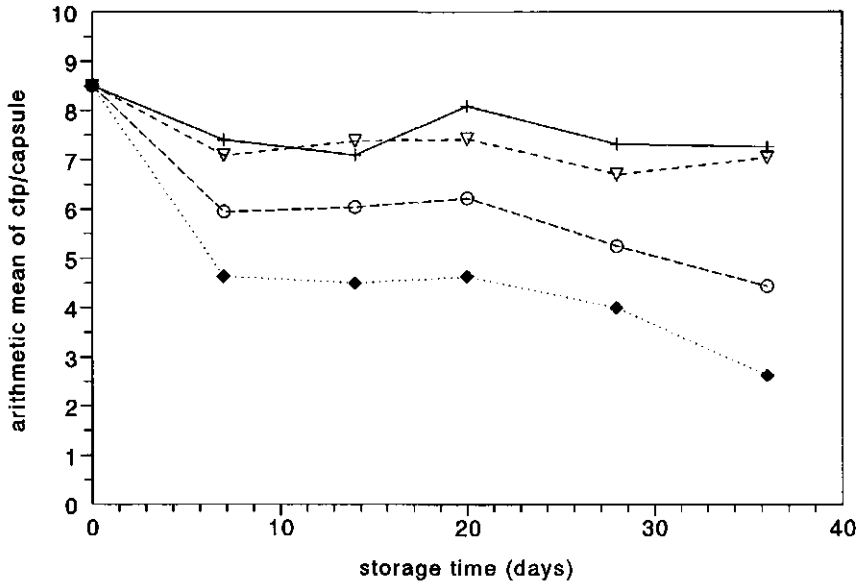

Figure 6. Level of *L. monocytogenes* in the RM as indicated by the arithmetic mean number of cfp per capsule in relation to storage time at various temperatures (-20 °C +, 5 °C ∇, 22 °C O, 30 °C ♦).

Table 6. Regression coefficients and corresponding *t*-values for *L. monocytogenes* stability tests.

Storage temperature	Regression coefficient[c]	t-value
- 20 °C[a]	0.00024	1.9
- 20 °C[b]	- 0.0029	1.5
5 °C[b]	- 0.0047	2.5[d]
22 °C[b]	- 0.0162	7.5[d]
30 °C[b]	non linear	n.d.[e]

a Stability test of batch L-4 at storage temperature (over a period of 96 weeks).
b Stability test (challenge test) of batch L-5 at higher temperatures (over a period of 5 weeks).
c regression coefficient = rate of change as the fraction of cfp per day.
d significant *t*-value ($p < 0.05$).
e n.d. = not determined.

Testing the representativity of the reference material

The strain used for the preparation of the RM was regarded as representative for *Listeria monocytogenes* as:

- it originated from food borne outbreak.
- it showed typical growth on Oxford agar.
- it showed typical reactions using the confirmation tests described by IDF (Anon., 1990).

After spray drying and subsequent storage at 5 °C the representativity was tested further for the effect of the following conditions:

- Osmotic shock

The results of the experiments using batch L-1 are presented in Table 7. Highly significant differences ($p < 0.001$) could be detected between the free and encapsulated milk powder on both TSA (*t*-test value 11.0) and Oxford agar (*t*-test value 12.8). Table 8 presents the results of the experiments using batches L-2 and L-3. A significant ($p = 0.036$) effect of osmotic shock was found for the combination of method 1 and batch L-2 (χ^2-test value 4.4). For the other three combinations of batch and method the effect was not significant ($p > 0.05$). Based on all results (irrespective of the batch and method used) a significant ($p = 0.024$) effect of osmotic shock was found (Cochran-Mantel-Haenszel statistic value 5.1).

- Heat injury

The results of the experiments using batch L-1 are also included in Table 7. Highly significant differences ($p < 0.001$) could be detected between the counts on TSA and Oxford agar for both free (*t*-test value 6.7) and encapsulated milk powder (*t*-test value 7.0). The results of the experiments using batches L-2 and L-3 are included in Table 8. Also for the detection of *L. monocytogenes* a highly significant ($p < 0.001$) difference in the number of positive isolations between method 1 (including pre-enrichment) and method 2 was found (Cochran-Mantel-

Haenszel statistic value 113). The significant difference was found for all combinations of the batches and encapsulated or free milk powder (χ^2-test values > 18).

Table 7. Geometric mean, mean $\log_{10}$ and $\log_{10}$ standard deviation of number of cfp per 0.1 ml volume of reconstituted RM of *L. monocytogenes* counted on Oxford agar and TSA for encapsulated and free milk powder.

	Encapsulated milk powder[d]		Free milk powder[e]	
	TSA	Oxford agar	TSA	Oxford agar
geom. mean[a]	61.7	43.7	23.4	8.3
mean $\log_{10}$ [b]	1.79	1.64	1.37	0.92
$\log_{10}$ s.d.[c]	0.05	0.05	0.11	0.17

a geom. mean = geometric mean of 10 values.
b mean $\log_{10}$ = mean of log transformed counts.
c $\log_{10}$ s.d. = standard deviation of log transformed counts.
d encapsulated milk powder = capsule reconstituted in 10 ml PS at 38°C for 30 minutes.
e free milk powder = capsule opened and reconstituted in 10 ml PS at 38°C by mixing for 5 seconds.

Table 8. Number of positive *L. monocytogenes* isolations (out of 50 capsules examined) for encapsulated and free milk powder using two different enrichment methods and two different batches of RM .

	Encapsulated milk powder[c]		Free milk powder[d]	
	method 1[e]	method 2[f]	method 1[e]	method 2[f]
batch L-2[a]	49[g] (98 %)[h]	28 (56 %)	42 (84 %)	20 (40 %)
batch L-3[b]	32 (64 %)	6 (12 %)	33 (66 %)	2 (4 %)

a mean level 16.2 cfp/capsule (based on the enumeration of 50 capsules).
b mean level 3.0 cfp/capsule (based on the enumeration of 50 capsules).
c encapsulated milk powder = capsule reconstituted in 10 ml PS at 38°C for 30 minutes.
d free milk powder = capsule opened and reconstituted in 10 ml PS at 38°C by mixing for 5 seconds.
e method 1 = pre-enrichment in TSBY for 18 h at 30 °C, followed by enrichment in LEB for 24 h and 48 h at 30 °C and isolation on Oxford agar.
f method 2 = enrichment in LEB for 24 h and 48 h at 30 °C and isolation on Oxford agar.
g number of positive isolations.
h percentage of positive isolations.

- Pre-warming of the enrichment broth

Table 9 presents the number of positive isolations after 22 h and 26 h incubation in LEB and ½ FB in relation to the pre-warming temperature (30 °C and 37 °C) of the broth. The results were obtained using batch L-5. A highly significant difference (p = 0.007) in the number of positive isolations was found between pre-warming at 30 °C and 37 °C (Cochran-

Mantel-Haenszel statistic value 7.2). However, the effect of pre-warming was medium related. For LEB a significant effect was found (Cochran-Mantel-Haenszel statistic value 8.2, p = 0.004), for ½ FB the effect was not significant (Cochran-Mantel-Haenszel statistic value 0.93, p = 0.34). No significant effects of incubation time (Cochran-Mantel-Haenszel statistic value 3.5, p = 0.06) or medium (LEB and ½ FB) (Cochran-Mantel-Haenszel statistic value 2.6, p = 0.11) were found.

Table 9. Number of positive *Listeria* isolations after 22 and 26 h incubation at 30 °C in LEB and ½ FB in relation to pre-warming of the broth.

Enrichment broth and incubation time		**Pre-warming temperature**	
		30 °C	**37 °C**
LEB	22 h	11/18[a] (56 %)	16/17 (95 %)
	26 h	15/18 (83 %)	17/17 (100 %)
½ FB	22 h	7/12 (58 %)	9/12 (67 %)
	26 h	9/12 (75 %)	10/12 (83 %)

a *number of positive isolations/total number of capsules examined.*

- Incubation time on recovery

The effect of incubation time on recovery using batch L-5 of the *Listeria* RM was tested in LEB and ½ FB and compared to the recovery of a heat treated culture (5 min at 56 °C) and non heat treated culture of the same *Listeria* strain. The level reached in the BHI used for heat treatment was $\log_{10}$ 9.2. After heating the level decreased to log 4.9 as counted on BHIA or log 1.8 as counted on BHIA supplemented with four % NaCl. The results of the recovery experiments are presented in Table 10. The levels of *L. monocytogenes* obtained with the heat treated culture were *ca* 2 $\log_{10}$ units lower than the level obtained with the RM. Comparable levels after 48 h incubation were obtained for the culture and RMs, indicating that maximum levels of growth were achieved within 48 h. Differences in growth between LEB and ½ FB were not observed, indicating that both media support growth of vital and sublethally injured *L. monocytogenes* equally well.

Table 10. Level of *L. monocytogenes* (log_{10} cfp.ml^{-1}) after 0, 7, 24, 31 and 48 h incubation at 30 °C in LEB and ½ FB broth inoculated with a culture, heat treated culture (5 min at 56 °C) and reference material.

Incubation time	Enrichment broth	Culture	Heat treated culture	RM
0 h	**LEB**	0.3	0.0[a]	-1.4
	½ FB	0.3	0.0	-1.4
7 h	**LEB**	2.0	<1	<1
	½ FB	2.0	<1	<1
24 h	**LEB**	6.3	<2	3.0
	½ FB	6.7	<2	3.2
31 h	**LEB**	7.5	2.7	4.6
	½ FB	7.4	3.0	5.2
48 h	**LEB**	9.1	6.7	8.2
	½ FB	8.3	6.6	8.7

a level added based on counts on BHIA.

DISCUSSION

The requirement for stability of reference materials is, for the RIVM/BCR microbiological RMs, met by spray drying of the micro-organisms. The use of spores of *B. cereus* for the preparation of an RM has additional advantages that are related to the resistance of spores against adverse conditions. This advantage is demonstrated in the stability of the material tested. Two stability tests were carried out, firstly at storage temperature (-20 °C) and secondly at higher temperatures to simulate the effect of temperature during shipment (also called challenge test). At none of the time/temperature combinations tested was a significant decrease in level of contamination detected. Thompson and co-workers (1978) reported the survival of *B. subtilis* after spray drying of milk and subsequent storage. Only the spores of *B. subtilis* survived the spray drying process but during storage of the powder the number of spores slowly decreased (*ca* 0.06 log_{10} units after four weeks and *ca* 0.2 log_{10} units after 32 weeks at 25 °C). For the *B. cereus* RM a decrease of 0.009 log_{10} units is expected after 32 weeks storage at 37 °C, based on the regression coefficient found in the challenge test.

For the *L. monocytogenes* RM the same stability tests were done but produced quite different results. Stability at storage temperature was good, but the stability decreased rapidly when the storage temperature was increased. Even at 5 °C a significant decrease in the level was observed. A temperature of 5 °C was used instead of the 37 °C used for testing the *B. cereus* RM as a limited stability at higher temperatures of the *L. monocytogenes* RM was expected. Doyle and co-workers (1985) studied the survival of two strains of *L. monocytogenes* stored at 25 °C. Four weeks after spray drying they observed a decrease of between 0.3 – 0.9 log_{10} units (depending on the type of milk and experimental trial) for the Scott-A strain and between 2.3 and 3.8 log units for the V-7 strain. The *L. monocytogenes* RM, prepared from the Scott-A strain, showed a rate of decrease of

0.35 log units (based on the regression coefficient at 22 °C and four weeks of storage) that is comparable to the results obtained by Doyle and co-workers (1985) for the same strain.

The *L. monocytogenes* RM needed a very long time for the HCMP to stabilise. The experiments presented were started almost three years after spray drying. Several stability tests started earlier did not give satisfactory results. This observation is in accordance with LiCari and Potter (1970) and Chopin (1978) who found a decrease in the die-off rate with increasing storage time for *Salmonella* and *S. aureus* respectively.

Based on the stability of the RM at higher temperatures transport conditions must be chosen that will have no influence on the RM. The *B. cereus* RM can be shipped without special precautions using (air)mail, but for the *L. monocytogenes* RM limits for time and temperature have to be set. The *Listeria* RM is shipped in a polystyrene box with a cold pack (frozen at -18 °C) and the transport time is limited to one week.

A second requirement for an RM is that it should be homogeneous. The homogeneity of microbiological RMs, prepared by spray drying and dilution of the HCMP obtained to achieve the desired level of contamination, is influenced by two factors. The first factor is the homogeneity of the HCMP itself and the second is the mixing process. The HCMP is produced from concentrated milk containing the target organism and, therefore, a homogeneous distribution of the micro-organism in this milk is essential. This, however, can be achieved relatively easily by mixing or stirring the contaminated milk and is, therefore, not regarded as a critical factor. Care should be taken to avoid large clumps of organisms as, for example, can be found after suspending pellets of organisms obtained by centrifugation or lumps of medium after harvesting organisms grown on solid media. In addition to having an adverse effect on homogeneity these can also clog the nozzle of the spray dryer.

The mixing procedure for the milk powder is more critical in relation to the homogeneity of the RM. The effect of the mixing procedure on the homogeneity of a batch of RM is illustrated by the following: The *L. monocytogenes* HCMP 2-1 was used for the preparation of three batches (L-1, L-2 and L-3) at various contamination levels (see also Table 1). For batch L-1 the HCMP was diluted 3,200 times, while batches L-2 and L-3 were diluted *ca* 10^6 times (one gram HCMP could be diluted to *ca* 10^3 kg mixed powder). Batches prepared from this HCMP, such as L-1, showed a much better homogeneity. The batch of RM (level 6×10^3 cfp per capsule) used for comparison of the recovery of *L. monocytogenes* on Oxford agar and PALCAMY (In 't Veld and de Boer, 1991b) had a $T_2/(l\text{-}1)$ of 3.1 (a value of one is expected in the case of a Poisson distribution). Batches, such as L-2 and L-3, prepared at a low level from the same HCMP showed much poorer homogeneity. The batch of RM used for the first *L. monocytogenes* comparative study (see also chapter 2) had a mean level of contamination of 11.6 cfp per capsule and a $T_2/(l\text{-}1)$ of 7.9, based on the enumeration of 99 capsules. A second batch of HCMP (HCMP 2-2) containing *ca* 4×10^2 cfp.g^{-1} was produced. Using this HCMP, homogeneous powder at a low level of contamination could be produced (batch L-4 had a T_{hom}/N value of 0.96). So, a high dilution factor of the HCMP adversely effects the homogeneity of the mixed powder.

Mooijman and co-workers (1995) showed that mixing in steps with a ratio of contaminated powder to sterile powder of 1 : 1 using a mortar and pestle improved the homogeneity of the batch. The use of a mortar and pestle also shortened the time needed

for the preparation of a batch. Each mixing step in the mortar takes *ca* one minute, mixing times using the Turbula mixer varied from 30 minutes up to four hours. The mortar can only be used with a total amount of mixed powder up to *ca* 500 gram, above that amount the Turbula mixer must be used. The improvement in homogeneity using the mortar is demonstrated for the *B. cereus* RM. A batch for certification (see chapter 4) was prepared (T_{hom}/N value on MEYP 0.71) which showed a better homogeneity (T_{hom}/N of 3.0 on MEYP) than the batch prepared using the Turbula mixer described in this chapter.

A third requirement for an RM is the representativity of the material. The behaviour of the strain after drying and storage was therefore evaluated. Due to the drying process the cells become subléthally injured by the high temperatures used for drying (heat injury) and by the drying process itself (osmotic injury). The lack of effect of heat injury and/or osmotic shock for the *B. cereus* RM is due to the use of spores in the RM. Thus the lack of effect of osmotic shock in particular can lead to a simplified use of the RM. For bacteria that are sensitive to osmotic shock a slow reconstitution of the milk powder (Van Schothorst *et al.*, 1979) and the use of gelatin capsules for the RMs (Beckers *et al.*, 1985) are essential for preventing death due to osmotic shock. Normally it takes 30 minutes to reconstitute a quantitative RM. For the *B. cereus* RM the contents of one capsule and the capsule parts can be added to the tube containing the PS and mixed for *ca* 5 seconds on a vortex mixer. This rapid reconstitution procedure was evaluated in a collaborative study (In 't Veld *et al.*, 1993). No significant difference between the normal (30 minutes at 38 °C) and the rapid reconstitution procedure could be found. However, a general remark from the participants indicated that opening of the capsule was difficult and easily led to spilling of the milk powder. It was, therefore, decided to advise the users of the RM not to open the capsule but to use the general reconstitution procedure for the quantitative RMs.

For the *L. monocytogenes* RM significant effects could be observed due to heat injury and osmotic shock (Tables 7 and 8). For heat injury significant effects were observed for both enumeration (using batch L-1) and detection (using batches L-2 and L-3) methods. The effect of osmotic shock was less for detection methods than for enumeration methods. For enumeration a highly significant difference was found, but for detection a significant difference was found when the results were tested irrespective of the batch and method used and for the combination of method 1 and batch L-2. The difference in effect of osmotic shock between enumeration and detection methods depends on the way the results are expressed (counts versus present or absent). A twofold decrease in the level is not similar to a twofold decrease in the number of positive isolations, as in principle only one organism should survive the effect of osmotic shock in order to give a positive detection result. Beckers and co-workers (1985) found a decrease in the percentage of positive *Salmonella* isolations from 100 % to 18 % using a batch of RM at a level of 6.5 cfp per capsule. The effect of osmotic shock for the *L. monocytogenes* RM was less pronounced than for the *Salmonella* RM. This difference might be related to a difference in cell wall composition between Gram positive and negative strains.

For germination of spores of some types of *Bacillus* and *Clostridium* strains a heat shock is necessary (Keynan *et al.*, 1964). The germination of the spores of the *B. cereus*

strain used for preparation of the RM was not increased by heat shock (Table 4). For these experiments saline solution was used as reconstitution medium. With this medium an effect on the rate of germination was found in relation to the time after spray drying. The longer the time after drying the slower the germination rate, indicating that the spores were becoming more dormant. Using other, nutrient richer, media (PS and BHI) as reconstitution media, the rate of germination was restored. Lysozyme can also affect the germination of spores. Suzuki and Rode (1969) found that spores of *B. megaterium* were affected by the action of lysozyme. However, lysozyme added in concentrations up to 200 $\mu g.ml^{-1}$ to TSA and MEYP did not affect the count of spores from the reference material.

The use of a non selective pre-enrichment was beneficial for the detection of *L. monocytogenes*. Based on the results presented in Table 8 a highly significant difference between the number of positive isolations between methods 1 (including pre-enrichment) and 2 (direct selective enrichment) was observed. However, the use of enrichment in a non selective broth is routine use for the detection of *Salmonella* (Anon., 1993b) but not for *L. monocytogenes* (Warburton *et al.*, 1992). The growth of *Listeria* in LEB and ½ FB using the RM or a heat treated culture is retarded in relation to the growth of a diluted culture (see Table 10). After 24 h incubation particularly the number of *Listeria* originating from the RM or heat-treated culture were at least 3 $\log_{10}$ units lower compared to the diluted culture. The growth of *Listeria* from the RM was better than for the heat-treated culture. The poor growth of injured *Listeria* cells in selective enrichment broths after 24 h incubation is confirmed by Lammerding and Doyle (1989), Bailey and co-workers (1989) and Wang and Hitchins (1994). Wang and Hitchins (1994) stated that severely damaged cells required at least 20 h to recover in TSB supplemented with yeast extract and even longer when selective agents were added. The tests carried out by Bailey and co-workers (1989) and as described in this chapter were performed without the addition of food samples. The addition of food samples will effect the recovery of *Listeria* even further. One of the problems is the decrease in pH of the medium, especially when it is not buffered as is the case for the LEB medium (Lammerding and Doyle, 1989). The effect of the addition of competitive micro-organisms is also described in chapter 2 .

The procedures for the detection of *L. monocytogenes* currently in use are all based on enrichment at a temperature of 30 °C. For good reconstitution of the gelatin capsule a temperature of *ca* 37 °C is needed. Table 9 showed that the pre-warming temperature of the broth has a highly significant effect on the number of positive isolations after *ca* 24 h incubation using LEB. It is not clear why the effect of pre-warming was not significant when using ½ FB, although for this broth pre-warming also resulted in more positives. The effect of pre-warming will, most likely, disappear when the incubation time is prolonged to 48 h. So the effect of pre- warming will be of importance when the incubation time of the (first) enrichment broth is not longer than 24 h as recommended for example in ISO 11290-1 for the detection of *Listeria monocytogenes* (Anon., 1997)

CONCLUSIONS

- Reference materials for *B. cereus* and *L. monocytogenes* can be produced that meet the criteria for homogeneity, stability and representativity.
- The procedure used for mixing the HCMP with the uncontaminated milk powder is critical for a homogeneous end product. Mixing in steps and the use of a mortar and pestle improve the homogeneity of the RM.
- The stability of the *L. monocytogenes* RM is not as good as that of the *B. cereus* RM. The rapid decrease in stability with a rise in storage temperature of the *L. monocytogenes* RM means that attention must be paid to the shipment conditions of this RM.
- The behaviour of the *L. monocytogenes* strain after drying was changed with respect to the occurrence of osmotic shock and heat injury. Osmotic shock is prevented by the use of gelatin capsules. The occurrence of heat injury requires the use of a resuscitation step in the enrichment procedure. Direct selective enrichment for the detection of *L. monocytogenes* with only a 24 h incubation period may be too short.
- For the *B. cereus* RM there were no apparent changes in behaviour of the strain after drying.

ACKNOWLEDGEMENTS

I wish to thank the Food Hygiene Laboratory of the Central Public Health Laboratory in London for their identification of *Bacillus cereus* and the *Listeria monocytogenes* strains.

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Potential use of microbiological reference materials for the evaluation of detection methods for *Listeria monocytogenes* and the effect of competitors: a collaborative study.

ABSTRACT

Reference materials (RMs) for *Listeria monocytogenes* were developed and tested for their suitability in three European collaborative studies. In the first study, the 40 participants each tested 20 RMs. In the second, the 42 participants each tested 25 RMs in combination with RMs containing competitive micro-organisms. In the third study, the 30 participants each tested a variable number of RMs in combination with food products. In all studies, RMs with *ca* 5 colony forming units (cfu) per capsule were used. In the third study RMs at two levels were used containing *ca* 5 and *ca* 100 cfu per capsule. Based on the number of cfu per capsule or the fraction of RMs not containing *L. monocytogenes*, the intended isolation frequency was calculated. All participants tested the materials according to their own procedures. These non-standard methods (NSMs) were divisible into six groups of comparable methods. In the first and second study a standardized method was also used by each participant. The results from the first study showed that most laboratories were able to isolate *L. monocytogenes* in the expected isolation frequencies. Using the standardized method 97 % of the RMs were found positive for *Listeria*. In the second study only *ca* 80 % of the RMs were found positive as a result of the addition of the competitors (*Enterococcus faecium, Bacillus subtilis, Lactobacillus plantarum* and *Lactococcus lactis* at a level of, respectively, 8×10^3, 11×10^3, 5×10^3 and 5×10^3 cfu per capsule). The groups of NSM methods based on the Food and Drug Administration's method for the detection of *L. monocytogenes* seemed to be less affected by the addition of the competitors than the groups of NSM methods based on the US Department of Agriculture's method. Addition of RMs to food products resulted in a substantial decrease in the isolation of *L. monocytogenes*. No relationship was apparent between the type of method used and the type of food product tested. It was concluded that the RMs are suitable for testing laboratory performance of the detection methods for *L. monocytogenes*.

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Published in **Food Microbiology**, 1995, **12**, 125-134.

INTRODUCTION

Listeria monocytogenes has become a micro-organism of interest to food microbiologists as a result of several food borne outbreaks (Fleming *et al.*, 1985; Gellin and Broome, 1989). As a consequence, an increase in research for improvement of enrichment broths and isolation agars for the detection of *L. monocytogenes* has occurred. Various new detection methods were developed of which the methods proposed by the Food and Drug Administration (FDA) and US Department of Agriculture (USDA) are the most widely used. The original FDA method was developed for testing dairy products and seafoods; the USDA method for testing meat products. The FDA method, developed by Lovett *et al.* (1987), uses tryptone soya broth (TSB) supplemented with 0.6% (w/v) yeast extract as a basal broth to which nalidixic acid (40 $mg.l^{-1}$), cycloheximide (50 $mg.l^{-1}$) and acriflavine (15 $mg.l^{-1}$) are added as selective agents. The broth is incubated at 30 °C for 24 and 48 h and *Listeria* is isolated on modified McBride agar. The USDA method, developed by McClain and Lee (1988), involves a two stage enrichment in a phosphate buffered broth to which nalidixic acid (10 $mg.l^{-1}$) and acriflavine are added for selectivity. The concentration of acriflavine is 12 $mg.l^{-1}$ for the first step and 25 $mg.l^{-1}$ for the second. Incubation is carried out at 30 °C for 24 h for each step; one ml of the first enrichment broth is inoculated in nine ml of the second broth. *Listeria* is isolated on lithium chloride-phenylethanol-moxalactam agar (LPM). The essential difference between both methods is the concentration of acriflavine and the application of a phosphate buffer. These two methods have been modified and have extensively been tested for their efficacies (Bailey *et al.*, 1990; Lammerding and Doyle, 1989; Lovett *et al.*, 1991; Noah *et al.*, 1991; Warburton *et al.*, 1991 and 1992).

Most methods developed so far use acriflavine as a selective substance. The exact concentration of acriflavine to be used in the enrichment broths is controversial as it also inhibits the growth of *Listeria*, especially of serotype 4b (Beumer *et al.*, 1988).

It is remarkable that the use of non-selective pre-enrichment, as commonly used for detection of *Salmonella*, has gained so little attention. A few studies have been published concerning the use of a non-selective pre-enrichment (Lammerding and Doyle, 1989) but it has not yet been widely accepted and used. A reason for this might be that the selectivity of the succeeding selective enrichment is too poor to allow a proper detection of *Listeria*.

In most of the comparative studies, either naturally contaminated samples without knowledge of the number of *Listeria* cells present or diluted cultures were used for inoculation of samples. Only a few (Lammerding and Doyle, 1989 and Bailey *et al.*, 1990) tested samples with a known low number of sublethally injured cells, as they are commonly found in the types of food products tested for the presence of *Listeria*.

For testing the performance of microbiological detection methods the use of reference materials should be considered. On behalf of the European Union's Measurement and Testing programme [former Community Bureau of Reference (BCR)], the National Institute of Public Health and Environmental Protection (RIVM) is developing such reference materials containing low numbers of sublethally injured cells. For the preparation of such materials micro-organisms are suspended in milk and subsequently spray dried. Due to this proces the micro-organisms become sublethally injured. Several reference materials have already been developed and evaluated (In 't Veld and Notermans, 1992; In 't Veld *et al.*, 1993). The effect of the injury on

recovery and the detection of *L. monocytogenes* has been tested by In 't Veld *et al.* (1991) and results indicated that the use of pre-enrichment is beneficial for the recovery of *L. monocytogenes*.

The reference material developed for *Listeria* was evaluated for its suitability as a quality control for *Listeria* detection procedures by collaborative studies.

MATERIALS AND METHODS

Three collaborative studies were organised in which the *Listeria* RMs were used. In the first collaborative study (CB-1) the *Listeria* RMs were tested alone. In the second study (CB-2) the RM was tested in combination with RMs containing competitive micro-organisms. In the third study (CB-3) the RMs were tested in combination with food products. An overview of the design of the collaborative studies is presented in Table 1.

Table 1. Overview of the design of the collaborative studies

Collaborative study no.	Materials analysed	Design		
		No. of participants	Detection methods	No. of samples analysed
CB-1	RM *Listeria*	40	SM[a] NSM[b]	25 25
CB-2	RM *Listeria* + RM competitors	42	SM[c] NSM	30 30
CB-3	RM *Listeria* + food products	30	NSM	voluntary

a Standard method used in CB-1 involving pre-enrichment in tryptone soya broth with yeast extract (TSBYE) (16-20 h at 30 °C), selective enrichment in *Listeria* enrichment broth (LEB) (24 h at 30 °C), isolation on Oxford agar (48 h at 37 °C).
b NSM, method of choice of participating laboratory.
c Standard method used in CB-3 involving pre-enrichment in phosphate buffered TSBYE (16-20 h at 30 °C), selective enrichment in phosphate buffered LEB (48 h at 30 °C), isolation on Oxford agar (48 h at 37 °C).
RM, reference materials.

Preparation of the *Listeria* reference materials

The *Listeria* RMs used in CB-1 were prepared as described by In 't Veld *et al.* (1991). For the batch used in CB-1, 0.5 g of the contaminated milk powder (CMP-1) was mixed with one kg sterilized (gamma irradiated; 10 kGy) milk powder (SMP) for four hours at room temperature in a 17 l stainless steel drum (mixer, type T 10b, W.A. Bachofen A.G. Maschinenfabrik, Basel, Switzerland). From the mixed powder 1.5 g was mixed further with one kg SMP. The final mixed powder was distributed into gamma-irradiated gelatin capsules (0.28 g/capsule).

In the preparation of the batch for CB-2, a second CMP (CMP-2) was used. The CMP-2 was prepared as follows; *Listeria* was grown in 10 ml of brain heart infusion broth (Difco 0037) for 24 h at 37 °C. This culture was diluted in peptone saline and one ml of a 10^{-4} dilution inoculated into 225 ml TSB (Oxoid CM 129) and incubated for 24 h at 37 °C. From the TSB culture 0.1 ml was transferred to three l milk (dry matter content 28 %, fat content 6.5 %). The milk was spray-dried as described by In 't Veld *et al.* (1991). Fifty grams of this CMP-2 was mixed with 1.6 kg SMP as described above.

For CB-3 two batches (A and B) were prepared. Batch A was prepared by mixing one g of the CMP-1 with 100 g SMP. Forty grams of the mixed powder were diluted further with 1.5 kg SMP and 170 of this mixed powder were diluted with 1.5 kg SMP. Batch B was prepared by mixing 30 g of the CMP-2 with one kg SMP, the mixed powder was further diluted with one kg SMP. Mixing apparatus and conditions were identical as described above.

The mean number of cfu of the mixed powder was determined after each mixing step by the procedure described by In 't Veld *et al.* (1991), except for batch B of CB-3. For this batch each capsule was reconstituted in a test tube (diameter 25 mm) with cap containing 10 ml peptone saline at 38 °C, and after 10, 20 and 30 minutes mixed for a few seconds on a Vortex mixer. After the final mixing one ml of the solution was transferred, in duplicate, to a Petri dish and 10 ml molten cooled tryptone soya agar (TSA) added. The plates were incubated for four h at 30 °C and then an overlayer of 10 ml double strength Oxford agar (Oxoid CM 856 plus SR 140) added. The plates were incubated for an additional period of 44 h at 30 °C.

Preparation of the materials containing competitive micro-organisms

The competitive micro-organisms material contained the following strains: *Bacillus subtilis* (ATCC 6051); *Enterococcus faecium* (NCTC 8213); *Lactococcus lactis* (ATCC 7962); *Lactobacillus plantarum* (ATCC 8014). The species to be used as competitive micro-organisms were selected in concert with the participating laboratories. The strains were cultured as described by In 't Veld *et al.* (1992). Spray-drying apparatus and conditions were identical as described for *Listeria.* For preparation of the batch of reference materials the following amounts of the CMPs were mixed with 1.5 kg SMP: 1.5 g CMP *B. subtilis*, 1.5 g CMP *E. faecium*, 6.5 g *L. lactis* (previous 1:100 diluted by mixing the CMP and SMP) and 2.5 g *L. plantarum* CMP. Mixing apparatus and conditions were identical as described above. The powder was filled in gamma irradiated gelatin capsules (0.28 g/capsule).

The contamination level of the mixed powder was determined after reconstitution of a capsule in a test tube (diameter 25 mm) with cap containing 20 ml peptone saline of 38 °C, and mixed after 5, 10, 15 and 30 minutes for a few seconds on a Vortex mixer. After the last mixing 0.1 ml volumes were spread, in duplicate, on TSA with yeast extract (TSAYE) and incubated for 24 to 48 h at 30 °C.

Bacillus subtilis could be distinguished by colonial morphology, *E. faecium* was confirmed by streaking a colony on TSA containing 50 ml l^{-1} egg yolk tellurite emulsion (Oxoid SR 54), blackening of the agar confirmed the organisms as *Enterococcus.* A distinction between *L. lactis* and *L. plantarum* was made on the basis of their different fermentation of mannitol and melezitose. *L. lactis* fermented melezitose and mannitol and *L. plantarum* only fermented mannitol. The mean number of cfu of the competitive micro-organisms sample was in total

2.9 x 10^4 per capsule. The levels for *E. faecalis*, *B. subtilis*, *L. plantarum* and *L. lactis* were respectively 8 x 10^3, 11 x 10^3, 5 x 10^3 and 5 x 10^3.

Design of the collaborative studies

For each study the participants received a detailed description of the procedures to be followed together with the dates on which the studies had to be carried out. A draft procedure was discussed beforehand with the participating laboratories in a plenary meeting. The reference materials were sent to the participants on dry ice by special delivery service for CB-1 and by (air)mail for CB-2 and CB-3. The materials were stored at 4 °C upon receipt. In CB-1 40 laboratories participated, in CB-2 42 and in CB-3 30 (Table 1).

In the first two studies (CB-1 and 2) the materials were tested by means of two methods. The first method (called standard method or SM) was standardized for all laboratories, the second method (called non-standard method or NSM) was the choice of the participating laboratory. In the third study no standardized method was used. The SM of CB-1 involved pre-enrichment in TSB supplemented with 0.6 % yeast extract (TSBYE) for 16 - 20 h at 30 °C. After incubation, 0.1 ml of the pre-enrichment broth was transferred into 10 ml of a selective enrichment broth with a reduced acriflavine concentration of 10 mg.l^{-1} (LEB according to International Dairy Federation (IDF) standard 143; Anon., 1990) and was incubated at 30 °C. After 24 h incubation the *Listeria* enrichment broth (LEB) was inoculated onto an Oxford agar plate (Oxoid CM 856 + SR 140) which was incubated at 37 °C and examined after 24 and 48 h incubation. For confirmation as *L. monocytogenes* the colonies were examined under Henry's illumination for a blue-grey colour and tested for catalase activity. The SM of CB-2 was similar to that of CB-1 except that the (pre)enrichment broth contained a phosphate buffer (8.64 g.l^{-1} Na_2HPO_4 plus 3.94 g.l^{-1} KH_2PO_4) and the selective enrichment was extended with a second isolation step after 48 h incubation. The confirmation procedure (In 't Veld *et al.*, 1992) included additional Gram staining, rhamnose/xylose fermentation, motility and haemolytic activity. The haemolytic activity was tested according to In 't Veld and de Boer (1991) by adding 100 µl of a 48 h culture grown in brain heart infusion broth at 37 °C to 100 µl of a two % sheep erythrocyte suspension in phosphate buffered saline (pH 7.4). The mixture was incubated in a microtiter plate for 45 minutes at 37 °C followed by two h at 4 °C.

In CB-1 25 individually numbered samples were tested for each of the two methods. For both methods five of the 25 samples were negative for *Listeria* and the numbers of these blanks were unknown to the participants. For CB-2 30 samples were tested including nine blank samples. For CB-3 each participant received capsules from both batches according to their own choice.

Analysis of the data

1. Calculations were made for each batch, except for batch B of CB-3, with the use of the binomial distribution to determine the chances of receiving capsules not containing *L. monocytogenes* amongst the intended positives capsules. These calculations were based on the 95 % confidence upper limit of the percentage of capsules not containing *L. monocytogenes* on the basis of enumerations of *L. monocytogenes* in the capsules. The

summarized chances (up to at least 95%) was used as the expected number of positive *L. monocytogenes* isolations out of a specified number of capsules analysed (corresponding to an α of $\leq$ 5%).

2. The homogeneity of the batch was calculated using the following formula for T_2 (Mooijman *et al.*, 1992):

$$T_2 = \Sigma \left(\frac{(Y_i - Y_+ / I)^2}{(Y_+ / I)} \right)$$

in which Y_i is the count from one capsule and Y_+ is the total count from all capsules. The T_2 will follow a χ^2 distribution with I-1 degrees of freedom (I being the number of capsules tested).

3. For each laboratory tests were made to determine whether different results were obtained with the SM and NSM methods used in CB-1 and 2. For this the χ^2 test (Wardlaw, 1985), with correction for continuity was used.
4. To investigate whether there were systematic differences between the SM and NSM methods, averaged over all laboratories, the data from CB-1 and CB-2 were analysed by fitting two different 'quasi likelyhood models' (McCullagh and Nelder, 1983). The first model included the effects of methods and laboratories. The second model included only the effect of laboratories. If the first model fitted the data significantly better than the second model, then it was concluded that there was a significant difference between the SM and NSM method.
5. The NSM methods could be divided into six categories of laboratories of comparable methods. The groups of methods are presented in Table 2. Some laboratories could not be assigned to any of the categories. To detect a difference between any of the six NSM methods, the Kruskal and Wallis test (Siegel, 1956) was applied. For pairwise comparison of the first group with the other groups the Mann Whitney U test was used, taking into account the presence of ties (Lehman and D'Abrerea, 1975).

The blank capsule results were treated separately from the artificially contaminated capsule results. The total number of false positive results were calculated from the blank capsule results.

RESULTS

Number of cfu, homogeneity, expected isolation frequency of the RM batches and the RM shipping times.

The mean number of cfu per capsule and the corresponding homogeneity of the batch is presented in Table 3 for all the batches used in the CBs. For testing presence/absence of *L. monocytogenes* it is important to know the fraction of capsules in which no *L. monocytogenes* can be detected. The fraction with the corresponding 95 % confidence upper limit is also presented in Table 3. Based on the upper limit the expected isolation frequency for a laboratory was determined.

The average shipping time of the reference materials for CB-1 was 3.8 days (ranging from 1 to 11 days) and for CB-2 the average time was 7.9 days (ranging from 2 to 31 days). No shipping times were recorded for CB-3. No relation between shipping time and isolation frequency in participating laboratories was observed (data not presented).

Table 2. Non-standard methods.

Group	Method[a]
1	Single step enrichment in *Listeria* enrichment broth (incubation for 24 and/or 48 h); isolation on selective agar of own choice[b].
2	Two step enrichment in LEB (incubation of 24 h for each step); isolation on selective agar of own choice[b].
3	Enrichment in non-selective tryptone soya broth with yeast extract (incubation for 24 h), second enrichment step in LEB (incubation for 24 - 48 h); isolation on selective agar of own choice[b].
4	Two step enrichment, first in UVM-1 (incubation for 24 h), second step in UVM-2 (incubation for 24 h); isolation on selective agar of own choice[b].
5	Identical to group 4, only instead of UVM-2, Fraser broth was used.
6	Single step enrichment containing thiocyanate as selective agent (incubation for 24 - 48 h); isolation on selective agar of own choice[b].

a Abbreviations see section on design of collaborative studies.
b On average the following isolations agars were used in order of frequency of use: Oxford agar, polymyxin-B-acriflavine-ceftazidime-lithium chloride-aesculin-mannitol agar, Modified McBride agar, McBride agar and lithium chloride-phenylethanol-moxalactam agar.

Isolation of L. monocytogenes using the contaminated capsules

Table 4 presents the average percentage of capsules found positive for *L. monocytogenes* by the participating laboratories and the percentage of laboratories finding at least the expected number of positive isolations (see expected isolation frequency presented in Table 3). In CB-1 and CB-2 five laboratories (12 - 13%) found a significant difference between the SM and NSM method. None of these laboratories found this difference in both CBs. The SM used in CB-1 gave, on average, significantly more positive isolations than the NSM method in CB-1. For CB-2, where the competitive micro-organisms were applied, no difference between the SM and NSM method could be detected.

Table 5 summarizes the percentage of positive isolations in relation to the NSM method used and the collaborative study. The method of group no. 6, using thiocyanate as selective agent, gave the lowest recovery of *L. monocytogenes*.

In CB-3 both batches of RMs were tested in combination with various types of food products. The relation between the type of food product and the number of cfu of the RMs used is presented in Table 6. The lowest recovery rate was found with soft cheeses, the highest with milk or milk products other than cheeses. The use of a higher inoculum (RMs of batch CB-3B) did result in more positive isolations. It has to be noted that there are differences between the number of laboratories and the number of food products analysed by them for the various combinations of method and food products.

Table 3. Data on presence and level of *Listeria monocytogenes* in the batches of samples used in the collaborative studies.

Data	Collaborative study batch number			
	CB-1	CB-2	CB-3A	CB-3B
cfus per capsule[a]	11.6	4.9	5.4	67
Homogeneity[b]	7.9	1.5	0.9	0.3
Number of capsules analysed	99	50	25	10
Fraction 'negatives'[c]	0.0	1.6	0.5	n.d.
Upper limit[d]	1.0	4.5	1.9	n.d.
Number of capsules analysed	356	192	25	n.d.
Expected isolation frequency[e]	19/20	18/21	18/20	20/20

a Mean number of cfu per capsule.
b Homogeneity expressed as $T_2/(I-1)$; T_2 is defined in Materials and Methods section.
c Fraction of 'negatives' is the fraction of capsules (%) in which no *L. monocytogenes* could be detected based on enumerations carried out by the organizing laboratory.
d Ninety-five percent confidence upper limit of the fraction of 'negatives' (%).
e Expected isolation frequency is the expected number of positive *L. monocytogenes* isolations out of a specified number of capsules tested.
n.d., not determined.

Isolation of L. monocytogenes using the blank capsules

In CB-1 four laboratories (10 %) found false positive isolations corresponding to 1.5 % of the blank capsules analysed. In CB-2 five laboratories (12 %) found false positive isolations corresponding to 1.1 % of the blank capsules. One laboratory found false positive results in both CBs. For CB-3 no blank capsules were analysed.

Table 4. Isolation of *Listeria monocytogenes* averaged over all laboratories for each collaborative study.

Collaborative study batch no. and method		% Positive isolations (number of capsules analysed)	Laboratories attaining ≥ expected isolation frequency (%)
CB-1	- SM	97 (800)	85
	- NSM	85 (800)	55
CB-2	- SM	79 (882)	45
	- NSM	78 (882)	53
CB-3A	- NSM[a]	88 (392)[a]	n.d.
	- NSM[b]	64 (1253)[b]	n.d.
CB-3B	- NSM[a]	98 (1253)[a]	n.d.
	- NSM[b]	73 (1238)[b]	n.d.

a RMs tested without food product.
b RMs tested in combination with food product.
SM, Standardised method; NSM, Non-standard method; n.d. = not determined.

Table 5. The percentage positive isolations obtained with the various types of Non-standard methods (NSMs).

Collaborative study batch no.	NSM group no[a]					
	1	2	3	4	5	6
CB-1[b]	94 %	94 %	100 %	74 %	80 %	46 %
CB-2[c]	99 %	98 %	76 %	60 %	58 %	24 %
CB-3A[d,e]	92 %	82 %	98 %	77 %	65 %	60 %
CB-3A[e,f]	69 %	75 %	68 %	48 %	58 %	44 %
CB-3B[d,e]	99 %	88 %	99 %	100 %	95 %	100 %
CB-3B[e,f]	70 %	85 %	77 %	64 %	66 %	67 %

a Number of group of NSM corresponds to numbers in Table 2.
b Number of laboratories per group of NSM are, respectively: 7, 9, 2, 8, 2 and 4.
c Number of laboratories per group of NSM are, respectively: 7, 6, 6, 4, 4 and 2.
d Only reference materials tested.
e Number of laboratories per group of NSM are, respectively: 7, 3, 7, 4, 4 and 1.
f RM tested in combination with food product.

Table 6. Effect of food type and reference materials contamination level on the isolation rate of *Listeria monocytogenes*.

Food added	RM contamination level[a]	
	(5 cfu)	(100 cfu)
Soft cheeses	36 %	36 %
Other cheeses	53 %	74 %
Meat/meat products	57 %	69 %
Poultry/poultry products	73 %	76 %
Fish/fish products	86 %	90 %
Milk/milk products other than cheese	90 %	92 %
Other foods	47 %	54 %

a Batches RM CB-3A and CB-3B.

DISCUSSION

For the production of a material suitable as a reference material, the variation in cfu between capsules must be within defined limits. The optimal variation in cfu between samples, in the ideal situation of a homogeneous solution, conforms to a Poisson distribution (Niemelä, 1983). The spray-drying and mixing of powders involved in the production of the RM will result in a larger variation between samples, conforming to a variation wider than the Poisson distribution (so called over-dispersion; Heisterkamp *et al.*, 1993). The variation in the number of cfu between the capsules was much higher for the RM batch used in CB-1, than for the other batches. This was probably caused by the extreme dilution of the CMP-1 used to obtain the intended contamination level. The use of CMP-2, containing lower numbers of $cfu.g^{-1}$ CMP, for the batches used in CB-2 and CB-3 (batch A), resulted in a much lower variation of cfu between the capsules. For batch B in CB-3, using CMP-1 which was less diluted than that used in CB-1, a reduced variation in cfu between the capsules was realized. The two batches of RMs used in CB-3 had a variation in cfu between capsules that did not differ significantly from a Poisson distribution.

A second important requirement for RMs is that its stability should be known. During the collaborative studies stability tests indicated a constant number of cfu during storage at 4 °C for a few months (results not presented). A two year stability test at -20 °C is currently in progress; after 1.5 year storage the level is still constant.

Due to the spray-drying process the cells become sub-lethally injured (by heat and desiccation). This sub-lethal injury will have an effect on the recovery of the organism. Also, in practice, food samples that contain sub-lethally injured organisms will be analysed. The degree of injury can vary widely as a result of several stress factors or combinations of them (e.g. heat,

drying, pH, wateractivity, freezing, etc.). Most collaborative studies are carried out with natural contaminated samples and/or with artificial contaminated samples inoculated with vital organisms. Only a few studies also analysed artificially inoculated samples containing sub-lethally injured organisms [heat treated cells by Bailey *et al.* (1990)]. Collaborative studies were carried out to test the possibility for use of the RM to evaluate the performance of the analytical test procedures (the third requirement for an RM). CB-1, in which the *Listeria* RMs were tested without competitive micro-organisms, clearly showed (see Table 4) that most of the laboratories were able to detect *Listeria*, especially when using the SM which included a pre-enrichment step. The lowest recovery was obtained using the NSM used by group 6. The thiocyanate selective agent in this detection procedure may have a negative effect on the growth of *L. monocytogenes*. The NSM based on the USDA method (NSMs of groups 4 and 5) gave somewhat lower recoveries than the methods based on the FDA method.

Addition of competing micro-organisms as an RM, on average reduced the recovery rate of *L. monocytogenes*. The use of a non-selective pre-enrichment step (SM and NSM of group 3) was not beneficial, even though for the SM a buffered enrichment broth was used to reduce a pH drop caused by the competitors (results not presented).

The results obtained by Lammerding and Doyle (1989), showed that a non-selective enrichment has a positive effect on the recovery of (sub-lethally injured) *Listeria* and were confirmed when no competitors were present. The addition of the competitors did not affect the FDA-related methods (NSMs of groups 1 and 2).

CB-3 tested the RM under conditions found in daily practice and whether the inoculum size effects recovery. Tables 5 and 6 show that the inoculum size only marginally influenced the isolation frequency. However, food matrices influenced the isolation rate enormously. On average, the isolation rate was 25 % lower when food products were added. The results with food products were lower than obtained in CB-2 for all groups of NSMs except group 6. This indicates that food matrix effects may be difficult to simulate by using an artificial competitive microflora. The difference between food samples and the artificial competitive microflora is related both to the type and level of artificial microflora present in the RM.

The differences between the groups of methods were also less when the food products were added (see Table 5). Also, the type of food product influenced the isolation rate (Table 6). Isolation of *L. monocytogenes* from soft cheeses seems to be difficult, while isolation from fish products and non-cheese milk products was easier. No relationship between the group of methods used and the type of food products was observed.

The one % false positive rate obtained in these studies is not unusual. In other collaborative studies, using the *Salmonella* RM, an overall false positive rate of *ca* 2.7 % was found. False positives may be caused by accidental switching of samples or cross contamination between blank and contaminated samples.

The results clearly demonstrate the suitability of testing RMs for evaluating the performance of detection procedures for *L. monocytogenes* in collaborative studies (for example the AOAC collaborative studies) using the RMs as a standard inoculum (level of contamination and homogeneity are known) of samples. It is also recommended that RMs should be analysed in combination with food products in order to measure the effect of the food matrix on the isolation of *L. monocytogenes* for the type of food under investigation. This

effect indicates the ability for recovering low numbers of (sub-lethally) injured *L. monocytogenes* from the type of food under investigation. In this way the performance can be checked and results obtained by other laboratories can be compared.

ACKNOWLEDGEMENTS

The authors thank:
Drs. B. Griepink and E. Maier (Chairpersons, Measurement and Testing Programme, Brussels, Belgium) for their support of the project [EU no: 2570/1/9/301/86/11/BCR-NL (10) or RIVM no 281008]; W.S. Ritmeester, J.A. Hobers (Laboratory for Water- and Food Microbiology, RIVM, Bilthoven, The Netherlands) for the preparation and testing of the RMs; Dr. J.A. Hoekstra, N.G.W.M. van Strijp-Lockefeer and S.H.W. Heisterkamp (Centre of Mathematical Methods of the RIVM, Bilthoven, The Netherlands) for data analysis; Dr. A.H. Havelaar for his project leadership; and the following persons for their participation in the collaborative studies:
Belgium - Prof. dr. J. Debevere, Rijksuniversiteit Gent, Gent, Dr. G. Vlaemynck, Rijkszuivelstation, Melle; Denmark - Dr. S. Qvist, Danish Veterinary Service, Frederiksberg, Dr. N. Skovgaard, Royal Veterinary and Agricultural University, Frederiksberg C; France - Prof. dr. M. Catsaras, Institut Pasteur de Lille, Lille-Cedex, Dr. M. Etienne, Ecole Nationale de la Sante Publique, Rennes Cedex, Dr. P. Colin, CNEVA - Unite Hygiene et Qualite, Ploufragan, Prof. dr. J.A. Nicolas, Veterinaire Departemental, Limoges, Dr. B. Corbion, Ministere de l'Agriculture, Paris, Dr. M. Catteau, Institut Pasteur de Lille, Lille Cedex, Dr. N. Richard, Laboratoire interregional de Montpellier, Montpellier Cedex; Germany - Dr. J. Krämer, University of Bonn, Bonn, Prof. dr. H.J. Sinell, Freie Universitat Berlin, Berlin, Prof. dr. G. Terplan, Universitat München, München, Prof. dr. P. Teufel, Institute for Veterinary Medicine of the Federal Health Office Berlin, Berlin, Dr. L. Leistner, Bundesanstalt fur Fleischforschung, Kulmbach; Greece - Prof. dr. J. Papadakis, Athens School of Hygiene, Athens, Dr. St. Malliaris, Institute of Food Hygiene, Athens; Ireland - Prof. dr. D. Collins, University College Dublin, Dublin, Dr. V. Young, Eastern Health Board, Dublin; Italy - Dr. V. Sanguinetti, Universita di Bologna, Bologna, Prof. dr. M. Stecchini, Istituto di Tecnologie Alimentari, Udine, Dr. D. Cesaroni, Settore Biotossicologico PMP, Bologna, Dr. G. de Felip, Istituto Superiore di Sanita, Rome; Luxemburg - Dr. F. Arendt, Laboratoire National de la Sante, Luxemburg; Portugal - Dr. M. Vicente da Cruz, Depart. de Tecnol. das Indust. Alimentares, Lisboa, Dr. M. Do Rosario Laranjeiro Novais, Instituto Nacional de Saude, Lisboa; Spain - Dr. M. Saco Galvany, Ministerio de Agricultura, Pesca Y Alimentacion, Barcelona, Dr. C. Allaert, Universitat de Lleida, ETS Enginyeria Agraria, Lleida, Dr. B. Sesma, Servicio Navarra de Salud, Pamplona-Iruna; Sweden - Dr. M. Peters, National Food Administration, Uppsala, Sweden; The Netherlands - Mr. H. Beckers, Nestle Nederland, Nunspeet, Mr. B. Hartog, CIVO-TNO, Zeist, Mr. H. Stegeman, RIKILT, Wageningen, Mr. R. Beumer, Landbouwuniversiteit Wageningen, Wageningen, Dr. H. Joosten, Coberco Research, Deventer; United Kingdom - Dr. R. Blood, Leatherhead Food Research Association, Leatherhead, Surrey, Dr. S. Johal, Laboratory of the Government Chemist, Teddington, Middlesex, Dr. R. Betts, Campden Food and Drink Research Association, Chipping Campden, Dr. C. Wray, Ministry of Agriculture, Fisheries and Food, Surrey, Weybridge, Dr. J.E.L. Corry, University of Bristol, Avon, Dr. D. Roberts, Central Public Health

Laboratory, London, Dr. S. Scotter, Ministry of Agriculture, Fisheries and Food, Norwich, Norfolk, USA - Dr. W. Andrews, Food & Drug Administration, Washington DC.

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The certification of a reference material for the evaluation of the ISO method for the detection of *Salmonella*

ABSTRACT

A reference material (RM) containing *Salmonella typhimurium* was certified as CRM 507 by the Standards, Measurements and Testing Programme of the European Commission. The material consists of a gelatin capsule filled with artificially contaminated milk powder. The material is certified for the evaluation of presence/absence methods based on the ISO 6579 procedure for the detection of *Salmonella*. In the certification study 11 laboratories determined the presence/absence of *Salmonella* from each of 50 capsules. They also determined the mean number of colony-forming particles (cfp) and the homogeneity of the batch of RM according to an enumeration procedure.

Certified values were calculated for both procedures separately. Based on the presence/absence procedure a fraction of capsules in which no *Salmonella* could be detected of 2.7 % (one-sided 95 % confidence upper limit 4.4 %) was certified, for the enumeration procedure this fraction was 0.61 % (one-sided 95 % confidence upper limit 1.6 %). The certified mean number of *Salmonella* cfp in one capsule is 5.9 (two-sided 95 % confidence interval 5.3 - 6.4). Data on the preparation, identification, stability (at storage and higher temperatures) and homogeneity of the material are presented.

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Published in the **Journal of Applied Bacteriology,** 1996, **80**, 496-504.

INTRODUCTION

Since 1987 the Laboratory of Water and Food Microbiology of the National Institute of Public Health and the Environment has been working on the development and evaluation of microbiological reference materials (RM) in cooperation with the European Commission's Standards, Measurements and Testing Programme (SM&T), formerly called the Community Bureau of Reference (BCR). The first reference material containing *Salmonella typhimurium* was developed several years before that. The first results with this RM were presented by Beckers *et al.* (1985). Several RMs have been developed so far for use in water and food microbiology. All of them have been evaluated in European collaborative studies, for example, RM containing *Salmonella typhimurium*, *Listeria monocytogenes* (In 't Veld *et al.*, 1995), or *Enterococcus faecium* (Mooijman *et al.*, 1992). The *Salmonella* RM has been evaluated in two collaborative studies (In 't Veld *et al.*, 1988, 1990). In the first study the *Salmonella* RM was examined without competitive micro-organisms, in the second study the RM was examined in combination with an RM containing competitors. In both studies the materials were evaluated by means of two presence/absence methods. The first method was based on the ISO 6579 procedure for the detection of *Salmonella* (Anon., 1990) and was standardized for all laboratories, the second method was the choice of the participating laboratory.

As the RM for *Salmonella* is intended for the evaluation of presence/absence methods, a low number of cells has to be present in the RM. Therefore the mean level of contamination was set at *ca* 5 colony-forming particles (cfp) per capsule. Due to the low mean number of cfp per capsule and the corresponding distribution of the cfp over the capsules there exists a small chance that a capsule will not contain any *Salmonella*. To check the level of contamination an enumeration procedure for the RM has been developed for this purpose. This enumeration procedure has also been evaluated in a collaborative study (In 't Veld *et al.*, 1989).

The results of all these studies indicated that the RMs fulfilled the requirements for an RM. These requirements are that the RM is representative for its intended use, stable within specified limits over a defined period of time and that the RM is homogeneous within defined limits (Griepink, 1989). As the results of the evaluations were so promising it was also decided to start a certification procedure for the RM. For certification a standardized procedure has to be followed (Anon., 1994a). The steps in this procedure are presented below: (1) production of a batch of reference material, (2) homogeneity testing, (3) stability testing, (4) the certification measurements (collaborative study), (5) evaluation of the results, (6) preparation of a certification report, (7) assistance to storage, distribution and stability monitoring.

For the certification measurements a group of selected laboratories examine a part of the batch of RM according to a strict analytical protocol. As the certification is based on a standardized method, the evaluation of results first focuses on the technical details to see whether the laboratory has strictly followed the protocol of this standard method. The results of the study are not known to the participants at the time of this technical discussion. This procedure is used to ascertain the highest possible degree of confidence in the results obtained in the certification study. After the evaluation of the technical results the data are analysed statistically as described by Heisterkamp *et al.* (1993). Finally the certification committee of the EC decides on the basis of the certification report if the material is up to the standard for certification. The certification of the RM containing *Salm. typhimurium* (CRM 507)

and an RM containing *Ent. faecium* (CRM 506) were the first two reference materials containing living microorganisms to be certified worldwide. The CRM containing *Ent. faecium* is used for the evaluation of enumeration procedures for faecal contamination of water (Mooijman *et al.*, 1994). This manuscript describes the method used and the results of this certification procedure for the *Salm. typhimurium* RM.

Some of the notations used in the certification report are not commonly used in microbiology. These notations were defined by the certification committee of the EC in accordance with meteorological principles and definitions. The same notations will be presented in this manuscript. For example the term colony-forming units is commonly used in microbiology but the word "unit" in metrology is reserved for SI units, therefore the term colony-forming particles (cfp) will be used instead.

MATERIALS AND METHODS

Preparation of the material

Salmonella typhimurium (ALM 40) was streaked for purity onto a sheepblood agar dish and incubated for 24 h at (37 ± 1) °C. A single colony was used for inoculation of two l heart infusion broth (prepared from individual ingredients). The broth was subsequently incubated at 37 °C for *ca* 24 h. After incubation the culture was divided over 250 ml centrifuge tubes and centrifuged for 15 min at 5000 rev.min^{-1} (2900 g_n). The pellet was resuspended in two ml of peptone saline solution and added to concentrated pasteurized full fat milk. The milk was spray dried after homogenization at the Agricultural University (Wageningen, The Netherlands) using a Stork pilot plant spray dryer at an inlet temperature of *ca* 140 °C and an outlet temperature of *ca* 80 °C. The resulting highly contaminated milk powder was stored at 5 °C.

To obtain the desired number of cfp in the contaminated milk powder, 30.8 g of the highly contaminated milk powder was mixed with 2.5 kg of sterile skim milk powder. The milk powder used for mixing was commercially available skim milk powder and was sterilized by γ-irradiation with a dose of 10 kGy.

The mixing of powder was done in a 17 l stainless steel drum with a Turbula type T 10 b mixing apparatus during four h at room temperature. The mixed powder was filled in gelatin capsules with 17 g of powder for 60 capsules using an aluminum filling apparatus sterilized for 15 minutes at 121 °C. The empty capsules were sterilized by γ-irradiation with a dose of 10 kGy. Fifty of the filled capsules were used for the determination of the number of cfp according to the procedure used in the certification study. Based on the results (level of contamination and homogeneity) of these capsules it was decided to use the batch for the certification study.

Stability studies

For testing the stability of the materials at storage temperature (-20 °C), every two months 50 capsules were enumerated according to the enumeration procedure used in the certification study. Also the stability of the materials stored at higher temperatures was determined at four different temperatures. The temperatures tested were: -20 °C (reference), 22 °C, 30 °C and 37 °C. Once a week, over a period of four weeks, 25 capsules for each storage temperature

were enumerated. At the start of the test 50 capsules were enumerated. The counts obtained for each storage temperature were analysed separately using generalized linear models (McCullagh and Nelder, 1983). The stability tests were carried out by several technicians at the same laboratory.

Homogeneity studies

The homogeneity is expressed as the variation in number of cfp between the capsules and is calculated by means of the T_2 test statistic which is based on Cochran's index of dispersion (Heisterkamp *et al.*, 1993).

The formula for calculating the T_2 value is presented below:

$$T_2 = \sum_{i=1}^{I} \frac{\left(z_i - \frac{z_+}{I}\right)^2}{\frac{z_+}{I}}$$

where z_i = number of colony forming particles of *Salmonella* in one capsule ($N_{S,cfp}$), z_+ = sum of $N_{S,cfp}$ in all capsules examined, I = total number of capsules examined.

In the case of a Poisson distribution (a homogeneous distribution of bacteria in a sample is approximately Poisson distributed (Heisterkamp *et al.*, 1993)) T_2 follows a χ^2-distribution with (I-1) degrees of freedom. In this case the expected T_2 value is the same as the number of degrees of freedom. Hence the measure of dispersion ($T_2/(I-1)$) is expected to be equal to 1. However, overdispersion is expected (Heisterkamp *et al.*, 1993) and hence the criterium for the acceptance of the prepared batch for use in the certification study was set at ≤ 2.

Certification study

For the certification study of the *Salmonella* RM two different procedures were used. The first procedure determined the presence or absence of *Salmonella* in one capsule, the second procedure determined the number of cfp of *Salmonella* in one capsule ($N_{S,cfp}$). Both procedures were carried out according to a detailed procedure presented in the analytical protocols (In 't Veld *et al.*, 1994).

Presence/absence procedure:

This procedure was based on the ISO 6579 procedure for the detection of *Salmonella* (Anon., 1990). Each laboratory determined the presence or absence of *Salmonella* in 50 capsules. Four of these individually identified capsules were blank control capsules. The code numbers of these capsules were unknown to the laboratories at the time of analysis. Each laboratory was free to select one selective enrichment broth and one type of isolation medium within the specifications of the ISO 6579. All dishes showing typical colonies were subjected to biochemical and/or serological tests for confirmation of *Salmonella*. If only a serological test

was carried out and the result was negative, an additional biochemical confirmation was requested.

The statistical analysis carried out on these data is described in detail by Heisterkamp *et al.* (1993). For comparison between laboratories the fraction of negatives obtained by the laboratories were tested for significant differences using Fisher's exact test using the mid-*p*-value.

Enumeration procedure:

The enumeration procedure was especially designed for the enumeration of low numbers of *Salmonella* in the RM. Each laboratory enumerated in total 50 capsules, the capsules were dissolved in two rounds. For each round also a control was tested, consisting of a Petri dish containing peptone saline to which no capsule was added. A summary of this procedure is presented below:

- dissolution of a capsule in 5 ml peptone saline in a Petri dish (dissolution conditions: (45 ± 5) min in a incubator at (38 ± 0.5) °C while shaking at *ca* 100 rpm);
- repair of *Salmonella* in Plate Count Agar (incubation time (4 ± 1) h at (37 ± 1) °C);
- selective growth of *Salmonella* by adding Violet Red Bile Glucose agar on top of the Plate Count Agar (incubation time: (20 ± 2) h at 37 °C).

After the second incubation period the number of typical colonies per capsule were reported. Each laboratory isolated *ca* 20 colonies from several dishes for confirmation of *Salmonella*. Confirmation was done as described for the presence/absence procedure. In earlier pilot studies (In 't Veld *et al.*, 1989), the dissolution step has been shown to be the most critical step in this procedure. Therefore much attention has been paid to this step. Examples of acceptable and not acceptable dissolved capsules were presented in the protocols.

The statistical analysis carried out on these data is described in detail by Heisterkamp *et al.* (1993). The dispersion in $N_{S,cfp}$ between capsules was calculated by means of the T_2 test statistic. For each laboratory the value of $T_2/(I\text{-}1)$ (I = number of capsules analysed) was compared to the overall measure of dispersion (T_{hom}/N) by means of a one-sided F-test, at the 5% level. For this a critical value for each laboratory was calculated as $F_{I\text{-}1,N} \bullet \Sigma T_{hom}/N$. The first five times of examinations carried out for the stability tests at -20 °C were used for the calculation of T_{hom}/N. This value is the sum of the T_2 values for each examination divided by the total number of capsules examined minus the number of examinations.

An overall comparison between the mean $N_{S,cfp}$ found by the laboratories was made by means of analysis of deviance. Grubbs' test was applied to detect laboratories with outlying averages.

The difference between the fraction of capsules not containing *Salmonella* as found for the enumeration test and by the presence/absence procedure was tested using Fisher's exact test.

RESULTS

Preparation of the material, homogeneity and stability

After mixing of the highly contaminated milk powder with the sterile milk powder 60 gelatin capsules were filled. Fifty of these capsules were examined to determine the number of cfp of

Salm. typhimurium in one capsule ($N_{S,cfp}$) and the homogeneity of the batch. The $N_{S,cfp}$ found was 5.04 and the measure of dispersion ($T_2/(I\text{-}1)$) was 1.25. The value for homogeneity was according to the criterion set ($T_2/(I\text{-}1) \leq 2$) and indicated that the counts did not differ significantly from a Poisson distribution ($\alpha \leq 0.05$). Therefore, it was concluded that the prepared batch was suitable for use in the certification study. The results (up to June 1995) of the stability test at storage temperature (-20 °C) of the batch is presented in Figure 1. Statistical analyses of the results did not indicate a decrease in $N_{S,cfp}$.

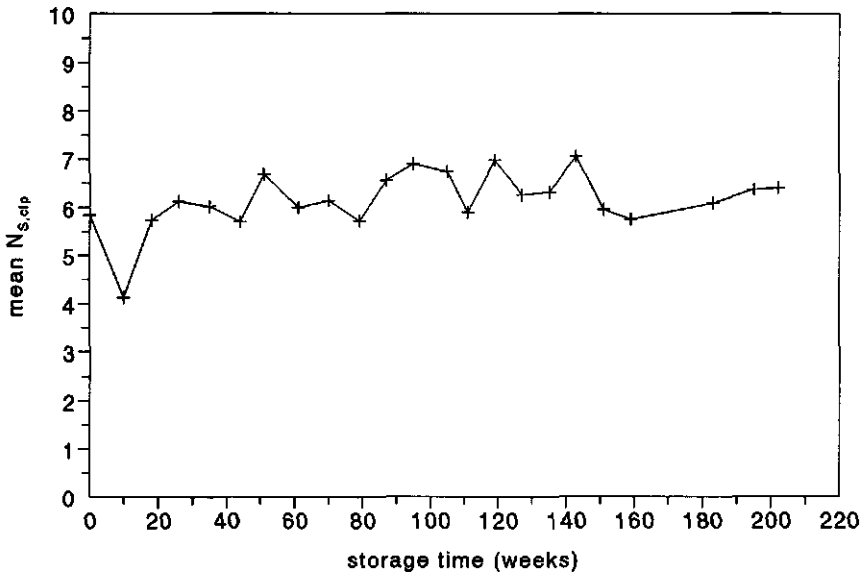

Figure 1. Stability of the mean number of colony forming particles of *Salmonella typhimurium* in one capsule ($N_{S,cfp}$) in relation to storage time at -20 °C.

The results of the first five stability test are presented in Table 1. The T_2 values of these tests were used to determine the overall measure of dispersion (T_{hom}/N). The T_{hom}/N was found to be 1.28 which is significantly different from the χ^2-distribution. It was, therefore, concluded that overdispersion existed. This overdispersion was taken into account when comparing the laboratory means.

Figure 2 presents the mean $N_{S,cfp}$ obtained at the higher storage temperatures (-20 °C, 22 °C, 30 °C and 37 °C). At -20 °C and 37 °C the results did not meet the linear assumption, so no regression coefficient could be determined. At 22 °C no significant decrease was observed, at 30 °C a significant decrease of three % per d was observed.

Table 1. Mean number of *Salmonella typhimurium* colony forming particles in one capsule ($N_{S,cfp}$), number of capsules analysed, T_2 value and measure of dispersion ($T_2/(l-1)$) found for enumerations of the stability test at storage temperature.

Stability test no.	Mean $N_{S,cfp}$	Number of capsules examined (l)	T_2	$T_2/(l-1)$
1	5.84	49	68.0*	1.42
2	4.13	48	49.3	1.05
3	5.74	46	60.9	1.34
4	6.13	48	70.4*	1.50
5	6.02	50	53.3	1.09

* significantly different from χ^2-distribution with (l-1) degrees of freedom (one-sided) at 95% confidence level.

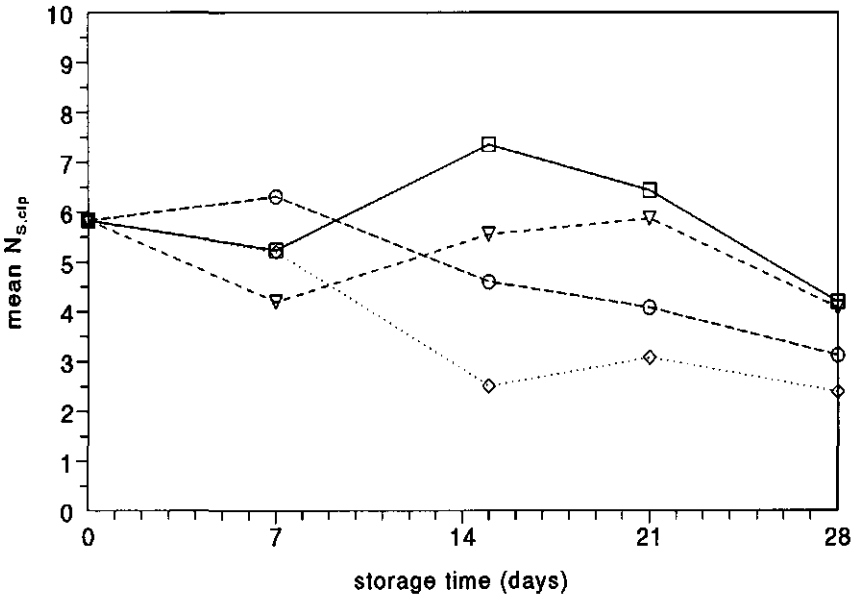

Figure 2. Stability of mean $N_{S,cfp}$ stored at various temperatures during four weeks. □, -20 °C; ∇, 22 °C; O, 30 °C; ◊, 37 °C.

Certification procedure

Presence/absence procedure:

The technical data of the laboratories were discussed to eliminate incorrect or doubtful results before the data were made available to the participants. Based on technical grounds the results of two laboratories were not used for certification as a result of errors made with the blank control capsules. Table 2 summarizes, for the remaining nine laboratories, the selective enrichment broth data (type of broth, pH, inoculation and incubation conditions) and the type of isolation medium used. All capsules showing typical colonies on the isolation agar were subjected to a confirmation for *Salmonella*. At least two colonies per capsule were used for this confirmation. All colonies (> 1000 colonies) tested by the laboratories gave a positive *Salmonella* identification. Four laboratories used biochemical and serological tests, three used only serological tests and two laboratories used only biochemical tests.

Table 2. Summary of the selective enrichment broths, pH, inoculation, incubation conditions and isolation media used for the presence/absence procedure.

Lab	Selective enrichment data						
	Broth[a]	Manufacturer	pH	Inoculation ratio[b]	Inc. temp. (°C)[c]	Total incubation time	Isolation medium[d]
1	RV	Oxoid, CM 669	5.2	0.1/10	42	47h 40min	BGA
2	MK	Individual ingredients	5.3	0.1/10	42.5	47h 00min	BGA
3	RVS	Oxoid, CM 866	5.0	1.0/100	41.5	48h 15min	Rambach
4	RV	Oxoid, CM 669	5.3	1/99	42	47h 43min	BGA + XLD
5	RV	Oxoid, CM 669	5.2	0.1/10	42	24h 00min	BGA
6	RV	Oxoid, CM 669	5.2	0.1/10	42.5	48h 30min	BPLSA
7	RV	Oxoid, CM 669	5.2	0.1/10	43	48h 15min	BGA
8	RV	individual ingredients	5.6	0.1/10	37	45h 45min	BGA
9	RV	individual ingredients	5.5	0.1/10	42	47h 15min	BGA

a RV, Rappaport Vassiliadis magnesium chloride malachite green broth; RVS, RV prepared with soya peptone; MK, Muller Kauffmann broth.
b inoculation ratio = volume (ml) of BPw used for inoculation/volume (ml) of selective enrichment broth.
c inc. temp. = incubation temperature.
d BGA, Brilliant Green Agar; XLD, Xylose Lysine Desoxycholate agar; BPLSA, Brilliant green Phenol red Lactose Sucrose Agar.

Table 3 presents the number of positive and negative capsules found by the remaining individual laboratories. The number of negatives found per laboratory (excluding the blank control capsules) varied between zero and three. No significant difference was found between laboratories for the fraction of negative capsules.

Table 3. Number of capsules found positive and negative for *Salmonella* in the presence/absence procedure (excluding blank control capsules).

Lab	Capsules found negative	Capsules found positive
1	1	45
2	2	44
3	1	45
4	0	46
5	2	44
6	1	45
7	3	43
8	1	45
9	0	46
total	11	403

The certified values for the fraction of capsules not containing *Salmonella* was calculated including its confidence limits on the basis of the nine sets of results. These values are presented in Table 4.

Enumeration procedure:

The technical data of the laboratories were discussed to eliminate incorrect or doubtful results before the data were made available to the participants. None of the laboratories found any *Salmonella* testing the blank controls for each round. Seven laboratories, which used a biochemical confirmation or a biochemical and a serological confirmation, confirmed all colonies tested (n = 238) as *Salmonella*. Four laboratories, which used only serological confirmation, confirmed 86 % of the colonies tested (n = 83) as *Salmonella*. The remaining 14 % were all confirmed as *Salmonella* by means of an additional biochemical confirmation. Three laboratories reported that for in total of seven capsules the exact $N_{S,cfp}$ could not be determined due to spreading of colonies in the dishes. Since these capsules contained *Salmonella* the results were used as a positive count capsule for the determination of the fraction of capsules not containing *Salmonella*.

Based on technical grounds the results of one laboratory were not used for certification as a result of an error of the incubation period. The mean $N_{S,cfp}$ of the remaining laboratories are presented in Table 5. A significant difference between laboratory means was found, although the Grubbs' test could not identify outlying laboratories.

Table 4. Certificate of analysis of *Salmonella typhimurium* CRM 507.

CRM 507, number fraction of negative capsules and number of cfp of *Salmonella typhimurium* in artificially contaminated milk powder			
quantity (test procedure)	certified value	confidence limit(s)	sets of accepted results
fraction of capsules in which no *Salmonella* could be detected (presence/absence procedure)	2.7 %	4.4 %[a]	9[b]
number of *Salmonella* colony forming particles in one capsule ($N_{S,cfp}$) (enumeration procedure)	5.9	5.3 - 6.4[c]	10[d]
fraction of capsules in which no *Salmonella* could be detected (enumeration procedure)	0.61 %	1.6 %[a]	10[e]

a one-sided 95% confidence upper limit.
b based on the results of 414 capsules.
c two-sided 95% confidence interval.
d based on the results of 485 capsules.
e based on the results of 492 capsules.

Table 5 also presents the values for the measure of dispersion found per laboratory. Based on the value of the overall measure of dispersion a critical value for $T_2/(l\text{-}1)$ was calculated per laboratory. None of the laboratories found a value higher than the critical value. Figure 3 presents the frequency distribution of the capsules examined by the 10 laboratories (in total 485 capsules).

Based on the results of the 10 laboratories the certified values for the mean $N_{S,cfp}$ and for the fraction of capsules not containing *Salmonella* were calculated including its confidence limit(s). The certified values are presented in Table 4.

Table 5. Mean $N_{S,cfp}$ per round, $T_2/(l\text{-}1)$ level found and critical value for each laboratory for the enumeration procedure.

Lab	Mean $N_{S,cfp}$			$T_2/(l\text{-}1)$	
	First round	Second round	Combined	Actual	Critical
1	7.32	7.36	7.34	1.560	1.796
2	6.21	6.16	6.19	1.613	1.811
3	4.33	4.81	4.57	0.941	1.838
4	5.52	5.68	5.60	1.050	1.796
5	6.04	6.96	6.54	1.031	1.796
6	5.18	4.75	4.94	1.639	1.796
7	7.81	6.12	6.66	0.868	1.796
8	6.58	4.88	5.76	1.783	1.796
9	5.68	5.44	5.56	1.066	1.796
10	6.19	4.96	5.52	1.342	1.816

DISCUSSION

Certification results

Analysis of the results of the stability test at storage temperature confirmed the stability of the material for almost four years. As long as this batch of certified RM (CRM) is available for use the monitoring of its stability will continue. The stability data at higher storage temperatures indicate that normal (air)mail without cooling of the RM is possible, if the transport time is limited to one week.

The values of $T_2/(l\text{-}1)$ found for the homogeneity of the batch are slightly higher than the theoretical optimum of 1. Recently a new procedure for (pre)mixing the highly contaminated milk powder with sterile milk powder using a mortar and pestle was introduced. Preliminary results obtained using this procedure indicate that the homogeneity of the mixed powder can still be improved.

The presence/absence procedure demonstrated that results obtained examining the batch of RM in various laboratories did not differ significantly from each other. As can be seen from Table 2 not all laboratories used the same selective enrichment broth nor the same isolation media and also the incubation conditions varied to some extent. So the variations in the experimental conditions within the ISO 6579 standard resulted in similar results.

The enumeration procedure, which was especially designed for this purpose, proved in the certification study that reproducible results can be obtained in various laboratories. The RM has a very low $N_{S,cfp}$ and the distribution of the cfp over the capsules is homogeneous. Although the

enumeration procedure will not often be used it was included in the certification study to demonstrate the reproducibility of the RM's homogeneity and level of contamination. As can be seen from Figure 3 there is a considerable variation in the number of cfp between capsules, which represents the normal (random) variation at such a low level of contamination. Some of the capsules did not contain any *Salmonella*. This is of special importance as these capsules can not be found positive using a presence/absence procedure.

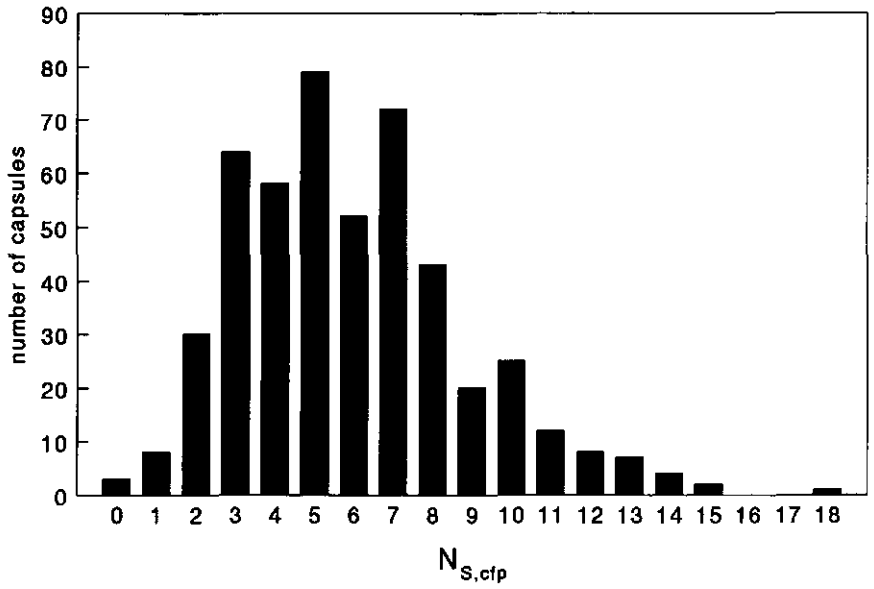

Figure 3: Frequency distribution of the enumeration of the RM, based on the examinations of 10 laboratories.

A significant difference between laboratories was found. However, none of the laboratories could be identified as an outlier.

The difference between the fraction of capsules not containing *Salmonella* as found by the enumeration tests (0.61 %) and by the presence/absence test (2.7 %) was not significant. Both values are certified according to the measurement procedure used. For a microbiological CRM method dependent certification is used as differences in procedures are likely to recover different parts of the bacterial population present in the CRM. For other CRM methods independent certification can be used, for example, when there exists a standard or SI unit to which the certified property can be related. In this way all the measurement procedures that are applied to this CRM have to lead to the same value.

Use of the CRM

The certified values presented in Table 4 cannot directly be used in practice. The certified values are based on the examination of a large number of capsules (more than 400 capsules), in practice a laboratory will examine only a small number of capsules. Depending on the number of capsules examined a certain number of capsules should be found positive for *Salmonella*. This is calculated by means of the binomial distribution (Wardlaw, 1985). Table 6 presents the relation between the number of capsules examined and the expected (probability ≥ 0.95) minimum number of capsules that should be found positive depending on the certified fraction of capsules not containing *Salmonella*.

Table 6. Expected minimum number of *Salmonella* isolations (95% probability) based on the number of capsules analysed and in relation to the measurement procedure.

Number of capsules examined	Fraction of negative capsules	
	0.61 %[a]	2.7 %[b]
5	5	4
6	6	5
7	7	6
8	8	7
9	8	8
10	9	9
11	10	10
12	11	11
13	12	12
14	13	12
15	14	13
16	15	14
17	16	15
18	17	16
19	18	17
20	19	18

a certified fraction of capsules in which no *Salmonella* could be detected according to the enumeration procedure.

b certified fraction of capsules in which no *Salmonella* could be detected according to the presence/absence procedure.

The more capsules that are examined simultaneously, the better the performance of the procedure can be judged. For example examining five capsules by the presence/absence procedure and finding four *Salmonella* isolations (which is an acceptable result) does not mean that when examining 10 capsules eight *Salmonella* isolations are acceptable (in this case at least nine *Salmonella* isolations are expected).

The performance of the presence/absence procedure for *Salmonella* as used for the certification study does not include the selectivity of the procedure (ability to isolate *Salmonella* in the presence of competitive micro-organisms). To test the influence of a certain (food)product (matrix effect) an RM is added to the homogenized food sample (In 't Veld *et al.*, 1992). The number of *Salmonella* isolations obtained will in most cases be lower than is expected without the addition of the food sample.

To compare the performance of various selective enrichment broths, it is advisable to inoculate the selective enrichment broths from the same Buffered Peptone water culture. If one of the selective broths obtains a positive *Salmonella* isolation (meaning that the capsule examined was contaminated) all procedures should obtain a positive *Salmonella* isolation. The overall performance (number of capsules found positive with at least one selective enrichment broth) should conform to the expected number of *Salmonella* isolations.

In the newsletter no. 4 of SM&T (Anon., 1994b) it is stated that a CRM is used to verify that a given method, as applied under given circumstances in a given laboratory, provides satisfactory results. In this case the given circumstances is the standardized method used to certify the material. A CRM is not used routinely for statistical control of methods as they are too expensive for that purpose. Instead of a CRM, internal quality control samples should be used or the non-certified RM obtained from outside the laboratory. These samples are used for routine quality control to demonstrate the quality of analysis from day to day. In this way no comparisons with other laboratories are made. A CRM is used incidentally to demonstrate that the performance is on the right level by comparing the laboratories' results to the certified value and, therefore, to other laboratories.

ACKNOWLEDGEMENTS

The authors wish to thank the following participants of the certification study: Dr. R. Betts, Campden and Chorleywood Food Research Association, United Kingdom; Mr. B.J. Hartog, TNO-Nutrition, The Netherlands; Dr. J. Kramer, University of Bonn, Germany; Dr. V. Sanguinetti, Universita di Bologna, Italy; Prof. M. Stecchini, Instituto di Tecnologie Alimentari, Italy; Mr. H. Stegeman, RIKILT-DLO, The Netherlands; Prof. Dr. P. Teufel, Bundesgesundheitsamt - Robert Von Ostertag Institute, Germany; Dr. I.M. Vicente da Cruz, Depart. de Tecnol. das Indust. Alimentares, Portugal; Mrs. G. Vlaemynck, Rijkszuivelstation, Belgium; Dr. C. Wray, Ministry of Agriculture, Fisheries and Food, United Kingdom. Dr. J.A. Hoekstra and Dr. S.H. Heisterkamp for the statistical analysis of the certification study. This work has been supported by the EC, Standards, Measurements and Testing Programme under contract no MAT1-CT92-0001.

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The certification of a reference material for the evaluation of methods for the enumeration of *Bacillus cereus*

ABSTRACT

A reference material containing *Bacillus cereus* was certified by the Community Bureau of Reference (BCR) for its number of colony forming particles (cfp) in 0.1 ml reconstituted capsule solution. To this end, a batch of *ca* 15,000 capsules was produced and tested for its homogeneity and stability. The variation in the number of cfp between capsules (homogeneity) was found to be not significantly different from a Poisson distribution. Stability was tested for extended periods at storage temperature (-20 °C) and at various higher temperatures up to 37 °C for four weeks to simulate transport conditions. Only at 37 °C did a small but significant decrease in the number of cfp occur. At -20 °C, no decrease in the number of cfp was observed over a period of *ca* four years. For certification, 12 laboratories determined the number of cfp on two agars Mannitol Egg-Yolk Polymyxin agar (MEYP, incubated at 30 °C) and on Polymyxin pyruvate Egg-yolk Bromothymol blue Agar (PEMBA, incubated at 37 °C). The certified geometric mean value on MEYP after 24 h of incubation was 53.4 cfp per 0.1 ml of the reconstituted capsule solution (95 % confidence interval 51.7 - 55.2) and on PEMBA 55.0 (95 % confidence interval 52.8 - 57.4). Based on these certified values user tables were constructed specifying the 95 % confidence limits when testing smaller number of capsules, as would be done in individual laboratories. Based on the information on homogeneity, stability and the certification study, the BCR decided to certify the material as CRM 528.

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Accepted for publication in the **Journal of Applied Microbiology**.

INTRODUCTION

Bacillus cereus is a spore forming organism frequently found in various types of food. Normally *B. cereus* is present in low levels but when high levels are present it is capable of producing two types of toxins (entero and emetic toxins) that can cause food poisoning. Several enterotoxins are known at present (Agata *et al.*, 1995; Beecher and Wong, 1994; Granum *et al.*, 1996) and one type of emetic toxin, called cereulide (Agata *et al.*, 1994). The best-characterised enterotoxin is the HBL-complex, a toxin consisting of three units (B, L_1 and L_2) that are all needed for activity. Enterotoxins are mostly produced in protein rich foods such as meat, emetic toxins in starch rich foods such as rice and pasta (Granum, 1997). To be able to produce toxins at a level that can cause food poisoning, *B. cereus* has to be present at high levels (> 10^5 cells per gram). In The Netherlands *ca* 19 % of all foodborne cases of disease with known aetiology are caused by *B. cereus* (Notermans and van de Giessen, 1993). Recently, the formation of enterotoxin by psychrotrophic strains of *B. cereus* in milk became a focus of interest (Christiansson *et al.*, 1989; van Netten *et al.*, 1990).

For the enumeration of *B. cereus* in foods several selective media are available: MEYP agar as described in ISO 7932 (Anon., 1993), PEMBA agar (Holbrook and Anderson, 1980) or KG agar (Kim and Goepfert, 1971). All three media contain egg yolk in order to exhibit the lecithinase reaction as a characteristic feature of *B. cereus*.

To be able to rely on results obtained in different laboratories quality assurance principles have to be applied by the laboratories. Reference Materials (RMs) and Certified RMs (CRMs) are useful tools in this respect. To be able to produce an (C)RM the number of organisms present must be distributed homogeneously over the samples and must remain stable over a long period of time. Spores were used for the production of the CRM containing *B. cereus* as they easily survive adverse environmental conditions such as heat, pH, low water activity etc. The production process for this CRM is comparable to other CRMs developed by the National Institute of Public Health and the Environment, such as *Salmonella* (In 't Veld and Notermans, 1992) or *Listeria monocytogenes* (In 't Veld *et al.*, 1995). The difference between an RM and CRM is the degree of accuracy of the (certified) values stating the number of organisms present in the material. In the ISO guide 35 (Anon., 1989) a CRM is defined as an RM that has been certified for its properties by means of a technically valid procedure and should be accompanied by a certificate issued by a certifying body. The certifying body in this respect is the Community Bureau of Reference (BCR) of the European Union.

MATERIALS AND METHODS

Preparation of the material

Bacillus cereus (ATCC 9139) was streaked for purity on sheep blood agar (SBA) and incubated for 24 h at (37 ± 1) °C. After incubation a single colony was suspended in 10 ml peptone saline solution (PS) by mixing on a vortex mixer. This suspension was used to inoculate 10 plates (0.3 ml per plate) containing the Polymyxin pyruvate Egg yolk Mannitol Bromothymol blue Agar (PEMBA) of Holbrook and Anderson (1980) prepared from original ingredients. The PEMBA plates were used for rapid sporulation of *B. cereus* and were incubated at (37 ± 1) °C. After 24 h incubation five ml PS was added to each plate and the

cells suspended with the use of a sterile glass spreader. The suspension from each plate was pipetted into a single tube and heated for 10 minutes in a waterbath at (80 ± 0.5) °C to inactivate vegetative cells.

The heated suspension was added to three litres of sterilised milk (evaporated to a dry mass concentration of 240 $g.l^{-1}$, dry fat mass concentration 40 $g.l^{-1}$), which was then spray dried using a Niro mobil minor spray dryer operated at an inlet temperature of *ca* 190 °C and an outlet temperature of *ca* 70 °C. The highly contaminated milk powder (HCMP) thus obtained was sealed in a polyethylene bag (0.2 mm thick) and stored at 5 °C until required.

To obtain the desired number of colony forming particles (cfp) in 0.1 ml of the reconstituted capsule solution, one g of the HCMP was mixed with a total of seven kg of sterile skim milk powder. The milk powder used for mixing was a commercially available skim milk powder sterilised by gamma irradiation with a dose of 10 kGy. The mixing of the HCMP with the skim milk powder was done in 13 steps. For each step equal amounts of contaminated milk powder were mixed with sterile milk powder, except for the last step in which *ca* four kg contaminated milk powder was mixed with *ca* three kg of sterile milk powder. The first eight steps were done using a mortar and pestle. Each of these steps consisted of mixing the powder for 15 - 20 seconds using the pestle, followed by remodelling of the powder using a paper card, this procedure was repeated three times. The remaining five steps were done in a 17 litre stainless steel drum with a Turbula type T 10 b mixing apparatus for one hour for each step.

The mixed powder was filled into gelatin capsules with an aluminium filling apparatus in a laminar air flow cabinet using 19 g powder for 60 capsules (0.317 g for each capsule). Before filling, the empty capsules were sterilised by gamma irradiation with a dose of 10 kGy. The first stage was to fill two sets of 60 capsules with powder obtained from different places in the mixed powder, in order to determine the level of contamination and homogeneity of the powder. Ten capsules from each of these two sets of 60 capsules were analysed in duplicate on MEYP and SBA. Before use the capsules were reconstituted as follows:

a: filling of test tubes (diameter 26 mm) with 10 ± 0.2 ml of peptone saline solution.
b: pre-warming of the tubes in a waterbath maintained at (38.5 ± 0.5) °C for 30 minutes.
c: addition of the capsules to the test tubes and mixing on a Vortex mixer for a few seconds 10, 20 and 30 minutes after the addition of the capsule, leaving the tubes outside the waterbath for as short a time as possible.
d: after the last mixing transferring the tubes from the waterbath to iced water.
e: using the dissolved capsule solution within 2 hours.

The plates were incubated at 30 °C for 24 hours.

After a first check whether or not the results met the criteria set, the entire batch of mixed powder was filled into gelatin capsules. Two capsules were taken from each filling of 60 capsules and were used to determine the variation in mass of the capsules (including the capsule itself). The variation in mass of the empty capsules is negligible in comparison to the filled capsules. From these capsules a further 20 capsules were selected at random to determine the level of contamination of the final batch on MEYP and SBA as described above.

Homogeneity studies

The variation in number of cfp between replicates from one reconstituted capsule (T_1) and between replicates from different reconstituted capsules of a single batch (T_2) were tested separately (Heisterkamp *et al.*, 1993). The formulas for the T_1 and T_2 test were published earlier by In 't Veld *et al.* (1993). In the case of a Poisson distribution T_1 and T_2 follow a χ^2-distribution with respectively $\{I \bullet (J\text{-}1)\}$ and $(I\text{-}1)$ degrees of freedom (I is the number of capsules, J is the number of replicates). In this case, the expected values of T_1 and T_2 are the same as the number of degrees of freedom. Hence, $T_1/\{I \bullet (J\text{-}1)\}$ and $T_2/(I\text{-}1)$ are expected to be equal to one. For the variation between replicates of different capsules of a single batch, the Poisson distribution is theoretically the smallest possible variation which could be achieved. However, overdispersion between capsules is expected and $T_2/(I\text{-}1)$ will usually be larger than one (Heisterkamp *et al.*, 1993). The homogeneity of the batch was determined based on the results of the stability test at higher temperatures. Only the results from the capsules stored at -20 °C were used. The five T_2 values for each medium at different points in time were summed to obtain the overall dispersion test statistic (T_{hom}) for SBA, MEYP and PEMBA. The number of degrees of freedom of T_{hom}, called N, is the sum of the number of capsules examined minus the number of homogeneity tests carried out (so $N = 25 - 5$). The value of T_{hom}/N is expected to be equal to one.

Stability studies

Two types of stability tests were done, a long term stability test at storage temperature (-20 °C) and a short term stability test at higher temperatures simulating transport conditions. For testing the stability of the materials stored at -20 °C, ten capsules were examined in duplicate at regular time intervals on MEYP and SBA. The stability of the material stored at higher temperatures was determined at four different temperatures. The temperatures tested were -20 °C (reference), 22 °C, 30 °C and 37 °C. Once a week, over a period of four weeks, five capsules from each storage temperature were examined in duplicate on MEYP and SBA. Analyses on PEMBA were also made in parallel to the stability test at higher temperatures but only for the capsules stored at -20 °C. The results were used to determine the overall measure of dispersion for PEMBA (see above under homogeneity studies). The counts obtained for each storage temperature were $\log_{10}$ transformed and analysed using linear regression.

Certification study

For the study twelve laboratories were selected, all experienced with the enumeration of *B. cereus* and with the reconstitution procedure for the RMs. Each participating laboratory received, by courier service, a parcel containing two series of eight numbered capsules each. The capsules were stored at -20 °C upon receipt. The study was carried out according to the fixed time schedule laid down in the analytical protocol. After reconstitution of each capsule the number of cfp of *B. cereus* in 0.1 ml capsule solution was determined for each capsule in duplicate on two agars (MEYP and PEMBA) and a third optional agar (Sheep

Blood Agar, SBA). The method for inoculation and incubation of the plates was described in a Standard Operating Procedure. The procedure for incubation and confirmation of *B. cereus* using MEYP was based on ISO 7932 (Anon., 1993) and that for PEMBA on method no L 00.00 - 25 (Anon., 1992) of the German Federal Food Law with confirmation according to ISO 7932. Laboratories incubated SBA according to their own procedures.

The MEYP plates were incubated for 48 h at 30 °C and the number of colonies counted after 24 h and 48 h. The PEMBA plates were incubated for 48 h at 37 °C and counted after 24 and 48 h. The plates were relabelled with random numbers before the first count, after 24 h incubation, was made. The colonies were marked on the base of the plates and after 48 h incubation only the additional colonies were counted and reported (the same random numbers were used for counting after both 24 and 48 h incubation). Each laboratory selected at random a number of colonies from each medium for confirmation. Confirmation tests were done as described in the ISO 7932.

The results of the analyses were reported on a test reporting form. Each laboratory was free to analyse the capsules in one or two series as long as the time for inoculation of all dishes in a series was not longer than 2 h. Each series of capsules contained one (blind) control sample consisting of a gelatin capsule filled with sterile milk powder.

The relationship between reconstitution time and the number of cfp per 0.1 ml sample on MEYP and PEMBA was tested in addition to the certification study by the organising laboratory. For this purpose 10 capsules were examined in two series of five capsules on separate days using separate batches of media. For each capsule, portions of one ml were taken after 30, 40, 50, 60, 75 and 90 minutes of reconstitution. The portions were directly put in iced water before inoculation of the plates. The incubation conditions were identical to those used for the certification study. The plates were counted after 24 h incubation.

Statistical analysis

The results from the blank capsules were excluded from the statistical analysis. The statistical analysis performed on the data is described in detail in a separate report (Heisterkamp *et al.*, 1993). A summary of the analysis carried out is presented below:

First the variation between the duplicate counts was calculated per laboratory, per medium and per incubation period by means of the T_1 test. It was expected that replicate counts would follow a Poisson distribution, in which case the T_1 test statistic would follow a χ^2-distribution with I (= the number of capsules examined) degrees of freedom ($\alpha = 0.05$). If the T_1 result of a laboratory was significantly different from a χ^2-distribution, the T_1 results of duplicates per capsule were further considered. Problems related to single capsules were concluded if the T_1 value per capsule was significantly different from a χ^2-distribution with one degree of freedom and at $\alpha = 0.05$ divided by I, where I is the total number of capsules examined for one medium (maximum 14) (Bonferroni's rule).

The variation between capsules was calculated per laboratory, per medium and per incubation period by means of the T_2-test. The T_2 value was divided by (I-1) (number of degrees of freedom for the T_2 test). For each laboratory the value of $T_2/(I\text{-}1)$ was compared to the overall measure of dispersion (T_{hom}/N) for that medium. For each laboratory the value of $T_2/(I\text{-}1)$ was compared to the T_{hom}/N for that medium by means of a one sided F-test

(Heisterkamp *et al.*, 1993) with *I*-1 and N = 20 degrees of freedom, with a probability of 95 %. The critical threshold was calculated as $F_{I-1,N} \bullet \Sigma T_{hom}/N$ for each laboratory separately depending on the number of capsules examined.

The laboratory means were compared for MEYP and PEMBA separately and per incubation period by using an analysis of variance (Snedecor and Cochran, 1967) on the $\log_{10}$ transformed results. The Grubbs' test (Anon., 1988) was applied to indicate outliers. For SBA no analysis of variance was carried out due to the limited number of laboratories using this medium.

From the analysis of variance the variance components were calculated per medium and per incubation period. These variance components were used to calculate the certified values and 95 % confidence limits for MEYP and PEMBA. The 95 % confidence limits for SBA were calculated as ± 2 times the standard deviation (based on $\log_{10}$ transformed counts). The mean values and confidence limits were back transformed to the original scale.

Technical discussion of analytical procedures

Before the results of the statistical analysis were presented to the participants a discussion on technical details of the analytical procedure (as laid down in the analytical protocol) was held. This discussion was used to determine whether deviations from the analytical protocol would influence the counts reported. If an influence was expected or could not be excluded the count(s) were not used in the final analysis of the data and thus in the calculation of the certified value(s).

RESULTS

Preparation of the material, homogeneity and stability

The highly contaminated milk powder (HCMP) contained *ca* 10^7 cfp.g^{-1} powder. After mixing, the level of contamination and homogeneity was checked against predefined criteria (see Table 1). The results of the first check indicated that all requirements were met, so the entire batch of mixed milk powder was filled into capsules. In total 15,600 capsules were produced. After the entire batch was filled into capsules a check was made to test the variation in weight of the filled capsules. The criterion for the variation in mass was: the standard deviation in mass divided by the average mass of a filled capsule should be less than 0.03. Two sets of respectively 210 and 310 capsules were weighed. The variation in mass was 0.017 and 0.015, respectively. From these capsules a further 20 capsules were selected at random to determine the level of the final batch on MEYP and SBA as described above. The results of this analysis are also presented in Table 1 together with the criteria for acceptance of the final batch.

Homogeneity studies

The T_2 values needed to determine T_{hom}/N were calculated for SBA, MEYP and PEMBA separately. All T_1 values, except three out of 15, were not significantly different from the χ^2-distribution. This means that the variation between analytical portions of one reconstituted capsule was according to a Poisson distribution.

Two out of 15 $T_2/(I-1)$ values were above the limit of 2. The factor T_{hom}/N was found to be 0.71 on MEYP, 1.14 on PEMBA and 1.27 on SBA. The mean dispersion values for each medium were smaller than two and none were significantly different from the χ^2-distribution. It was, therefore, concluded that the variation between samples of different capsules was according to a Poisson distribution.

Table 1. Results and criteria of testing the batch of milk powder directly after mixing (first check) and after filling of all capsules.

Medium		Arithmetic mean count[a]	T_1[b]	$T_2/(I-1)$[e]
MEYP	first check:			
	set 1	57.0	8.78[c]	0.97
	set 2	59.3	16.0[c]	2.19
	both	58.1	24.8[d]	1.56
SBA	first check:			
	set 1	60.3	6.14[c]	0.83
	set 2	60.5	13.5[c]	0.56
	both	60.4	19.7[d]	0.66
MEYP	all capsules	51.6	15.0[d]	1.24
SBA	all capsules	52.5	13.5[d]	0.77

a criterion for number of cfp: between 40 and 70 cfp per 0.1 ml reconstituted capsule.
b criterion for T_1: not significantly different from χ^2 (limits footnotes c and d).
c 95 % confidence interval: 3.25 - 20.5.
d 95 % confidence interval: 9.59 - 34.2.
e criterion $T_2/(I-1)$: ≤ 2.

Stability studies

The results of the analyses from the start in January 1994 up to November 1997 on MEYP are presented in Figure 1. The results for SBA (t-value = 0.50) did not indicate a significant ($\alpha = 0.05$) change in number of cfp over the period tested when using linear regression. However, on MEYP (t-value 2.52) a small but significant ($p = 0.012$) increase in the number of cfp was found. The rate of increase observed was 0.00015 $\log_{10}$ units per week, corresponding to an average increase of one cfp per year.

Figure 2 presents the results of the stability test at higher temperatures using MEYP. Only at 37 °C a significant decrease of the mean number of cfp of 0.34 % per day was observed. Based on these results it is concluded that shipment of the materials at ambient temperature will have no effect on number of cfp.

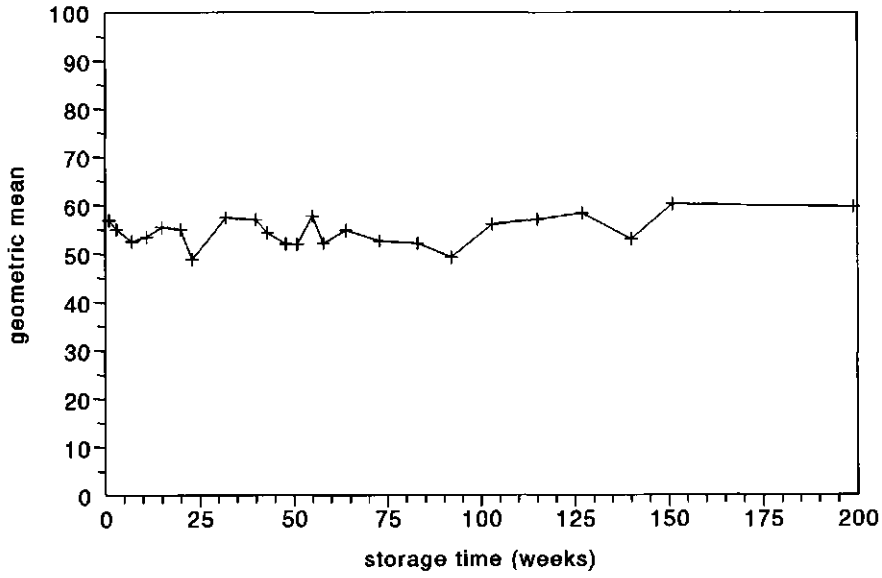

Figure 1. Results of stability tests at storage temperature (-20 °C) on MEYP over a period of 200 weeks.

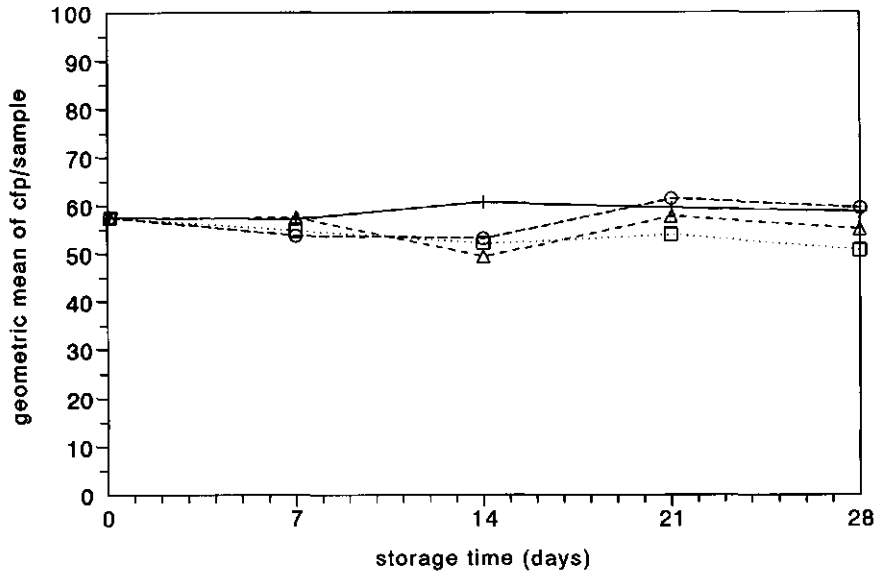

Figure 2. Results of stability tests at various temperatures (- 20 °C +, 22 °C Δ, 30 °C O, 37 °C □) over a period of four weeks.

Relationship reconstitution time and number of cfp

The results of the tests to determine the relationship between reconstitution time and the number of cfp per 0.1 ml sample are presented in Figure 3. The results are averaged for the two sets of five capsules each.

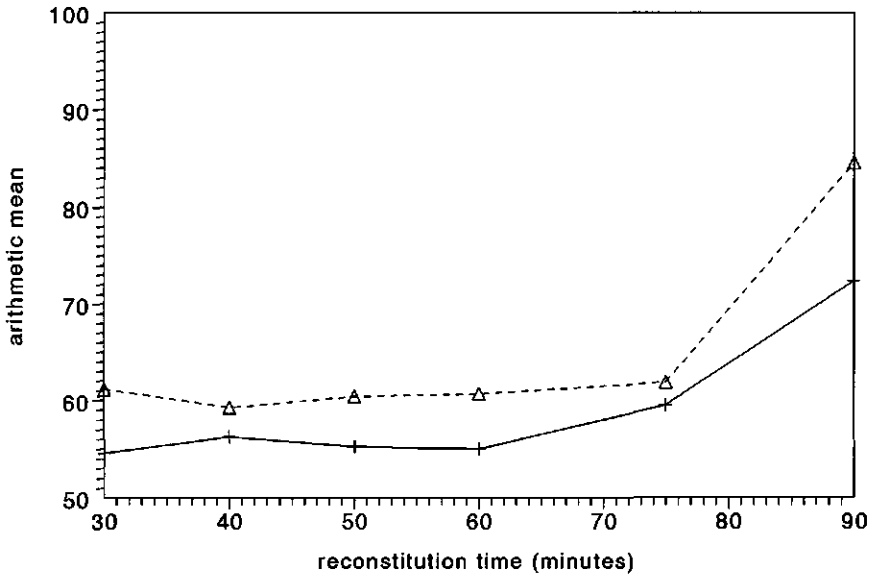

Figure 3. Relation between reconstitution time at 38 °C and the arithmetic mean of the number of cfp per 0.1 ml sample on MEYP (+) and PEMBA (<).

Certification study

Technical discussion

Before use, the capsules needed to be reconstituted according to the protocol. The period for reconstitution specified in the protocol was 30 minutes. The laboratories used between 30 and 40 minutes for reconstitution, except for one laboratory that used *ca* 67 minutes. The results from the latter laboratory were not used for certification because the period was too long and multiplication of the spores could not be excluded (see Figure 3). No other deviations from the protocol were identified that (could have) influenced the results.

The results reported for the two blank capsules did not show any false positive counts. Confirmation tests on selected colonies according to the ISO 7932 standard showed that all colonies tested were *B. cereus*. Laboratories tested a total of 120 colonies from both MEYP and PEMBA and 40 colonies from SBA.

Statistical analysis

The results of the T_1 tests, T_2 tests and the geometric means are presented in Table 2 for MEYP and PEMBA agar and in Table 3 for SBA. Results are based on the examination of 14 capsules per laboratory.

Table 2. Results per laboratory on MEYP and PEMBA counted after 24 h incubation.

Labcode	MEYP			PEMBA		
	T_1	$T_2/(I\text{-}1)$	Geometric mean	T_1	$T_2/(I\text{-}1)$	Geometric mean
1	5.37	1.83[b]	57.5	6.50	1.69	62.2
2	22.1	1.73[b]	53.9	11.4	1.14	55.8
3	7.20	1.09	50.9	14.0[d]	2.16[d]	52.4[d]
4	8.57	0.52	55.2	11.0	2.23	55.6
5	14.7	1.40	50.5	17.7	1.48	54.9
6	25.0[a]	1.09	53.8	15.0	0.37	53.1
7	10.3	2.78[b]	53.9	4.98	1.94	54.3
8	12.3	0.60	49.2	6.53	0.83	50.0
9	21.7	1.01	55.1	11.6	1.67	57.6
10	31.8[a]	1.59	56.5	13.8	1.99	58.8
11	18.3	1.98[b]	51.5	21.8	3.11[c]	51.8

a significantly different from χ^2-distribution (critical value 23.7 at $I = 14$ and $\alpha = 0.05$).
b significantly different from T_{hom}/N (critical value $T_{hom}/N \bullet F_{(I-1),N} = 1.59$ at $I = 14$ and $\alpha = 0.05$).
c significantly different from T_{hom}/N (critical value $T_{hom}/N \bullet F_{(I-1),N} = 2.57$ at $I = 14$ and $\alpha = 0.05$).
d *results based on the examination of 13 capsules.*

Table 3. Results of the laboratories using SBA.

Labcode	T_1	$T_2/(I\text{-}1)$	$T_{hom}/N \bullet F_{(I-1),N}$	Geometric mean
1	12.8	0.78	2.85	51.5
4	8.29	0.97	2.85	48.4
11	13.0	1.05	2.85	52.8
12	19.7	2.52	2.85	47.7

The results presented are obtained after 24 h incubation, results obtained after 48 h incubation were similar (results not presented).

For various laboratories it was shown that the T_1 values were significantly different from the χ^2-distribution. For laboratory 3 it was shown that the result of the T_1 value of one capsule on PEMBA was a problem, the results of this capsule were excluded for certification due to problems with pipetting the capsule solution (formation of foam in the tube). For the other laboratories with a significant T_1 value no capsule could be identified as causing problems. For these laboratories the results were therefore considered as unexplained deviations and therefore retained for analysis.

The results of $T_2/(I\text{-}1)$ on MEYP and PEMBA are presented in Figure 4. For MEYP three laboratories found a higher value for $T_2/(I\text{-}1)$ than the critical threshold, these results were related to the exceptionally low value for T_{hom}/N for this medium. For PEMBA one laboratory found a higher value for $T_2/(I\text{-}1)$ than the critical threshold, this result might be explained by random variation of $T_2/(I\text{-}1)$.

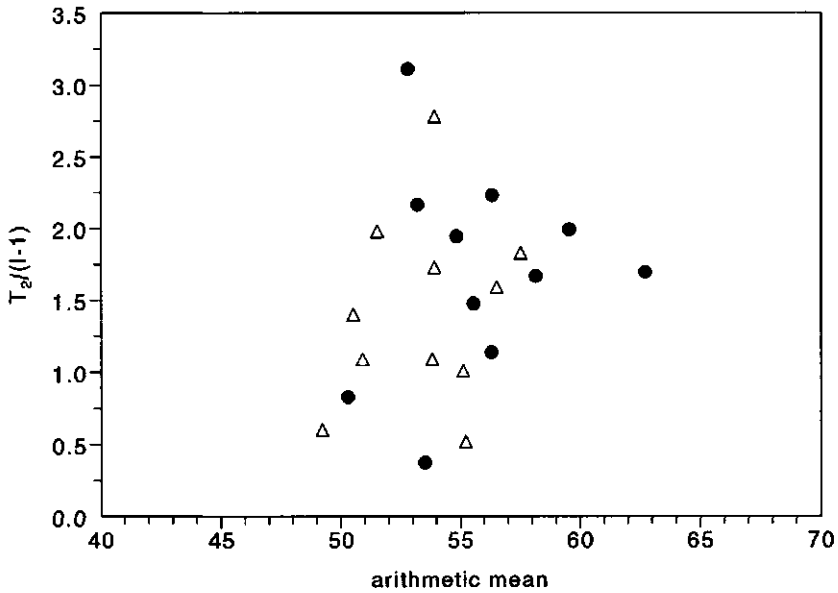

Figure 4. Relation between dispersion test statistic ($T_2/(I\text{-}1)$) and arithmetic mean number of cfp per 0.1 ml sample found on MEYP (Δ) and PEMBA (•) after 24 h incubation.

Significant differences between laboratory means existed for MEYP and PEMBA after both 24 and 48 h incubation. However, no outlier was indicated in the group of laboratories. Thus it was concluded that the results were obtained from a sufficiently homogeneous group of laboratories.

The certified values are presented in Table 4. The indicative values on SBA resulted in a geometric mean number of cfp of 50.1 after 24 h incubation (95 % confidence interval 48.4 - 51.7) based on the examination of 56 capsules in four laboratories.

Table 4. Certified values of CRM 528 on MEYP and PEMBA after 24 and 48 h incubation.

Procedure	Certified value[a]	95 % Confidence limits		Sets of accepted results
		lower limit	upper limit	
MEYP (ISO 7932) after 24 h incubation	53.4	51.7	55.2	11[b]
MEYP (ISO 7932) after 48 h incubation	53.7	52.1	55.4	11[b]
PEMBA (L 00.00 - 25)[d] after 24 h incubation	55.0	52.8	57.4	11[c]
PEMBA (L 00.00 - 25)[d] after 48 h incubation	55.8	53.6	58.0	11[c]

a this value is the geometric mean of 11 accepted sets of data, independently obtained by 11 laboratories.
b comprising the results of 154 capsules.
c comprising the results of 153 capsules.
d German Federal Food Law method number.

DISCUSSION

Certification results

The batch of the *B. cereus* RM fulfilled the requirements of a CRM to a high degree. The homogeneity test of the material indicated no significant difference from a Poisson distribution, irrespective of the type of medium used. However, in the certification study a number of laboratories found a significantly higher dispersion than expected from the results of the producing laboratory, especially on MEYP. In Table 2 it can be seen that the values for $T_2/(I\text{-}1)$ are, for most laboratories, higher than the value of one for a Poisson distribution. The average value for $T_2/(I\text{-}1)$ on MEYP was 1.4 and on PEMBA 1.7. However, only one out of the 11 laboratories found a value for $T_2/(I\text{-}1)$ significantly higher than one on MEYP and one laboratory on PEMBA. So, the homogeneity of the material is good which can also be derived from the narrow confidence interval of the certified values. Also, the variance components indicate that the variation resulting from differences between capsules is small compared to the variation due to replicates. The variation between laboratories is also small and comparable to the variation between capsules. This is reflected in the small differences between the mean counts per laboratory (see also Table 2).

Bacterial counts of the stability test at storage temperature were stable for almost four years. The increase in the number of cfp observed on MEYP is regarded as of no or minor importance as the increase is very small. As long as this batch of certified RM (CRM) is available for use the monitoring of its stability will continue. The stability data at higher

temperatures indicate that normal (air)mail without cooling of the RM is possible for shipment to other laboratories, if the transport time is limited to one week.

The reconstitution procedure is regarded as the main critical factor to obtain reproducible results. In the certification study one laboratory used 65 – 70 minutes for reconstitution of the capsules whereas the protocol specified 30 – 40 minutes. Additional tests indicated that up to 60 min no increase in the number of cfp occurs, but after 75 min a substantial increase was observed. Hence, the results of this laboratory were excluded from further analysis. These data also indicate that the time limits in the protocol are not that critical and can be exceeded as long as the total time needed for reconstitution is less than one h.

Use of the CRM

The certificate states the certified values with their 95 % confidence limits. These certified values are obtained from a large number of examinations carried out during the certification study. As the user of a CRM will examine only a small number of capsules, user tables are prepared for the interpretation of results in a single laboratory presenting the 95 % confidence limits for different combinations of capsules and replicates per capsule that are likely to be used in practice. These limits are presented in Figure 5.

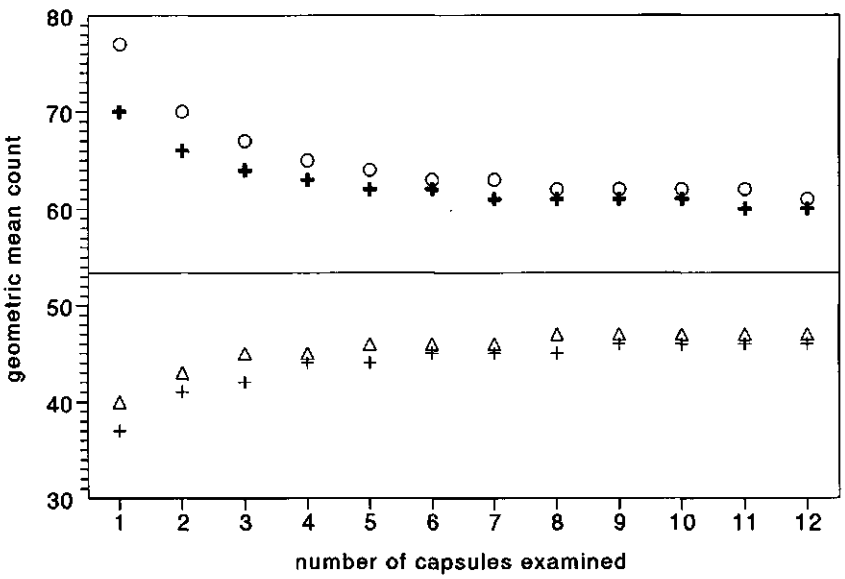

Figure 5. The 95 % confidence limits of the geometric mean of number of cfp per 0.1 ml on MEYP after 24 h incubation for different combinations of capsules and replicates, calculated from the certified value and the variance components of *Bacillus cereus* CRM 528.

The limits are calculated using the certified value and variance components per method on the $\log_{10}$ scale. Back-transformation of the obtained values for the upper and lower limit will give the limits on the normal scale. Due to the back-transformation the values on the original scale represent geometric mean values. The limit values are rounded to whole counts. For the lower limit values are rounded to the lowest whole count, for the upper limit values are rounded to the highest whole count.

In Figure 5 it becomes clear that the examination of more than a few capsules does not lead to a substantial improvement of the confidence limits. It is therefore recommended to test four capsules in duplicate each time the CRM is used.

From all the results presented it can be concluded that the enumeration of *B. cereus* on MEYP or PEMBA using the CRM is highly reproducible or, in other words, is very precise.

ACKNOWLEDGEMENTS

The authors wish to thank the following participants of the certification study: Dr. R. Betts, Campden and Chorleywood Food Research Association, United Kingdom; Mrs. M. de Buyser, Laboratoire Central d'Hygiène d'Alimentaire, Ministère de l'Agriculture, France; Mr. B.J. Hartog, TNO-Nutrition, The Netherlands; Dr. J. Kramer, University of Bonn, Germany; Dr. V. Sanguinetti, Universita di Bologna, Italy; Prof. M. Stecchini, Istituto di Tecnologie Alimentari, Italy; Mr. H. Stegeman, RIKILT-DLO, The Netherlands; Prof. Dr. P. Teufel, Bundesgesundheitsamt - Robert Von Ostertag Institute, Germany; Dr. I.M. Vicente da Cruz, Depart. de Tecnol. das Indust. Alimentares, Portugal; Mrs. G. Vlaemynck, Rijkszuivelstation, Belgium; Dr. C. Wray, Central Veterinary Laboratory, Ministry of Agriculture, Fisheries and Food, United Kingdom. Mrs. N.G.W.M. van Strijp-Lockefeer and Mrs. J.A. van Dommelen for the statistical analysis of the certification study. This work has been supported by the EC, Standards, Measurements and Testing Programme under contract no MAT1-CT92-0001.

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An alternative method for the preparation of highly contaminated milk powder for use in the production of microbiological reference materials.

ABSTRACT

Highly contaminated milk powders (HCMP), for the preparation of microbiological reference materials, were produced using the STREA-1 fluid bed dryer. To achieve this concentrated milk containing the target micro-organism, was sprayed onto sterile milk powder held fluidised by means of air at 40 °C, a process known as spray granulation. Firstly, the optimal drying conditions for the homogeneity of an HCMP using *Bacillus cereus* spores were determined and evaluated. Then several other organisms (*Listeria monocytogenes, Salmonella enteritidis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Campylobacter jejuni*) were dried using this procedure and also tested for their homogeneity and stability when stored at both -20 °C and 22 °C. *C. jejuni* did not survive the drying procedure at all, no organisms could be recovered from the milk powder the day after drying. The homogeneity of the HCMPs varied widely. The time used for spraying the milk onto the powder was critical for obtaining homogeneous HCMPs. At 22 °C a non linear decrease in the number of cfp was found for most strains; this was not as good as material prepared by silica gel drying and/or spray drying. Stability at -20 °C was satisfactory and was comparable to freeze drying. Production of an HCMP by using the STREA is therefore regarded as an alternative method to production of HCMP by spray drying.

INTRODUCTION

Microbiological reference materials are prepared from highly contaminated milk powder (HCMP) produced by spray drying a bacterial culture in evaporated milk. The HCMP thus obtained is stored for a period of time to allow stabilisation of the number of viable bacteria in the powder. The time needed for stabilisation depends on the organism and varies widely. A stable *Bacillus cereus* RM was produced directly after spray drying, while that for *Listeria monocytogenes* took more than one year (see also chapter 1). After the HCMP has been stabilised it can be stored for many years at -20 °C and used as a stock powder for the preparation of batches of RM. These are prepared by dilution (mixing) of the HCMP with sterile milk powder until the desired contamination level is obtained. Batches of RM produced over time from the same HCMP show similar or improved stability with increasing age of the HCMP, i.e. the rate of decrease in the number of cfp in the RMs is constant or even declines with the age of the HCMP.

The spray drying process has been proven successful for various organisms, for example *Salmonella typhimurium* (see chapter 3), *Bacillus cereus* (see chapter 4), *Listeria monocytogenes* (In 't Veld *et al.*, 1996), *Enterococcus faecium* (Mooijman *et al.*, 1994), *Enterobacter cloacae* (Mooijman *et al.*, 1995) and *Escherichia coli* (Mooijman *et al.*, 1996). However, some strains proved to be very difficult or impossible to stabilise by spray drying. Among these strains are *Pseudomonas aeruginosa*, *Aeromonas* spp. and *Campylobacter jejuni.*

The survival of bacteria during spray drying is essentially related to the outlet temperature of the spray dryer and the type of bacteria (Chopin *et al.*, 1977a/b; Fu and Etzel, 1995) but the injury to bacterial cells during spray drying is not identical to the injury from heating alone. Lievense and co-workers (1990) reported that bacterial inactivation following fluidised bed drying of *Lactobacillus plantarum* was a result of two mechanisms: thermal inactivation and inactivation due to dehydration. Thus low temperatures (≤ 40 °C) were recommended for drying *L. plantarum* in order to reduce the effect of thermal inactivation. For the production of milk powder spray dryers are usually operated at outlet temperatures ranging between 65 °C and 100 °C. Thus a temperature of *ca* 70 °C was used for the preparation of our HCMP. Lowering the outlet temperature for spray drying results in a higher water content and thus a higher water activity of the final powder (Chopin *et al.*, 1977 b). A higher water content or storage at higher relative humidity has an adverse effect on the survival of bacteria after drying (Thompson *et al.*, 1978; Palumbo and Williams, 1990). Chopin and co-workers (1978) found the greatest survival of various strains of *Staphylococcus aureus* when spray dried in milk and stored at 25 °C at a water activity (a_w) of 0.1. Survival at an a_w of 0.2 was almost the same as that at a_w 0.1; at an a_w of 0.3 survival was much reduced.

Sugars added to the heating menstruum also have an effect on the heat resistance of organisms. Corry (1974 and 1976a) demonstrated the effect of various sugars and polyols (at concentrations up to *ca* 1.5 M) on the heat resistance of *Salmonella* and osmophilic yeasts. Sucrose gave the greatest increase in heat resistance for the strains tested; the effect was related to the degree of plasmolysis of the cell and therefore to the degree of water extraction from the cell (Corry, 1976 b). Sugars can also be accumulated or produced

by micro-organisms themselves under conditions of osmotic stress in order to prevent plasmolysis of the cell. Not only are sugars accumulated or produced by micro-organisms as a reaction to osmotic stress, but also polyols, amino acids or betaines. All these compounds, accumulated or produced by micro-organisms, are referred to as compatible solutes (organic compounds compatible with cellular enzymatic activities and counter-balancing osmotic pressure) according to Brown (1976). Trehalose is considered to have a special role (Crowe *et al.*, 1984) as this sugar is present in some organisms (for example in the spores of certain fungi and *Saccharomyces cerevisiae*) that can survive complete dehydration. Janning (1995) tested the effect of the addition of sugar to milk containing various micro-organisms on the resistance of the organisms towards drying onto silica gel. For all organisms tested an improvement in survival just after drying and/or an improvement in the stability of the dried organisms stored at 25 °C was observed. In general though sucrose gave the best results, better than trehalose, although trehalose was used at a lower concentration due to solubility problems.

It would be advantageous to have an alternative method to spray drying that could produce a stock material (comparable to an HCMP produced by spray drying) for the production of RMs without the need to use high drying temperatures and thus reduce the amount of heat injury to the bacteria. Janning and co-workers (1994) developed a silica gel drying system as a model for determining the susceptibility of bacteria to drying at ambient temperature. Small quantities (1 g) of silica-gel were inoculated with a fixed volume (0.1 ml) of a suspension of a test strain suspended in concentrated milk. Various types of micro-organisms were dried by this method ranging from strains which it is known can be effectively dried by spray drying (for example, *Enterococcus faecium*) to strains which are difficult to stabilise by spray drying (for example, *Pseudomonas aeruginosa*). In general this silica-gel drying method, compared to spray drying, resulted in better survival of organisms through the drying process itself and also in comparable rates of reduction after drying for all the strains tested. For example, the regression coefficient of *E. coli* dried on silica-gel (Janning *et al.*, 1994) was -0.034 $\log_{10}$ cfp per day at 22 °C (tested over a period of *ca* 100 days). For the *E.coli* CRM produced by spray drying (Mooijman *et al.*, 1996) this was -0.02 $\log_{10}$ cfp per day at 22 °C (tested over a period of 28 days). The main advantage of the silica-gel system is the ease of drying a number of different strains at the same time, which is not possible with the spray drying method. Although the silica gel drying method showed promising results as a screening test it could not be regarded as an alternative to spray drying as only a limited number of samples can be prepared at any one time and the production of a stock powder is not feasible.

Freeze drying is often used for the preservation of micro-organisms (Heckley, 1985) and has been proven as a method that can be used for a wide range of micro-organisms. Freeze dried micro-organisms are contained in vials or ampoules under vacuum. Although freeze drying is commonly used for the production of samples used in proficiency testing (see also chapter 7), stability of these samples is needed for only a relatively short period of time (up to a few months). Little quantitative data are available on the survival of freeze dried organisms over time. Peterz and Steneryd (1993) tested the stability of freeze dried samples containing a mixture of organisms over a period of *ca* one year. The greatest decline in

numbers of organisms observed was *ca* 0.8 $\log_{10}$ units for coliforms; only for *Salmonella* (based on MPN determinations) and spores of *Clostridium perfringens* was no significant decrease observed. In principle freeze dried material can be used as a stock material for the production of an RM, however, the mixing properties of freeze dried material with milk powder were expected to be difficult and the stability of the freeze dried strain is likely to be changed or even disappear after removal of the vacuum from an ampoule or vial and subsequent mixing of the material.

A third alternative method is fluid bed drying. Fluid bed drying is a technique that uses (heated) air for both drying and to keep the product fluidised. The advantage of such a system is the relatively low temperature used for drying. The disadvantage is that liquids cannot be dried without the addition of a support material which then allows it to be extruded into a granulated product (Lievense, 1991); various types of polysaccharide are used as support material. Little is known about which micro-organisms can be dried by this method. Most of the research on fluid bed drying of micro-organisms is related to the drying of yeasts (Taeymans *et al.*, 1986; Hill, 1987a) which can be dried without the addition of a support material. Lievense and co-workers (1990) described the fluid bed drying of *Lactobacillus plantarum* using potato starch as support material.

A special form of fluid bed drying, called spray granulation or fluid bed spray drying, involves spraying a liquid on to a support material; an agglomeration occurs and the support material is then dried in the fluid bed both during and after the liquid is added. Spray granulation has been used for drying lactic acid bacteria (Hill, 1987b; Roelans and Taeymans, 1990; Zimmerman and Bauer, 1990). Various substances such as different types of flour, lactose, skim milk powder and even sodium chloride were used as support material (Roelans and Taeymans, 1990; Zimmerman and Bauer, 1990; Hill 1987b).

This chapter describes the spray granulation of various micro-organisms using milk powder as support material for the production of HCMPs. The HCMPs thus prepared, using the STREA-1 (Niro Aeromatic, Bubendorf, Switzerland), were tested for both homogeneity and stability.

MATERIALS AND METHODS

The experiments were divided into three parts. The aims of these parts were:

1. to determine the optimal operational conditions for drying the bacterial suspension.
2. to determine the homogeneity of the powders thus obtained and the recovery of the micro-organism both before and after dilution with sterile milk powder using an HCMP containing *B. cereus* spores.
3. to prepare and test the stability of HCMPs containing different test strains.

Determination of the optimal drying conditions with the STREA

The STREA dryer used (see Figure 1) consists of a glass column containing the milk powder. The milk powder is held fluidised by means of heated air; the amount of air used and the temperature are both controlled. The column contains a nozzle through which contaminated milk is sprayed on top of the fluidised milk powder. The air leaves the column

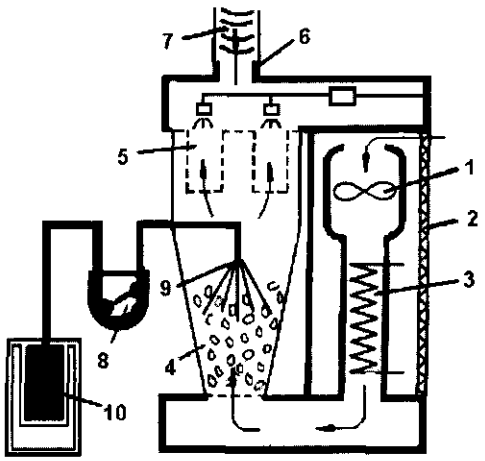
through four filter bags that are regular cleaned by means of blowing compressed air into the bag (blow back dwell). The inlet and outlet temperatures are recorded. The entire apparatus is placed in a laminar down flow cabinet to ensure sterility of the air used for the drying operation.

In the first part of the study the operational conditions required for drying a bacterial culture onto milk powder were determined. The criteria for the process conditions were:

- outlet temperature should not exceed 40 °C.
- a_w of the HCMP should be less than 0.3.

The process parameters to be determined were:

- temperature of drying (the relationship between inlet and outlet temperature).
- nozzle pressure and pump speed.
- pressure required for cleaning the outlet air filter bags (blow back dwell).
- quantity of milk suspension to be added to the milk powder.
- degree of fluidisation of the milk powder during drying.



1 = fan
2 = inlet air filter
3 = heating device
4 = fluidised milk powder
5 = outlet air filter bags
6 = air outlet
7 = outlet air pipe
8 = pump
9 = nozzle
10 = milk suspension

Figure 1. STREA spray granulation apparatus.

Determination of the homogeneity and recovery of *B. cereus* from the HCMP

Preparation of the HCMP

For preparation of the HCMP a spore suspension of *Bacillus cereus* was used. This suspension was prepared by inoculation of *B. cereus* (ATCC 9139) (0.2 ml of a reconstituted capsule from a previously prepared reference material) onto ten plates of Polymyxin pyruvate Egg yolk Mannitol Bromothymol blue Agar (PEMBA) (Holbrook and Anderson, 1980), prepared from original ingredients. The PEMBA plates were used for rapid

sporulation of *B. cereus* and were incubated at (37 ± 1) °C. After 24 h incubation five ml Peptone Saline solution (PS) were added to each plate and the cells suspended by means of a sterile glass spreader. The suspension from each plate was pipetted into a sterile test tube and heated for 10 minutes in a waterbath at (80 ± 0.5) °C to inactivate vegetative cells. The number of colony forming particles (cfp) of *B. cereus* per ml was determined both before and after the heat treatment. Counts were made on Mannitol Egg Yolk Polymyxin agar (MEYP) according to ISO 7932 (Anon., 1993) incubated for (24 ± 2) h at (30 ± 1) °C. Based on these counts spore suspensions were made by adding the appropriate quantity of spore suspension to 50 ml portions of sterilised milk (Friesche Vlag Halvamel, milk evaporated to a dry mass concentration of 240 $g.l^{-1}$, dry fat mass concentration 40 $g.l^{-1}$), to give spore levels of *ca* 10^5, 10^7 and 10^8 cfp per ml milk suspension. These suspensions were dried under the process conditions previously determined and described on page 96 (Drying conditions with the STREA). The milk powder used was γ-irradiated milk product 17 (Nestlé, Amsterdam, The Netherlands). The resulting HCMPs were named A, B and C for the low, medium and high levels of inoculation respectively.

Homogeneity

The homogeneity of the powder obtained by the spray granulation method was tested as described below. Part of the HCMPs were filled into gelatin capsules (0.27 g per capsule) in a laminar down flow cabinet. Ten capsules from each HCMP were reconstituted and after appropriate tenfold dilutions, duplicate counts per capsule were made on MEYP agar (0.2 ml spread plates for HCMP A, 0.1 ml spread plates for HCMP B and C). Reconstitution was performed as described in chapter 1 for the *B. cereus* RM. The resulting counts were analysed for their dispersion between duplicate counts (T_1 test) and the dispersion of the average counts between capsules (T_2 test). The formulae for the calculation of the T_1 and T_2 tests were presented in chapter 1.

Since the homogeneity is also influenced by mixing HCMP with sterile milk powder, the homogeneity test was repeated after dilution of the HCMP B and C with sterile milk powder to a mean number of *ca* 10^4 cfp per capsule. The milk powder used for mixing was the same as that used for drying the milk suspension. Mixing the HCMP with γ-irradiated skim milk powder (smp) was done in a series of steps, resulting in a mixed milk powder (mmp) for each step. For HCMP-B the following scheme was used:

I. 10 g HCMP-B + 10 g smp = mmp_{B-1}
II. 20 g mmp_{B-1} + 20 g smp = mmp_{B-2}
III. 40 g mmp_{B-2} + 40 g smp = mmp_{B-3}
IV. 80 g mmp_{B-3} + 20 g smp = mmp_{B-4}

For HCMP-C the following scheme was used:

I. 1 g HCMP-C + 1 g smp = mmp_{C-1}
II. 2 g mmp_{C-1} + 2 g smp = mmp_{C-2}
III. 4 g mmp_{C-2} + 4 g smp = mmp_{C-3}
IV. 8 g mmp_{C-3} + 8 g smp = mmp_{C-4}
V. 16 g mmp_{C-4} + 16 g smp = mmp_{C-5}
VI. 32 g mmp_{C-5} + 32 g smp = mmp_{C-6}
VII. 64 g mmp_{C-6} + 26 g smp = mmp_{C-7}

All steps were performed using a sterile mortar (volume 1.7 litre) and pestle. Each step consisted of mixing the powder for 15 - 20 seconds using the pestle, followed by remodelling of the powder using a paper card (sterilised by γ-irradiation, dose 10 kGy). This procedure was repeated three times for each step. All mixing steps were undertaken in a laminar down flow cabinet. The final mixed powders (mmp_{B-4} and mmp_{C-7}) were filled into gelatin capsules. Ten capsules from each HCMP (B and C) were reconstituted and duplicate counts per capsule were made on MEYP agar. The counts were analysed by the T_1 and T_2 tests.

Preparation and stability testing of the first series of HCMPs

Preparation of HCMP

Using the drying conditions described for *B. cereus*, HCMPs were prepared with the following strains:

1. *Pseudomonas aeruginosa* (strain identification ALM 32; origin unknown).
2. *Salmonella enteritidis* (phage type 2, rifampicin resistant, strain identification LBA 88-8993; National *Salmonella* Centre, Bilthoven, The Netherlands).
3. *Escherichia coli* (strain identification WR 1; Mooijman *et al.*, 1996).
4. *Listeria monocytogenes* (strain identification Scott-A; see chapter 1).
5. *Staphylococcus aureus* (strain identification ALM 25; food poisoning isolate).
6. *Campylobacter jejuni* (PHLS strain identification FQA No. 043; raw chicken isolate).

1. The *P. aeruginosa* strain was streaked for purity onto a Tryptone Soya Agar (TSA) plate and incubated for (24 ± 2) h at 37 °C. After incubation a single colony was suspended in each of four tubes containing 10 ml Brain Heart Infusion broth (BHI) and incubated for (24 ± 2) h at 37 °C. From this BHI culture a suspension of the strain was made in 100 ml evaporated sterile milk supplemented with 2 M sucrose. Sucrose was used as it has been shown to improve stability of various organisms either dried onto silica gel (Janning, 1995) or spray dried (Moriamez, unpublished results). The target level of inoculation of the milk suspension was 10^6 cfp.g^{-1} HCMP. The BHI culture was first concentrated by centrifugation for 15 minutes at 10,000 g_n. The pellets from all four tubes were resuspended in the milk containing 2 M sucrose.

2. The *S. enteritidis* strain was cultured as described for *P. aeruginosa* except that the TSA and the BHI was supplemented with 50 μg.ml^{-1} rifampicin.

3. The *E. coli* strain was also cultured as described for *P. aeruginosa* except that two pellets from the centrifuged BHI culture were used for inoculation of the milk containing 2 M sucrose and two for inoculation of 100 ml milk without sucrose.

4 and 5. The *L. monocytogenes* and the *S. aureus* strains were cultured as described for *P. aeruginosa* but without concentration of the BHI culture by centrifugation. The full five ml of BHI culture were directly inoculated into milk containing 2 M sucrose.

A second HCMP (HCMP-II) was produced for both *L. monocytogenes* and *S. aureus*. The *L. monocytogenes* strain was cultured as described above except that thirteen BHI tubes were used instead of four and 50 ml of milk instead of 100 ml. The *S. aureus* strain was cultured as described for the second HCMP produced for *L. monocytogenes* except that the BHI contained 10 % NaCl.

6. The *C. jejuni* strain was cultured from the freeze dried Food Microbiology EQA sample no. 0031. The sample was reconstituted in 50 ml Buffered Peptone water, 0.1 ml of the suspension inoculated onto a Bolton agar plate (modified CCD agar; Hutchinson and Bolton, 1984) and incubated under microaerobic conditions (85 % N_2 and 15 % CO_2) for (48 ± 4) h at 42 °C. After incubation a colony was streaked for purity onto a fresh Bolton agar plate and incubated as described above. This plate was used for the inoculation of eight tubes containing 10 ml BHI. The tubes were incubated as described for the Bolton agar. After incubation the cultures were concentrated by centrifugation (15 min at 10,000 g_n) and the pellets from four tubes used for inoculation of 100 ml evaporated milk containing 2 M sucrose and the other four for inoculation of 100 ml evaporated milk without added sucrose.

A total of ten HCMP were prepared using the procedures described above and stored at -20 °C. Part of the HCMP was used for filling gelatin capsules (0.24 g/capsule) and for the determination of the a_w (measured at 25 °C).

Stability tests

The stability and homogeneity of the HCMP were tested by storing the capsules at 22 °C and -20 °C. The stability at -20 °C was determined by monitoring the number of cfp in the material over a period of *ca* 11 months and at 22 °C over 5 - 6 weeks. For the *S. enteritidis, S. aureus* and *E. coli* (dried with sucrose) HCMP the stability at 22 °C was determined again over a period of *ca* three weeks after initial storage of the capsules for *ca* seven months at -20 °C. The stability of the HCMP-II containing *L. monocytogenes* and *S. aureus* were determined for *ca* five months at -20 °C and for *ca* four weeks at 22 °C.

After preparation the HCMPs were stored for one day at -20 °C before the capsules were filled and stored at 22 °C and -20 °C. For both the HCMPs-II the capsules were filled and stored at -20 °C and 22 °C on the day of preparation. After *ca* 200 days storage at -20 °C a number of capsules of *S. enteritidis, E. coli* (with sucrose) and *S. aureus* were stored at 22 °C to check whether the stability at 22 °C had changed.

For each measurement five capsules were reconstituted and diluted in PS according to the procedure described for *B. cereus* in chapter 1 and 0.1 ml volumes examined in duplicate on TSA (except for *C. jejuni*). The plates were incubated for (24 ± 2) h at 37 °C. For *C. jejuni* counts were made on Bolton basal agar incubated for (48 ± 4) h at 42 °C. From these counts the corresponding values for the T_1 and T_2 test statistics were calculated. The T_2 values for each time point of the stability test at -20 °C were summed and divided by the total number of capsules examined minus the number of time points, to obtain a value for the homogeneity of the material (called T_{hom}/N).

Based on the stability test data at -20 °C a regression analysis was performed ($\log_{10}$ transformed counts) using the Genstat (release 5.2) programme. A piecewise linear model was used for the regression analysis as described by Pindyck and Rubinfeld (1981). The model used is a continuous one marked by one structural break (significant change in slope). The method is summarised below, further details can be found in Janning (1995). The following equation represents the piecewise linear model used:

$$X = \beta_1 + \beta_2 t + \beta_3 (t - t_s) D_t + \varepsilon \quad (1)$$

where: $X = \log_{10}$ number of cfp per capsule
t = storage time (days)
t_s = time point at which a structural break occurred
β_1 = intercept of the first line segment
β_2 = slope of first line segment
$\beta_2 + \beta_3$ = slope of the second line segment
$D_t = 1$ if $t > t_s$
$D_t = 0$ otherwise
ε = error component

For the first line segment (before the structural break) D_t is 0. Formula 1 can then be written as:

$$X = \beta_1 + \beta_2 t + \varepsilon \quad (2)$$

After the structural break (second line segment) D_t is 1. Formula 1 can then be written as:

$$X = \beta_1 - \beta_3 t_s + (\beta_2 + \beta_3) t + \varepsilon \quad (3)$$

where: $\beta_1 + \beta_3 . t_s$ = intercept of second line segment
$\beta_2 + \beta_3$ = slope of the second line segment

To detect the occurrence of a structural break a two tailed Students *t*-test ($\alpha = 0.05$) was used to test whether β_3 differed significantly from 0. When no structural break was detected the equation was reduced to a single straight line (formula 2). Tests were done to determine whether the slope of this line differed significantly from zero by means of a two tailed Students *t*-test ($\alpha = 0.05$). When β_3 differed significantly from zero a structural break occurred at time point t_s. Further tests were made to determine whether the slope of the second line fragment ($\beta_2 + \beta_3$) differed significantly from zero by means of the F-test ($\alpha = 0.05$). The inverse of the regression coefficient ($\beta_2 + \beta_3$) was used to calculate the time needed for a one $\log_{10}$ reduction in the number of cfp.

Preparation and homogeneity of the second series of HCMPs

A second series of HCMPs was produced for the strains *S. enteritidis* (HCMP-II and III), *L. monocytogenes* (HCMP-III and IV) and *L. innocua* (strain identification ALM 105, origin unknown). The *S. enteritidis* and *L. monocytogenes* strains were cultured as described for the preparation of the first series of HCMPs, except that for *L. monocytogenes* 500 ml BHI was used and after incubation the culture was concentrated by centrifugation as described for *S. enteritidis*. *L. innocua* was cultured as for *L. monocytogenes* but using only 250 ml BHI. Two HCMPs were produced for both *S. enteritidis* and *L. monocytogenes* but only one for *L. innocua*. The drying conditions were identical to those for drying for the first series of HCMPs, except for the time used to spray the milk suspension onto the milk powder. For the first series the spraying time was 2 - 3 minutes, for the second *ca* 20 minutes. This increase

was achieved by lowering the feed rate of milk suspension (pump set at 0.5 ml.min^{-1}) and by spraying at intervals (15 seconds on and off for *S. enteritidis* and 30 second cycles for the *Listeria* strains).

After production the HCMPs were stored at -20 °C for a few days and used to fill gelatin capsules (*ca* 0.28 g per capsule). Homogeneity was determined based on the examination of two sets of 10 capsules of each organism. The capsules were reconstituted, appropriately diluted and duplicate counts made on:

a) PCA with an overlay of Violet Red Bile Glucose agar (VRBG) for *S. enteritidis* (see method described in chapter 3) and
b) PALCAM agar for the *Listeria* strains.

The counts obtained were used to calculate the T_1 and T_2 values for each individual set and the combined sets of 10 capsule per HCMP.

RESULTS

Drying conditions with the STREA

Preliminary experiments indicated that the powder obtained from Nestlé (milk product 17) gave better results than skim milk powder. The skim milk powder quickly formed large wet lumps that could not be held fluidised when the sterile evaporated milk was added. The difference in composition between milk product 17 and skim milk powder is that the former contains free flowing agents and sucrose which prevent the formation of large lumps of powder during the spraying of the evaporated milk. The maximum amount of milk powder that could be kept fluidised was 500 grams. Table 1 presents the relationship between the inlet temperature (as set on the apparatus) and the outlet temperature obtained.

Table 1. Relationship between inlet and outlet air temperature and the effect of addition of milk and drying on the water activity of milk product 17.

Temperature		Water activity of milk product 17		
inlet air[a]	outlet air[a]	untreated	after addition of 10 ml evaporated milk	after drying[b]
40	30	0.21	0.34	0.25
50	35	0.21	0.34	0.19
60	40	n.t.[c]	n.t.	n.t.

a variation in temperatures of ± 5 °C.
b water activity after pre drying for 30 minutes, addition of 10 milk and post-drying for 15 minutes.
c n.t. = not tested.

In order to obtain a lower final a_w the milk powder was kept fluidised both before (pre-drying) and after the addition of the milk culture (post-drying). The results of pre-drying for 30 minutes and post-drying for 15 minutes at inlet temperatures of 40 °C and 50 °C are

presented in Table 1. As the a_w of the final HCMP should be less than 0.3 and the temperature for drying should be as low as possible (to prevent injury due to heat), drying at 40 °C was selected for further experiments. The use of 10 ml as a volume of evaporated milk to be sprayed onto the powder could not be modified because a larger volume of milk led to the formation of lumps of milk powder. Spraying 10 ml evaporated milk onto the powder took 2 - 3 minutes. The remaining process conditions selected were: nozzle pressure of one bar and a pressure for cleaning outlet air filters (blow back dwell) of two bar.

Thus the following main process conditions were used for further experiments: 10 ml evaporated milk (corresponding to 10.75 g) sprayed onto 500 gram of milk product 17 at an inlet temperature of 40 °C. The powder was both pre-dried for 30 minutes and post-dried for 15 minutes.

Homogeneity of and recovery of *B. cereus* from the HCMP

The number of cfp in the *B. cereus* spore suspension in PS was determined both before and after heat treatment (10 min at 80 °C). The number of cfp before heat treatment, as determined on MEYP agar, was 1.2×10^9 ml^{-1} and after heat treatment 8.3×10^8 ml^{-1}. This indicates that sporulation was almost complete (*ca* 70 % of the cells). This spore suspension was used to prepare HCMPs A, B and C. The results of the drying experiments are described in Table 2.

Table 2. Preparation of HCMP containing *B. cereus* spores.

HCMP	Number of cfp in milk suspension (ml^{-1})	Milk suspension added during drying (g)	Expected number of cfp in HCMP (g^{-1})
A	2.3×10^5	8.7	4.0×10^3
B	9.6×10^6	9.0	1.7×10^5
C	7.1×10^7	8.2	1.1×10^6

The results presented in Table 2 for the number of cfp of *B. cereus* in the HCMPs represents the expected levels. These levels were calculated based on the amount of milk added to the powder and the number of cfp per ml of the milk.

The HCMPs were filled into gelatin capsules to determine the number of $cfp.g^{-1}$ after drying. To determine the recovery of the spores from and the homogeneity of the HCMPs ten capsules of each were enumerated in duplicate on MEYP agar after reconstitution of the capsules and appropriate tenfold dilution of the capsule suspension. The results of these tests are presented in Table 3. The recovery was calculated as the ratio between observed and expected number of cfp. None of the T_1 values calculated (results not presented) exceeded the critical value for the T_1 test, which means that variation between duplicate counts from a capsule suspension conformed to a Poisson distribution. The homogeneity of the powder is expressed as $T_2/(I\text{-}1)$, where $(I\text{-}1)$ is the number of degrees of freedom. In the case of a Poisson distribution the expected value for $T_2/(I\text{-}1)$ is 1.

The effect of mixing the HCMP with sterile milk powder on the homogeneity of the mixed powders was tested for HCMP-B and C. The results of enumeration of 10 capsules in duplicate on MEYP agar are presented in Table 4.

Table 3. Number of cfp, homogeneity and recovery of *B. cereus* from the HCMPs.

HCMP	Number of cfp per capsule	Number of cfp in HCMP (g^{-1})	$T_2/(I-1)^b$	Recovery[c] (%)	a_w
A	1.6×10^3	5.8×10^3	1.05	145	0.22
B	5.0×10^4 [a]	1.9×10^5	0.61	110	0.27
C	2.5×10^5	9.5×10^5	1.61	88	0.27

a average of 9 instead of 10 capsules.
b I = number of capsules examined.
c recovery from the drying procedure expressed as observed number of cfp per gram HCMP after drying divided by the expected number of cfp per gram HCMP.

Table 4. Number of cfp and homogeneity of *B. cereus* HCMP-B and C after mixing with sterile milk powder

Mixed milk powder	Number of cfp of *B. cereus* per capsule	Dilution factor of HCMP	Recovery after mixing (%)	$T_2/(I-1)$
mmp_{B-4}	6.8×10^3	10	136	1.00
mmp_{C-7}	2.6×10^3	100	104	1.03

Preparation of the first series of HCMPs

Table 5 summarises the results of the preparation of the HCMPs with all the test strains and the expected numbers of cfp per gram HCMP (assuming 100 % survival). This expected level was used as the reference for calculating the percentage survival directly after drying and also during storage at -20 °C. The reference for calculating the percentage survival at 22 °C was the level of the organism in the HCMP stored at -20 °C at the time the monitoring experiments were started.

Survival of the test strains in the first series of HCMP

The survival of the test strains directly after drying (initial decrease) is presented in Table 6. Figures 2 and 3 present the results of monitoring at -20 °C for *S. enteritidis, P. aeruginosa, E. coli* (dried from milk supplemented both with and without sucrose), *L. monocytogenes* (HCMP-I and II) and *S. aureus* (HCMP-I and II).

Table 5. Preparation of first series of ten HCMPs.

Test strain	Number of cfp per ml milk suspension	Sucrose added to milk	Milk suspension added during drying (g)	Expected number of cfp per g HCMP	a_w
S. enteritidis	1.6×10^8	yes	8.72	2.7×10^6	0.26
L. monocytogenes					
- HCMP-I	1.1×10^8	yes	8.54	1.9×10^6	0.25
- HCMP-II	2.5×10^9	yes	9.15	4.2×10^7	0.23
E. coli	3.5×10^8	yes	8.70	6.1×10^6	0.24
	2.2×10^8	no	9.15	3.9×10^6	0.25
S. aureus					
- HCMP-I	3.8×10^7	yes	8.45	6.4×10^6	0.24
- HCMP-II[a]	2.9×10^8	yes	8.15	4.7×10^6	0.23
P. aeruginosa	6.6×10^7	yes	8.29	1.1×10^6	0.26
C. jejuni	3.5×10^5	yes	8.73	6.1×10^6	n.d.
	1.3×10^8	no	8.78	2.3×10^6	n.d.

a cultured in BHI containing 10 % NaCl.
n.d. = not determined

Table 6. Survival of the test organisms in the first series of ten HCMPs directly after drying.

Test strain	Observed number of cfp per g HCMP	Time between drying and testing (days)	Survival[b] (%)
S. enteritidis	6.3×10^4	1	2.3
L. monocytogenes			
- HCMP-I	2.3×10^5	1	12
- HCMP-II	6.3×10^6	4	15
E. coli	4.4×10^5	1	7.2
	$2.4 \times 10^{4\,a}$	1	0.62
S. aureus			
- HCMP-I	3.6×10^4	1	5.6
- HCMP-II	3.3×10^6	4	69
P. aeruginosa	1.9×10^4	1	1.7
C. jejuni	$< 10^2$	1	< 0.01
	$< 10^{2\,a}$	1	< 0.01

a no sucrose added to the milk for drying.
b survival = ratio between observed and expected number of cfp per gram HCMP.

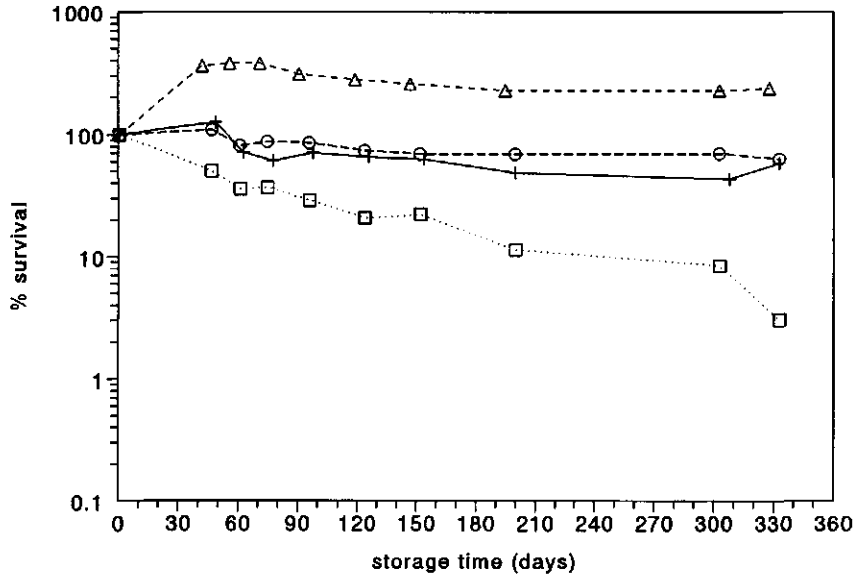


Figure 2. Survival of *S. enteritidis* (+), *P. aeruginosa* (Δ), *E. coli* dried from milk supplemented with 2 M sucrose (O) and *E. coli* dried from milk without added sucrose (□) after initial decrease and storage at -20 °C.

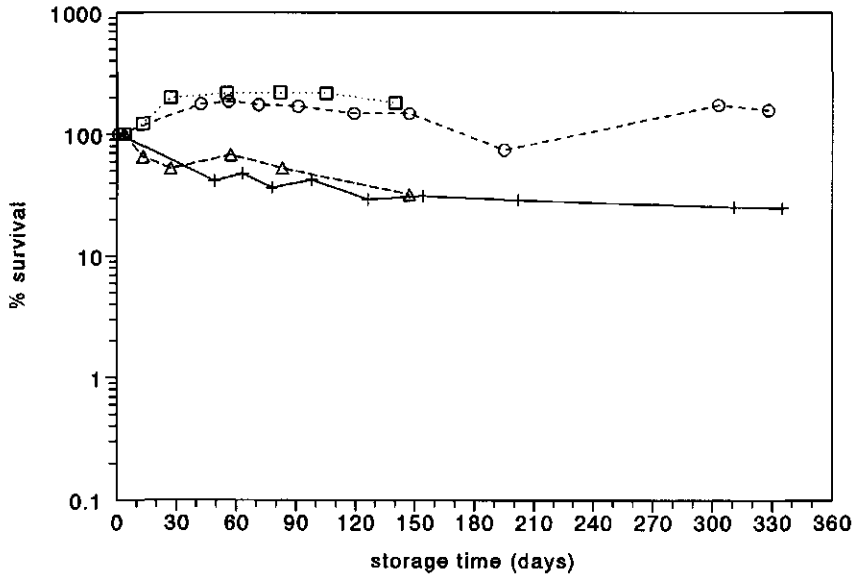


Figure 3. Survival of *L. monocytogenes* HCMP-I (+), *L. monocytogenes* HCMP-II (Δ), *S. aureus* HCMP-I (O) and *S. aureus* HCMP-II (□) after initial decrease and storage at -20 °C.

The results of the linear regression analysis are presented in Table 7 and are based on the counts from the -20 °C storage temperature. The data from day 195 for the *S. aureus* HCMP-I were not used for the regression analysis as these counts were exceptionally low and were most probably due to an unidentified error made during the microbiological analysis. For all strains, with the exception of *E. coli*, a structural break was observed during monitoring. A significant decrease in the number of cfp was observed for most strains (except for *S. aureus*) even after a structural break. Table 7 presents the rate of decrease after the structural break. It should be noted that the regression analysis did not always give a good fit. For a good fit the percentage explained variation should be about 80 % or higher, lower values mean a poorer fit of the model to the data.

Table 7. Regression analysis of counts from the first series of HCMPs stored at -20 °C.

Test strain	**t_s[a] (day)**	**Regression coefficient[b] ($days^{-1}$)**	**Time for 1 log_{10} reduction (days)**	**% explained variation**
S. enteritidis	49	- 0.00065[c]	1539	60.4
L. monocytogenes	49	- 0.00086[c]	1210	79.2
L. monocytogenes (HCMP-II)	27	- 0.0019[c]	519	67.9
E. coli (with sucrose)	0	- 0.000589[c]	1699	44.4
E. coli (without sucrose)	0	- 0.00377[c]	265	89.3
S. aureus	42	- 0.00011[d]	9049	59.6
S. aureus (HCMP-II)	27	- 0.00032	3175	87.9
P. aeruginosa	42	- 0.00077[c]	1295	88.3
C. jejuni	n.d.	n.d.	n.d.	n.d.

a time at which a structural break occurred.
b regression coefficient ($\beta_2 + \beta_3$) of the second line segment (after t_s).
c regression coefficient differed significantly from 0 ($\alpha = 0.05$).
d excluding the results of day 195.
n.d. = not determined.

Figures 4 - 7 present the results of monitoring at 22 °C for the strains mentioned above. No linear regression analysis was performed on these data as too few data were available for a proper analysis.

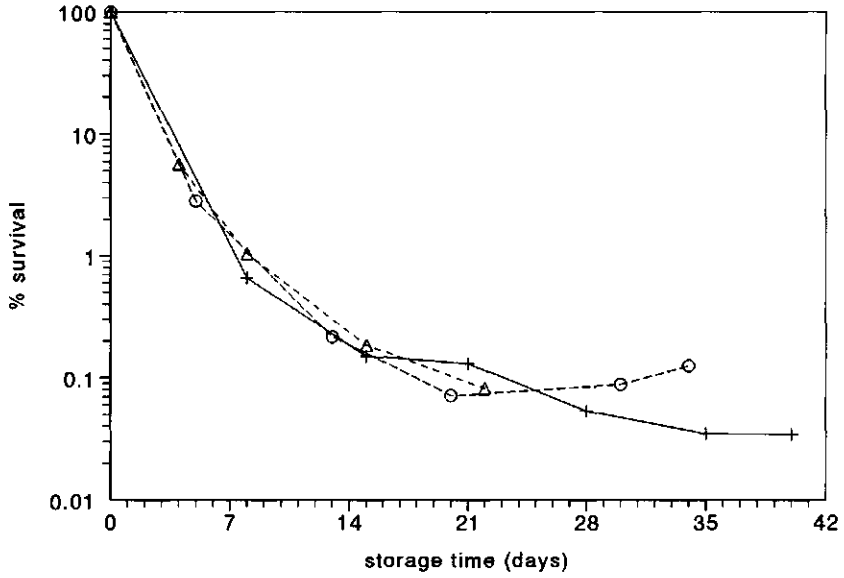


Figure 4. Survival of *S. enteritidis* and *P. aeruginosa* at 22 °C after storage of the HCMP at -20 °C for different times (1 day (+) or 200 days (Δ) for *S. enteritidis* and 1 day for *P. aeruginosa* (O)).

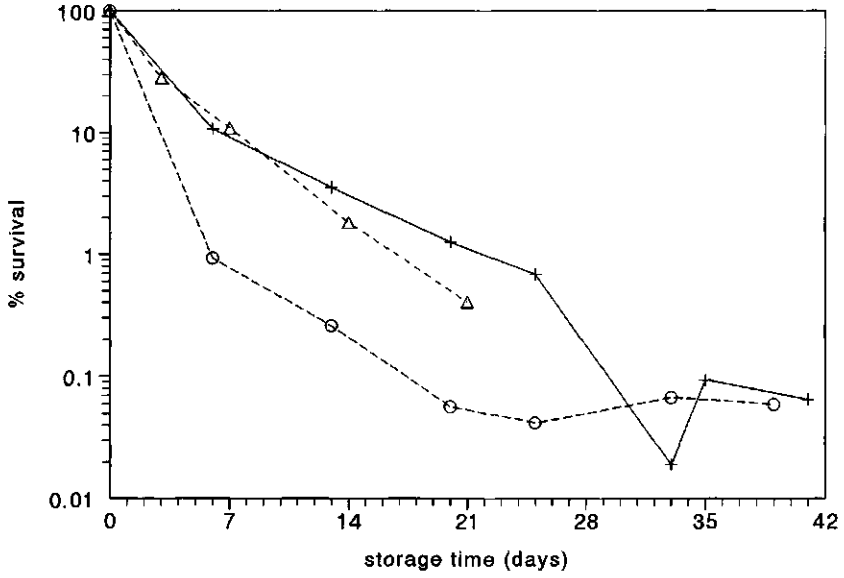


Figure 5. Survival of *E. coli* at 22 °C after storage of the HCMP at -20 °C for different times (1 day (+) or 195 days (Δ) for HCMP dried from milk supplemented with 2 M sucrose and 1 day for the HCMP dried from milk without added sucrose (O)).

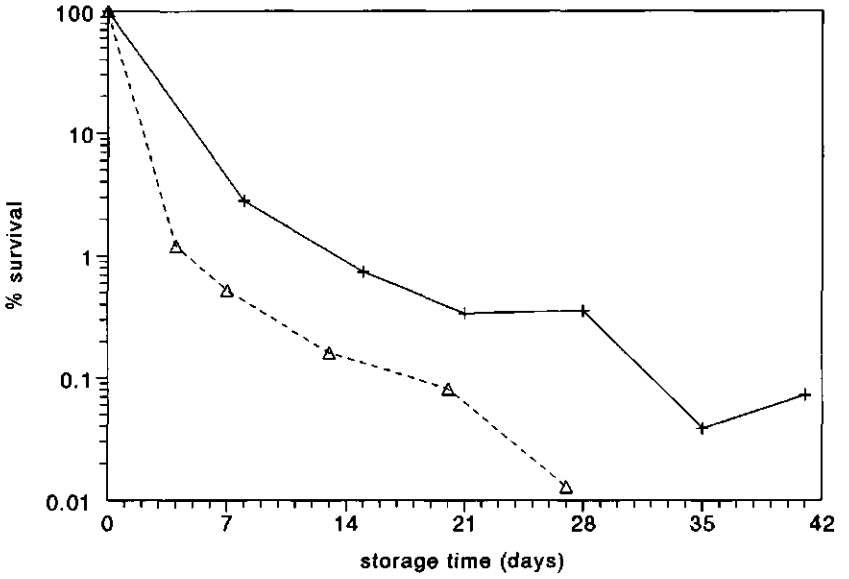


Figure 6. Survival of *L. monocytogenes* at 22 °C after storage of the HCMP at -20 °C for different times (1 day for HCMP-I (+) and 4 days for HCMP-II (Δ)).

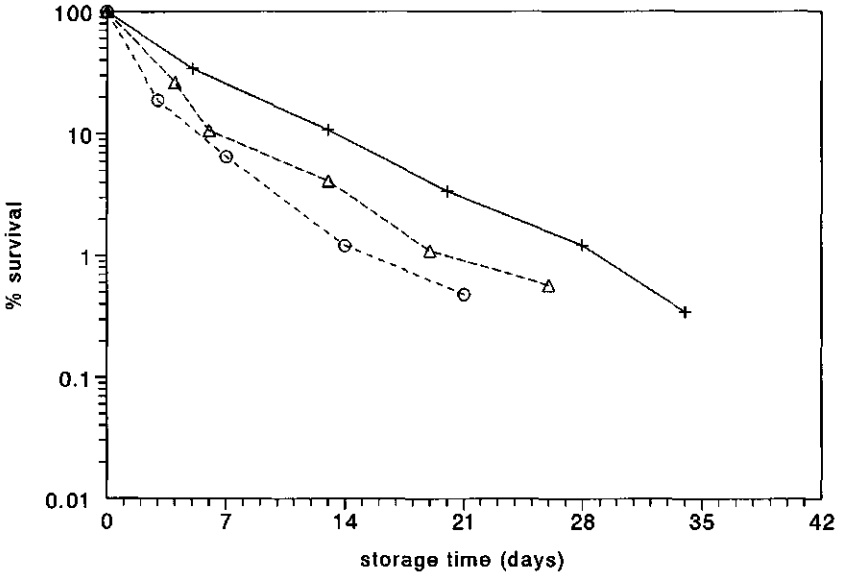


Figure 7. Survival of *S. aureus* at 22 °C after storage of the HCMP at -20 °C for different times (1 day (+) or 195 days (O) for HCMP-I and 4 days for HCMP-II (Δ)).

Homogeneity of the first series of ten HCMPs

A significant T_1 value was found on 13 of the 72 occasions an analysis was performed. To obtain an estimate for the dispersion of the mean counts between capsules (homogeneity of the batch) the T_{hom}/N values were calculated for each HCMP based on the counts obtained at the -20 °C storage temperature. All data were used for the calculations without exclusion of that with a significant T_1 value. The procedure for the calculation of T_{hom}/N is described in chapter 4. Table 8 presents an overview of the homogeneity of the various HCMP.

Table 8. Homogeneity of the ten HCMPs from the first series (data from storage at -20 °C).

HCMP containing	n[a]	ΣT_2	N^b T_{hom}	T_{hom}/N
S. enteritidis	10	265	40	6.6
L. monocytogenes	10	57.5	40	1.4
L. monocytogenes (HCMP-II)	6	88.7	24	3.7
E. coli (with sucrose)	10	139	40	3.5
E. coli (without sucrose)	10	64.9	40	1.6
S. aureus	10	121	40	3.0
S. aureus (HCMP-II)	7	78.3	28	2.8
P. aeruginosa	9	70.9	36	2.0
C. jejuni	n.d.	n.d.	n.d.	n.d.

a number of time points.
b N = degrees of freedom for T_{hom} test.
c n.d. = not determined.

Homogeneity of the second series of five HCMPs

The results of the homogeneity tests of the five HCMPs prepared in the second series are presented in Table 9. The values for the T_1 tests are not presented as none of the T_1 values found was significantly higher than would be expected for a χ^2-distribution. The high value for T_{hom}/N of the *L. monocytogenes* HCMP-III is caused by the difference in means found for the individual sets of 10 capsules. For the first set the mean of the counts on the plates was 80.0 and for the second set 106.2 cfp. No explanation for the difference in means could be found. The counts were made at the same time as those for *L. monocytogenes* HCMP-IV, using the same batch of PALCAM plates.

Table 9. Results of examination of the second series of five HCMPs.

Test strain	Arithmetic mean[a]	$T_2/(I-1)$		T_{hom}/N[b]
		first set	second set	
S. enteritidis				
HCMP-II	3.0×10^4	1.68	1.17	1.50
HCMP-III	4.5×10^4	0.68[c]	1.35	1.04
L. monocytogenes				
HCMP-III	9.3×10^7	2.05[d]	1.16	5.36[e]
HCMP-IV	4.3×10^7	2.15[d]	1.63	1.90[e]
L. innocua	1.7×10^6	0.75	1.55	1.50

a arithmetic mean of the number of cfp per capsule (containing *ca* 0.28 g HCMP).
b T_{hom}/N is the sum of the T_2 values of both sets divided by the total number of capsules examined minus 2.
c examination of seven capsules.
d significantly different from χ^2-distribution (critical value for $T_2/(I-1)$ of 1.88 with $I = 10$ and $\alpha \approx 0.05$).
e significantly different from χ^2-distribution (critical value for T_{hom}/N of 1.60 with $N = 18$ and $\alpha = 0.05$).

DISCUSSION

Spray granulation conditions

The process conditions selected for the preparation of HCMP are directed at minimising the bacterial injury due to heat. For this reason an inlet temperature of 40 °C was chosen, even though a higher inlet temperature would result in powders with a lower water activity. The temperature chosen is similar to those used for drying various *Lactobacillus* species by Hill (1987b), Roelans and Taeymans (1990), Zimmerman and Bauer (1990) and Lievense (1990). The use of various types of support material is also described by Hill (1987b). The choice of which support material was used depended on the particular application of the dried material as a starter culture. The use of milk powder as support material was described by Hill (1987b) for the production of a yogurt starter culture and was also used in the experiments described by Roelans and Taeymans (1990).

The pre- and post-drying times used in the process compensated for most of the increase in water activity due to the addition of 10 ml of milk suspension to the milk powder. The increase in a_w could be reduced further by leading the air used for drying over anhydrous silica gel in order to lower the water content of the air.

The first step in the evaluation of the drying process was to test the homogeneity and the recovery of the bacteria after drying. For this purpose a spore suspension of *B. cereus* was first used as no die off during the drying process was expected. Using the spore suspension three HCMPs were prepared at various contamination levels. The recovery of the organisms after drying was high, indicating that the spores survived well and did not form aggregates during the drying process, which, after reconstitution, would only yield a single colony that would really consist of an aggregate of spores. The homogeneity of the powders

before and after mixing (see Tables 3 and 4) was good (results based on the analysis of 10 capsules). No significant difference from a Poisson distribution ($T_2/(I\text{-}1) = 1$) could be detected. No data on the homogeneity of spray granulation of *Lactobacillus* species are currently available in the literature.

Drying of the first series of ten HCMPs

Preparation of HCMPs containing various non-spore forming bacteria gave varying results with respect to survival directly after drying (initial decrease) and upon storage of the HCMP. The bacterial strains used were selected because they had proved to be sensitive to the spray drying process and/or to show poor stability after spray drying.

Initial decrease

The recovery after the first analysis time (after 1 - 3 days storage at -20 °C) is used as an indication of the survival of the strain through the drying process. It appeared to be impossible to dry *Campylobacter* by the spray granulation method. Only one day after drying the organisms could not be cultured from the powder. The mere addition of the *Campylobacter* culture to milk containing 2 M sucrose resulted in a decrease in the number of cfp in the milk suspension by almost 3 $\log_{10}$ units (data not presented). Because of this extremely high initial decrease (i.d.) no further efforts were made to improve drying conditions by this method for *Campylobacter*. Drying of *Campylobacter* onto silica gel also resulted in no detection of the organism after one day of storage at 22 °C (Janning, 1995). It is well known that *Campylobacter* is sensitive to dry conditions, but the strain used for drying was isolated from a freeze dried material, indicating the capability of this strain to survive dry conditions. The difference in the resistance of *Campylobacter* to spray granulation and to freeze drying may be related to the sensitivity of the organism to oxygen; this is minimised in freeze drying and storage of the material under vacuum.

All other strains survived the spray granulation process. The survival directly after drying varied from 69 % survival for the *S. aureus* HCMP-II to 1.7 % for *P. aeruginosa*. The difference in i.d. observed for the two *S. aureus* HCMPs is the effect of the addition of 10 % NaCl to the BHI used to culture the strain and prepare the HCMP-II. The values found for the i.d. using spray granulation are different from the those observed for silica gel drying (Janning *et al.*, 1994). Although there was a difference in the storage temperatures used before the first analyses of the samples (silica gel samples were stored at 22 °C and the spray granulation samples at -20 °C), this difference cannot explain the observed difference in i.d. Large differences between the i.d. for silica-gel drying (> 5 $\log_{10}$) and spray granulation (1.7 $\log_{10}$) were particularly found for *P. aeruginosa*. However, this difference is again related to the addition of sucrose to the milk used for drying. Janning (1995) also tested the effect of sucrose on the drying of *P. aeruginosa* on silica gel. Using 2 M of sucrose an i.d. of 1.3 $\log_{10}$ (5 % survival) was found. The difference observed in i.d. between spray granulation and silica gel drying of *S. enteritidis* is also most likely to be the effect of the addition of sucrose. However, no explanation can be found for the better i.d. of *E. coli* dried on silica gel.

The percentage survivals found with spray granulation of *Lactobacillus* species (Hill, 1987b; Roelans and Taeymans, 1990; Zimmerman and Bauer, 1990) are > 10 % and, therefore, comparable to the i.d. values found for *L. monocytogenes* and *S. aureus*. The results for i.d. obtained with spray drying are expected to be higher than those obtained with spray granulation due to the higher temperatures used for spray drying. However, when low outlet temperatures (65 - 70 °C) and a high solids content of the milk (40 % total solids) are used comparable survival rates can be obtained. For example, LiCari and Potter (1970a) observed a 0.6 $\log_{10}$ reduction (25 % survival) for *S. tennessee* at an outlet temperature of 65 °C. For other *Salmonella* serotypes higher i.d. values (1 - 3 $\log_{10}$ units) were observed at an outlet temperature of 65 - 67 °C (LiCari and Potter, 1970a; Miller *et al.*, 1972). Thompson and co-workers (1978) observed a survival of 0.46 % for *E. coli* dried at 71 °C which is comparable to the values found for *E. coli* spray granulation dried in milk without added sucrose. Chopin and co-workers (1977a/b and 1978) observed i.d.s for *S. aureus* ranging between 0 and 10 % using various strains dried at an outlet temperature of *ca* 70 °C.

Survival during storage

The survival during storage of the first series of HCMPs was determined at a storage temperature of -20 °C (normal storage temperature for RMs) and at 22 °C (temperature normally used to determine the stability of dried organisms).

Regression analysis was performed on the results from the survival studies on storage at -20 °C. The first fact to be determined was whether a structural break occurred (test if β_3 differed significantly from 0). For all HCMPs, except the two *E. coli* HCMPs, a structural break could be detected. The time needed for this break (t_s) varied between 27 and 49 days storage. For the two *E. coli* HCMPs no structural break could be detected indicating that the rate of decrease was constant over the period tested. After the structural break tests were performed to determine whether ($\beta_2 + \beta_3$) differed significantly from zero (no decrease or increase in the level of contamination over time). Only for the two *S. aureus* HCMPs did the regression coefficient not differ significantly from zero. For the other strains a negative slope and thus a rate of decrease was found. It is difficult to compare the data obtained at a storage temperature of -20 °C with data from the literature as in the latter stability was determined at ambient temperature. A comparison of the regression coefficients with those obtained by Peterz and Steneryd (1993) for their freeze dried RMs stored at 4 °C, indicates a comparable rate of decrease. For *S. aureus*, *Salmonella* (compared to freeze dried sample B) and *E. coli* (dried from milk supplemented with sucrose) the rates of decrease are better for spray granulation than for freeze drying. With *E. coli* (dried from milk without added sucrose) and *Salmonella* (compared to freeze dried sample A) the opposite was observed.

For most of the strains dried the rate of decrease at 22 °C was non-linear. After a certain period of time the rate of decrease was reduced. However, at this time a very low level of contamination of the HCMP was reached, of the order of less than 100 cfp.g^{-1} HCMP. This level corresponded to a decrease of 4 - 5 $\log_{10}$ units compared to the expected level of contamination of the HCMP. A reduced rate of decrease after a certain period was also observed for *Salmonella* stored at high temperatures (25 °C to 55 °C) by LiCari and Potter (1970b). In the first four weeks after spray drying and storage at 25 °C a reduction in the level of contamination for various *Salmonella* strains of 1 - 2 $\log_{10}$ was observed. For

spray granulation of *S. enteritidis* a larger decrease was observed of 2 $\log_{10}$ after one week of storage at 22 °C.

The addition of sucrose to the milk containing *E. coli* had resulted in the improvement of the i.d. and the rate of decrease at -20 °C (see Figure 5). The time needed to reach a 2 $\log_{10}$ reduction in the number of cfp.g^{-1} HCMP was *ca* three weeks compared to less than one week in the absence of sucrose. However, after 4 - 5 weeks storage at 22 °C a similar reduction in the number of cfp in both HCMPs was obtained. The level in the *E. coli* HCMP dried from milk without sucrose stabilised after *ca* three weeks storage, but the level in the HCMP dried from milk supplemented with sucrose still decreased. A comparison of the stability at 22 °C of *E. coli* dried using spray granulation with silica gel drying (Janning *et al.*, 1994) or spray drying (Thompson *et al.*, 1978; Mooijman *et al.*, 1996) indicates that stability using spray granulation is less than for silica gel or spray drying. Also for *P. aeruginosa* spray granulation gave poorer results compared to silica gel drying.

The time needed for a 2 $\log_{10}$ reduction of *S. aureus* stored at 22 °C in spray granulation dried material was *ca* three weeks. This value corresponds to the values observed by Chopin and co-workers (1978) for various spray dried *S. aureus* strains at an a_w of 0.3 and stored at ambient temperature. Lower water activities (0.1 and 0.2) improved survival of the *S. aureus* strains.

Mooijman and co-workers (1992) observed a relationship between the stability of batches of RM prepared from the same HCMP (containing *Enterobacter cloacae*) and the age of the HCMP. The older the HCMP the better the stability of the material. A similar effect was observed by LiCari and Potter (1970) and Chopin (1978) who noted a decrease in the rate of destruction with increasing storage time of the milk powder. These findings could not be confirmed when the stabilities at 22 °C of the HCMPs for *S. enteritidis, E. coli* and *S. aureus* were monitored. Stability at 22 °C was monitored directly after drying and again after *ca* 200 days of storage at -20 °C. Only small differences in the rate of decrease were observed.

Homogeneity of the STREA HCMPs

The homogeneity of the first series of ten HCMPs (see Table 8) expressed as T_{hom}/N varied widely. Although homogeneous powders could be produced using *B. cereus* spores (see Table 3 and 4), all other values for the homogeneity test statistic (T_{hom}/N) were significantly different from a χ^2-distribution at $\alpha = 0.05$. At $\alpha = 0.01$ only the value for *L. monocytogenes* (HCMP-I) was no longer significant. For the production of an RM a value for $T_2/(I\text{-}1) \leq 2$ is regarded as acceptable (see also chapters 3, 4 and 7). Using this criterion for T_{hom}/N three HCMPs had acceptable homogeneity (*L. monocytogenes* HCMP-I, *E. coli* (dried from milk without added sucrose) and *P. aeruginosa*). In order to improve the homogeneity of the HCMPs a second series of trials was undertaken. For these trials the time taken to spray the milk suspensions onto the support material was increased by reducing the feed rate and spraying at intervals. The results of the homogeneity tests for these HCMPs (see Table 9) indicated that the homogeneity was improved. Except for *L. monocytogenes* the values found for T_{hom}/N were not significantly different from the χ^2-distribution at $\alpha = 0.05$. For the *L. monocytogenes* HCMP-IV the T_{hom}/N value was < 2,

which is regarded as acceptable. These results indicate that homogeneous HCMPs can be produced by spray granulation although particular attention must be paid to the method and timing of spraying on the milk suspension.

CONCLUSIONS

- It is possible to prepare homogeneous HCMP using spray granulation under the process conditions determined in combination with a longer spraying time of the milk suspension onto the powder in order to improve homogeneity of the HCMP.
- Results for survival through the drying process (initial decrease) were good and comparable to silica gel drying or to spray drying under the most favourable conditions for survival of the organisms.
- The results of the stability tests at 22 °C are not as good as those for silica gel drying or spray drying.
- The drying procedure for spray granulation is less labour and time intensive compared to spray drying.

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Use of reference materials for quality assurance of laboratory examinations

ABSTRACT

Laboratories using reference materials (RMs) have to make a choice from different options: certified or non-certified RMs; quantitative or qualitative tests; occasional or routine use. This chapter presents the protocols that were developed for these combinations and the statistical methods for evaluation of the results.

Shewhart control charts are suitable for the evaluation of results obtained from the routine use of quantitative RMs. To construct these charts, log transformed counts are used to calculate the mean and control limits. The use of Kalman filtering is proposed in order to use Shewhart type control charts with data obtained from unstable RMs.

For qualitative RMs it is recommended that one capsule at a time is examined for routine use. The results are then judged for each individual capsule (they should be positive) and for a series of capsules (the number of positives can be compared to the expected number of positive isolations as calculated for the occasional use of qualitative RMs). The fraction of negatives for a quantitative RM or CRM should be at or very close to zero percent as the result for each analysis should be positive.

Certified RMs are used occasionally and are mainly intended for the determination of trueness in a laboratory. The certificate provided with a CRM gives the mean expected value and its 95 % confidence limits. Based on these results user tables are constructed presenting the 95 % confidence limits for the number of capsules (and replicates per capsule in the case of quantitative CRMs) likely to be examined in practice. The results of power analyses are given to demonstrate the minimum difference between the certified value and the true laboratory mean (for quantitative CRMs) or the fraction of negatives (for qualitative CRMs) that can be detected. For the qualitative CRM a fraction of negatives of 0 % is necessary in order to minimise the number of capsules needed to detect a certain fraction of negatives found in the laboratory (p_{lab}).

For the occasional use of non-certified RMs additional information is required on the variation between laboratories in order to interpret results. The laboratory variance component of a CRM can be used to adjust the confidence limits of the RM obtained in one laboratory.

INTRODUCTION

The previous chapters focused on the characteristics of the reference and certified reference materials developed. The next two chapters will focus on the possibilities for their use in laboratories (chapter 6) and whether the requirements of the users of the materials correspond to their properties (i.e. the quality of the reference materials described in chapter 7). Both chapters focus on the users of the RMs and CRMs as the quality of the material and their possible use are interrelated. The definitions of and possible uses of RMs are given in the international standard ISO guide 30 (Anon., 1992). However, this document gives only a general description, as it has to be valid for a wide range of RMs and their usage in microbiology is not considered specifically. Also it does not deal with the use of RMs for presence/absence testing. More information on microbiological quality assurance is presented by Lightfoot and Maier (1998).

The use of RMs is a part of the total quality assurance measures and is referred to as analytical quality control (Lightfoot and Maier, 1998). This requires that in addition to use of RMs attention must also be paid to the correct use and/or maintenance of laboratory protocols, equipment, materials etc. The use of RMs is directed towards establishing the standard of performance of methods, apparatus, technicians etc. Three types of analytical quality control (QC) can be identified. First line quality control is performed by the technician and at a high frequency (referred to in this chapter as routine use of RMs). The objective of first line QC is to demonstrate equal performance of a method over time. Second line QC is the responsibility of a person not directly linked to the examination itself and is performed less frequently. The objective here is to demonstrate comparable performance of technicians, apparatus etc. Third line QC is the responsibility of laboratory management and is intended to demonstrate equivalent performance between laboratories (for example by participation in proficiency testing schemes and/or in inter-laboratory collaborative studies). Protocols were therefore developed for the use of the microbiological RMs and CRMs and are described in more detail in this chapter. Two types of RM can be distinguished, those intended for the evaluation of enumeration procedures (quantitative RMs) and those for the evaluation of detection procedures (qualitative RMs). Both quantitative and qualitative materials are available as non certified RMs and certified RMs.

The level of contamination (for quantitative RMs) or fraction of negatives (for qualitative RMs) provided with each batch of RM is stated in relation to the measurement method as it cannot be related to a standard (for example an SI unit). A range of measurement methods that differ in measurement principle do not exist in relation to these materials, the principle of the microbiological methods used to examine the RMs is based on the multiplication of the organism in a liquid or on a solid growth medium before enumeration or detection.

The possible uses of RMs and CRMs can be summarised as (Mooijman *et al.*, 1992):

1. for first line quality control.
2. as standard stable material for collaborative studies.
3. for determining the influence of matrix ingredients and competitive micro-organisms on the isolation of a particular organism.
4. for developing and validating methods and media for the detection and enumeration of micro-organisms.

5. for comparing the performance of different laboratories.
6. for testing accuracy in individual laboratories.

The six uses listed above can be further subdivided into two major groups. The first being the use of RMs on a routine basis (item 1) and the second the use of RMs and CRMs on an occasional basis (items 2 - 6).

USE OF REFERENCE MATERIALS ON A ROUTINE BASIS

RMs can be used for the quality control of routine microbiological examinations. For this RMs are examined at the same time as routine samples on a regular basis and the results evaluated by the technicians themselves based on predetermined criteria. This is first line quality control or a first line check and is intended to evaluate the quality of examination over time.

Routine use of quantitative reference materials

Control charts can be constructed for the routine use of quantitative RMs. The results obtained from the RMs (representing the information on the current measurement process) are compared to limits established after consideration of the inherent measurement process variability. The most commonly used type of control chart is the Shewhart chart. These charts, developed by Shewhart (1931), are a statistical device used for the study and control of repetitive (production) processes (Duncan, 1974). They are graphs with, in general, the observations made over the course of time plotted on the horizontal axis, a quality characteristic on the vertical axis and control limits (statistically based) for judging the measurement process. The quality characteristic can be a sample mean, a sample range or a sample standard deviation. Shewhart (1931) suggested that the control chart may serve:

- to define the goal or standard for a process that management strive to attain.
- as an instrument for attaining that goal.
- as a means of judging whether or not the goal has been reached.

Such charts can therefore be used in specification, production and inspection (Shewhart, 1931). Control charts are aimed at differentiating between natural variation (due to chance) and variations caused by assignable or special causes. Such assignable causes may be related to analytical problems in a particular measurement or series of measurements and the cause should be investigated and identified. Combinations of quality characteristics can be presented on different charts from the same observations, for example combinations of means and ranges (Anon., 1991). For production processes the observations plotted onto the graphs are based on the analysis of more than one sample. Therefore, it is possible to plot the range or standard deviation for each observation. When examination of more than one sample is impracticable or impossible (as in a microbiological laboratory) a decision can be made to use only one sample per observation on the graph.

The use of control charts with averages as the quality characteristic is useful for controlling the average of the process, i.e. the average number of cfp in a standard volume using RMs over a period of time. The statistics used in combination with these control charts are described in the ISO 8258 standard (Anon., 1991). However, these statistics assume a

Normal distribution of the data, which is not the case for microbiological RMs (Niemelä, 1983). Heisterkamp and co-workers (1991) showed, both on a theoretical and an experimental basis, that a logarithmic transformation of the microbiological counts resulted in data that behave approximately Normally. The analytical results are therefore $\log_{10}$ transformed before the mean and standard deviation (s.d.) is calculated. For calculation of the s.d. the following formula is used.

$$s.d. = 0.8865 \bullet \overline{R} \quad \text{and} \quad \overline{R} = \frac{1}{I-1}\sum_{i=2}^{I} x_i - x_{i-1}$$

where: s.d. = standard deviation.
$\overline{R}$ = average moving range.
I = total number of capsules examined.
x_i = $\log_{10}$ transformed count of the I^{th} capsule.

The s.d. is calculated on the basis of the difference between two succeeding $\log_{10}$ transformed counts. This method of calculating the s.d. is preferred when only one sample is examined for each observation on the graph (Anon., 1991). It will result in a more robust estimate of the s.d. compared to the usual way of calculating the s.d. It also means that this estimate of the s.d. is likely to be less affected by variations in counts due to assignable errors (Van Dommelen, 1995) and results in more robust control limits. From the mean and standard deviation the following control chart limits are calculated on the logarithmic scale:

- warning limits : $\underline{x} \pm 2 \bullet s.d.$
- action limits : $\underline{x} \pm 3 \bullet s.d.$

After calculation of the mean and the control chart limits these values are back-transformed to the original scale in order to obtain a control chart for the original scale. As a result of this transformation, single counts obtained from an RM can be plotted directly on the control chart without calculation of the $\log_{10}$ value. The back-transformation will lead to an asymmetrical distance for the upper and lower warning and control limits around the geometric mean level. The geometric mean and the upper and lower control limits are drawn on the control chart as horizontal lines (see Figure 1). Each time an RM is examined the count is plotted on the graph, using the X-axis as the order of examination. The back-transformation of the mean and the control charts limits is not essential but is merely used to facilitate the notation of new observations.

The geometric mean and the upper and the lower control limits are calculated from the results of examination of the first 20 RMs. Each RM having been examined singly and preferably on different days and by different technicians to cover within laboratory variations. The counts of these RMs should be plotted on a graph, preferably a separate graph, in order to check whether these counts meet the criteria stated below (i.e. that the analytical process is under control). If the result of one or more of these 20 counts does not meet the criteria, the cause(s) for this should be identified and a decision made about the validity of the count(s). If the cause can be identified (assignable cause) then this count should be disregarded and the geometric mean and control limits recalculated from the remaining counts. If the cause cannot be found then the count should be regarded as belonging to the normal variation of the process and should therefore be included in the calculation of the geometric mean and control limits.

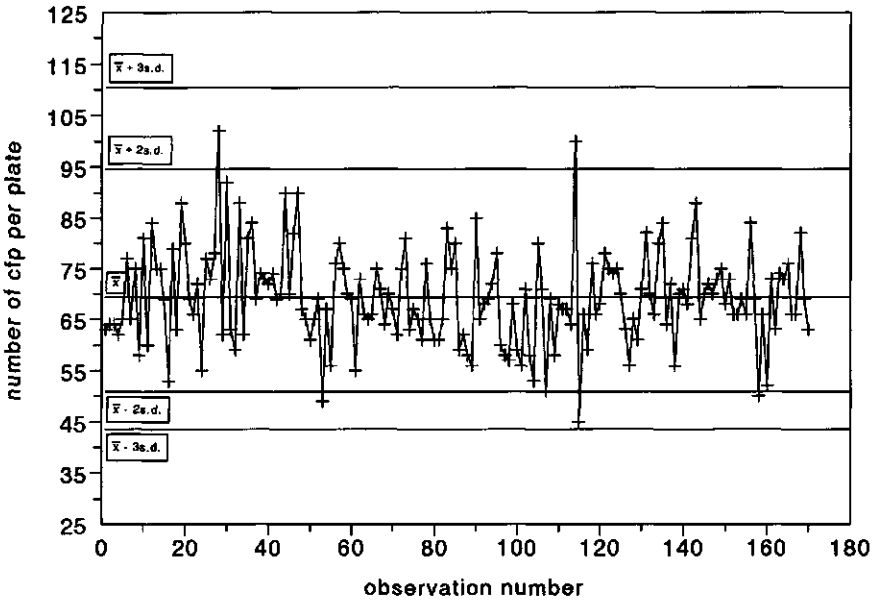


Figure 1. Shewhart control chart using data obtained with the *B. cereus* RM.

When the measurement process is under control, that is when any variation in counts is basically due to chance, the counts obtained with the RMs, in 95 % of the cases, fall between the lower and upper warning limits and in 99.7 % of cases between the lower and upper action limits. When the variation in the counts does not conform to the pattern that might reasonably be produced by chance variation, then it is concluded that the process is out of control, meaning that there are one or more assignable causes. Several tests for detecting out of control situations have been developed. The purpose of each test is to detect a particular non-random pattern in the points plotted on the control chart. These tests have been evaluated by Nelson (1984; 1985). The following criteria are used for interpreting the microbiological control charts (Nelson, 1984; Van Dommelen, 1995):

1. there is a single violation of the action limit ($\bar{x} \pm 3 \bullet s.d.$).
2. two out of three consecutive observations exceed the same warning limit ($\bar{x} \pm 2 \bullet s.d.$).
3. there are nine observations in a row on the same side of the mean.
4. six consecutive observations are steadily increasing or decreasing.

When a measurement process is under control, the chance of incorrectly obtaining a false signal that the process is out of control is less than 0.5 % for each of the tests mentioned above. The overall probability of obtaining a false signal from one or more of these tests is *ca* 1 %. However, in practice these tests should be considered as simple practical rules for action rather than tests with specific associated probabilities (Nelson, 1985).

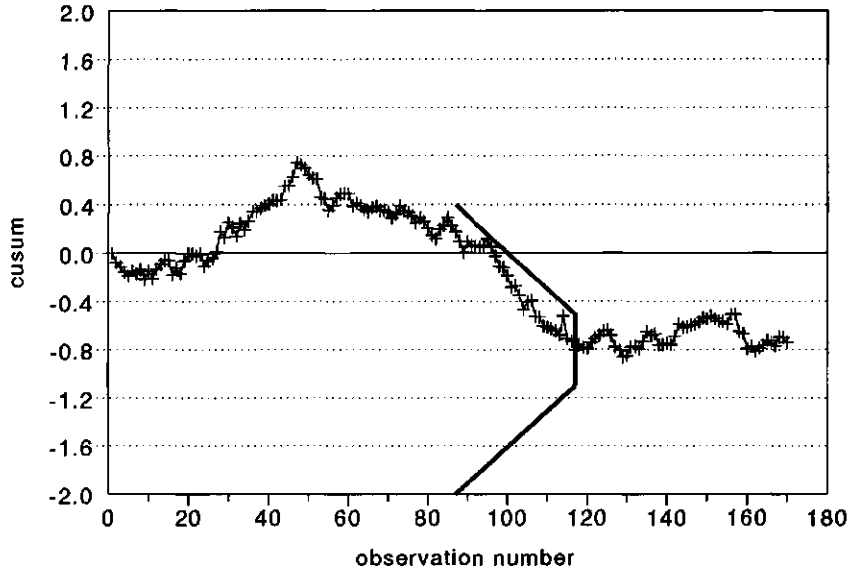
When a measurement process is out of control, the cause(s) should be identified and a decision made on the validity of the count(s). The various tests are sensitive to different

kinds of special cause of error in the measurement process and can be of help in identifying the cause(s). Nelson (1985) gave an overview of each test and their sensitivity for different sources of error. Test 1 is sensitive to a single aberration in the process such as a mistake in calculation, an error in measurement, malfunctions of equipment and so on. Test 2 is sensitive to a shift in the process average although it is also somewhat sensitive to an increase in variation. Test 3 is sensitive to a shift in the process average and test 4 is a test for trend or drift. No simple rules can be defined for judging the validity of results as this will depend on many factors, which are not always strictly related to the measurement process itself, such as the availability of replicate samples or the consequences of using the incorrect results.

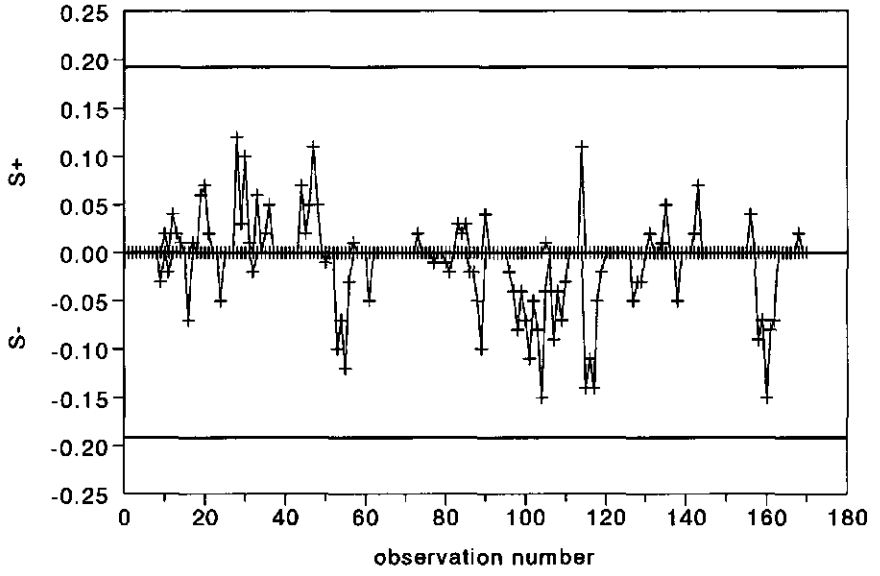
In general Shewhart control charts are more sensitive for detecting incidental changes in the measurement process than for detecting small shifts in the average value of the process (trends). An alternative is the use of Cusum charts for a more rapid detection of trends. The essence of Cusum charts (Cusum stands for cumulative sum) is that the values for each observation, after they have been subtracted from a reference value (e.g. the process mean), are summed (Anon., 1997). To be able to judge whether or not a process is out of control a, so called, V-mask is used. The most convenient form of V-mask is the truncated V-mask. The V-mask consists of three lines, one vertical and two sloping lines, called decision lines. The length of the vertical line is 10 times s.d. and the middle of this line is placed on top of the observation of interest, normally the last observation. The decision lines start at both ends of the vertical line and have a slope of $\pm$ (0.5 • s.d.) per observation interval. Using this V-mask it is possible to judge whether or not a previous observation falls outside the mask, which indicates an out of control situation. An example of a Cusum chart with truncated V-mask arbitrarily placed at observation number 117 is presented in Figure 2a. The data is the same as that used in Figure 1 for the Shewhart control chart. The average of the first 20 ($\log_{10}$ transformed) observations was used for the calculation of the reference value, the same observations were also used to calculate the s.d.

Another type of Cusum chart is described by Van den Berg and co-workers (1996). This type of chart consists of two charts, one for the positive deviations (S^+ chart) and one for the negative deviations (S^- chart). Each chart has its own reference value (k^+ and k^- for the S^+ and S^- charts respectively) that are 0.8 s.d. units above (for the S^+ chart) and below (for the S^- chart) the reference value. Negative Cusum values are plotted as zero on the S^+ chart and the reverse for the S^- chart. Each chart has an action limit defined as the reference value plus or minus (3.2 • s.d.). Once the action limit has been exceeded the corresponding value in the S^+ or S^- chart is reset to zero. An example of such a chart using the same $\log_{10}$ transformed data as for the other Cusum chart is presented in Figure 2b.

Neither Shewhart nor Cusum charts can be used when the material examined is not fully stable. The stability of an RM is difficult to achieve and may not be obtained for all types of micro-organism. For those less stable materials alternative control charts are needed that take into account a change in the level of contamination. A possibility is to adapt the method of calculating the control limits used in a Shewhart control chart by applying the principle of Kalman's filtering (Strejc, 1981). The model developed by Kalman is described in more detail



a: Cusum chart with truncated V-mask.



b: Combined S^+ and S^- Cusum charts.

Figure 2. Examples of Cusum charts using $\log_{10}$ transformed data obtained with the *Bacillus cereus* RM.

in annex A. In simple terms Kalman's filtering produces an estimate of the value for the next observation based on the previous observations. To use Kalman's filtering the time interval between two observations should be more or less constant as the estimates produced are not related to a specific time interval but to the different observations.

An example of a Shewhart control chart with and without Kalman's filtering is presented in Figure 3. The data were obtained with the RM for *Staphylococcus aureus* (enumerated on Baird-Parker agar) as developed by the Dutch Food Inspectorate in Groningen (see also chapter 7) and were kindly supplied by the Dutch Food Inspectorate in 's Hertogenbosch. The RMs were examined over a period of *ca* one year and the level of contamination decreased from *ca* 80 cfp/plate to *ca* 65 cfp/plate. The control limits for the chart without the use of the Kalman filtering were calculated as described previously. The data presented are just a part of the total number of observations as there were too many points to include in one chart. The data were transformed by taking the root of each value before Kalman's filtering was applied. Root-transformation of counts is appropriate in the case of Poisson distributed counts. The standard deviation needed to calculate the control limits was derived from the estimate of the mean as, for a Poisson distribution, the variance is equal to the mean. However, overdisperison from the Poisson distribution is expected and also included. The overdispersion was estimated based on the previous observation in a similar manner to the estimate of the value according to Kalman's filtering. The calculations for the estimates for Kalman's filtering need to be done with computer software (for example with a spreadsheet programme such as Excel or Lotus 1-2-3). In general the use of software makes it easier to calculate control limits and can be of help in detecting an out of control situation irrespective of the type of control chart.

The control limits for a Shewhart chart are calculated on the first 20 observations. The control limits for charts with Kalman's filtering are calculated each time an observation is plotted on the chart, but it will take a number of observations before the estimates produced by Kalman's filtering are comparable to the limits calculated without Kalman's filtering. The number of observations needed will depend on the initial values for overdispersion and level of contamination. With the data sets examined, to determine the effect of the Kalman's filtering, 20 observation were more than enough to give comparable control limits to the ones calculated without Kalman's filtering.

The detection of an out of control situation using a Shewhart chart with Kalman's filtering will be more difficult than for the original Shewhart chart due to the fluctuating control limits. However, this alternative chart allows the use of a quantitative control (although not stable in time) which is preferable to the use of any type of qualitative control as the latter is less sensitive in detecting an out of control situation. The criteria for detecting out of control situations for the original Shewhart chart cannot be identical for a Shewhart chart with Kalman's filtering. Tests 1 and 2, as presented earlier, can be used for charts with Kalman's filtering but tests 3 and 4 cannot be applied as they are directed towards the detection of trends.

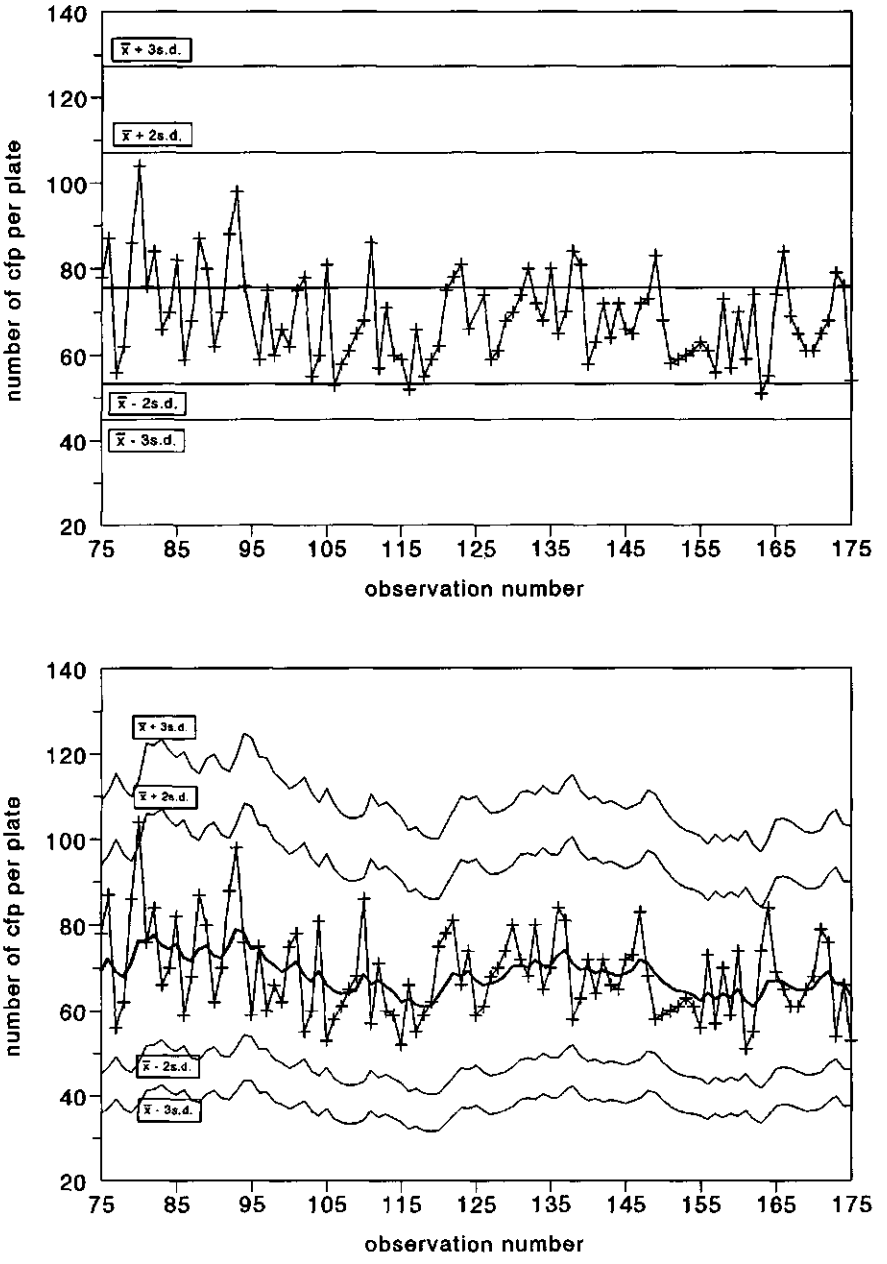


Figure 3. Shewhart control chart without (upper figure with control limits based on the first 20 observations) and with (lower figure) Kalman filtering using part of the data obtained with an "unstable" RM containing *S. aureus*.

Qualitative reference materials

Control charts for processes in which the data are not Normally distributed are also possible. In the ISO 8258 standard (Anon., 1991) examples of control charts are presented that are based on binomial and Poisson distributions. These distributions could also apply to results obtained using the qualitative RMs. However, many replicates have to be made to obtain a single observation for such control charts. This will not be possible in practice using RMs. The alternative is to examine a single capsule for the presence of the target organism with each series of examinations. The result of this examination has to be positive, even though there is a small chance that a capsule will not contain the target organism. As long as the fraction of capsules in which the target organism is not present is small (< 5 %), all capsules examined should be found positive.

Some laboratories examine two capsules at a time instead of one in routine use. This is discouraged as laboratories tend to accept one negative isolation result out of the two capsules examined. They assume that the one negative is the result of a truly negative capsule and thus there is nothing wrong with the detection procedure. However, because the probability of a capsule not containing the target organism is small at the mean level of contamination of the RM, the probability of one capsule not containing the target organism and one that does will still be very small. For example, when the percentage negative capsules is 2.0 % the chance of finding one negative and one positive capsule is just 4 %.

This can be illustrated by the following example from a laboratory using the *Salmonella* RMs. Each time samples were tested for the presence of *Salmonella* the laboratory examined two *Salmonella* RMs as positive control samples, this was done in total 162 times. Out of the 162 test times on 23 occasions one capsule was found positive and the other negative with a particular selective enrichment broth. Based on the above premise the laboratory would tend to accept that these 23 cases were due to a truly negative capsule. However, this laboratory did not use just one selective enrichment broth but three; all were inoculated from the same pre-enrichment culture. With the other two enrichment broths they found only 12 instances where there was one positive and one negative capsule. So on 11 occasions the one enrichment broth showed a poorer performance than the other two, but results with this broth would have been accepted if it had been used alone.

In order to judge performance over a longer period of time (overall performance) the number of negative isolations are added together and compared with the expected number for that batch of RMs. The expected number of negative (or positive) isolations is calculated from the fraction of negative capsules and the total number of capsules examined by using the binomial distribution. The calculation is detailed in the section on the occasional use of qualitative CRMs (see page 130). As long as only a single capsule is used on each test occasion the interpretation for the occasional use of RMs and CRMs can be followed as no day to day variation can be calculated. In order to facilitate the interpretation of overall performance, the results of each analysis of an RM (+ or -) can be plotted on a chart.

USE OF REFERENCE MATERIALS ON AN OCCASIONAL BASIS

As the results of experiments performed occasionally have to be judged per experiment, they need to be compared to a reference value. RMs are supplied with an indication of their level of contamination (quantitative RMs) or their fraction of capsules not containing the target organism (qualitative RMs). These values are based on the results of examination of a small number of capsules in a single laboratory. Certified RMs are supplied with a certified reference value which is based on the results from a number of laboratories. The results obtained by a laboratory using CRMs are therefore compared to the results of all the laboratories involved in the process of certification of the material. However, CRMs are very expensive and not widely available. Their main importance is to enable a laboratory to demonstrate its trueness. In most other cases of occasional use CRMs will not be necessary but RMs can be used instead. The use and limitations of quantitative and qualitative RMs and CRMs for occasional use are described below.

Occasional use of quantitative reference materials

Certified reference materials

The procedure for certification of a quantitative RM is described in chapter 4 for the *Bacillus cereus* RM. The certificate accompanying the CRM gives the certified geometric mean with 95 % confidence limits that are based on the examination of a large number of capsules. For example, the results from the examination of 171 capsules were used for calculation of the certified values for the *B. cereus* RM on MEYP agar. As the user of a CRM will examine only a small number of capsules user tables must be prepared. In these tables the 95 % confidence limits are given for different combinations of numbers of capsules and replicates per capsule likely to be examined in practice. These limits are calculated using the certified value and the variance components using the following formulae (Heisterkamp *et al.*, 1993):

$$\hat{\sigma}^2_{\bar{x}} = \hat{\sigma}^2_L + \frac{\hat{\sigma}^2_{cap}}{I} + \frac{\hat{\sigma}^2_{sub}}{I \bullet J},$$

$$LL = \hat{\mu} - t_{1-\alpha/2,L-1} \bullet \hat{\sigma}_{\bar{x}} \quad \text{and} \quad UL = \hat{\mu} + t_{1-\alpha/2,L-1} \bullet \hat{\sigma}_{\bar{x}}.$$

where: I = number of capsules examined.
J = number of replicates examined per capsule.
L = number of laboratories used for the determination of the certified value.
LL = lower limit.
UL = upper limit.
$t_{1-\alpha/2,L-1}$ = Student t-value at (1 - α/2) fractile and L-1 degrees of freedom.
$\hat{\sigma}^2_{cap}$ = variance component for capsules.
$\hat{\sigma}^2_L$ = variance component for laboratories.
$\hat{\sigma}^2_{sub}$ = variance component for replicates.
$\hat{\sigma}^2_{\bar{x}}$ = variance for a combination of number of capsules and replicates examined.
$\hat{\mu}$ = certified value.

The limits are calculated using the certified value and variance components per method on a $\log_{10}$ scale. Back-transformation of the values obtained for the upper and lower limit will give the limits on the normal scale. Due to the back-transformation the certified values (mean, upper and lower limit) on the original scale represent geometric values. The limit values are rounded to whole counts. For the lower limit values are rounded to the lowest whole count while for the upper values are rounded to the highest whole count.

A user table presents the upper and lower limits a laboratory user must find with respect to number of capsules and replicates examined. Based on the counts obtained the user calculates the geometric mean and compares this value to the limits presented in the user table for that specific combination of capsules and replicates examined. The user table for the *Bacillus cereus* CRM 528 on MEYP and PEMBA incubated for 24 h at 30 °C is presented in Table 1. The upper and lower confidence limits on MEYP are also presented in Figure 5 in chapter 4 to illustrate the effect of the number of capsules examined on the confidence limits. Table 1 below and the Figure 5 in chapter 4 both show that the effect of the increase in the number of capsules examined is limited once *ca four* capsules are examined.

Table 1. The 95 % confidence limits of the geometric mean number of cfp per 0.1 ml on MEYP (certified value 53.4) and PEMBA (certified value 55.0) after 24 h incubation at 30 °C (MEYP) or 37 °C (PEMBA) for different combinations of capsules and replicates of the *Bacillus cereus* CRM.

Number of capsules examined	Number of replicates examined	MEYP		PEMBA	
		lower geometric mean	upper geometric mean	lower geometric mean	upper geometric mean
1	1	37	77	38	79
	2	40	70	40	74
2	1	41	70	42	72
	2	43	66	43	69
3	1	42	67	43	70
	2	45	64	45	67
4	1	44	65	44	68
	2	45	63	46	66
5	1	44	64	45	67
	2	46	62	46	66
6	1	45	63	45	66
	2	46	62	46	65
7	1	45	63	46	66
	2	46	61	47	65
8	1	45	62	46	66
	2	47	61	47	65
9	1	46	62	46	65
	2	47	61	47	64
10	1	46	62	46	65
	2	47	61	47	64

Although provisional conclusions can be drawn from the user table about the number of capsules that should be examined, another point must be taken into consideration, the power of the analysis. In order to explain what the power is (see also Kish, 1965), more information on the theory of testing statistical hypotheses is needed. For this a comparison between the result of an experiment, in this case the examination of a CRM in a laboratory, and the true situation is made. The true situation is based on the certified value and is denoted as $\hat{\mu}$ while the result of an experiment is denoted by μ_{lab}. The hypothesis is tested to determine whether μ_{lab} is equal to $\hat{\mu}$ or not. Four different situations can be distinguished which are presented in Table 2 together with their statistical probabilities.

Table 2. Possible combinations between the results of an experiment (μ_{lab}) and the certified value ($\hat{\mu}$) and their statistical chances.

		Result of the experiment	
		$\mu_{lab} = \hat{\mu}$	$\mu_{lab} \neq \hat{\mu}$
True situation	$\mu_{lab} = \hat{\mu}$	1 - α	α
	$\mu_{lab} \neq \hat{\mu}$	β	1 - β

For the results presented in a user table the true situation of $\mu_{lab} = \hat{\mu}$ is tested. The 95 % confidence limits are also presented in the user table, meaning that α is set at 5 %. Thus in 5 % of the experiments performed the wrong conclusion is made, meaning that the geometric mean value found by the laboratory is not equal to the certified value although it is equal in reality. This is called a type I error. For the power of analysis a similar formulation can be given, only in this case the true situation of $\mu_{lab} \neq \hat{\mu}$ is tested. The power (denoted by 1 - β) can be defined as the chance that the geometric mean value found by the laboratory is not equal to the certified value when in reality they indeed are different. Where they actually are different it is called a type II error (β).

Van Dommelen (1995) elaborated on this power analysis for CRMs. The power of the analysis should theoretically be as high as possible. For power analysis of microbiological RMs a value of 0.8 is used. Higher values will lead to a greater difference between the upper and lower limits presented hereafter or more capsules will need to be examined to detect a certain difference.

Before a recommendation can be made on the number of capsules that need to be examined, a decision should be made on how large the difference between the geometric mean value from the experiment and the certified value at maximum may be. The smaller the difference accepted the greater the number of capsules that need to be examined in order to draw conclusions with the same power. The size of the difference must be chosen in relation to what difference is acceptable in practice or what difference will have a consequence for the judgement of results. Figure 4 shows the relationship between the power of the test and the geometric mean value that can be detected as different from $\hat{\mu}$ examining 1, 2, 5 and 10 capsules respectively in duplicate for the *B. cereus* CRM.

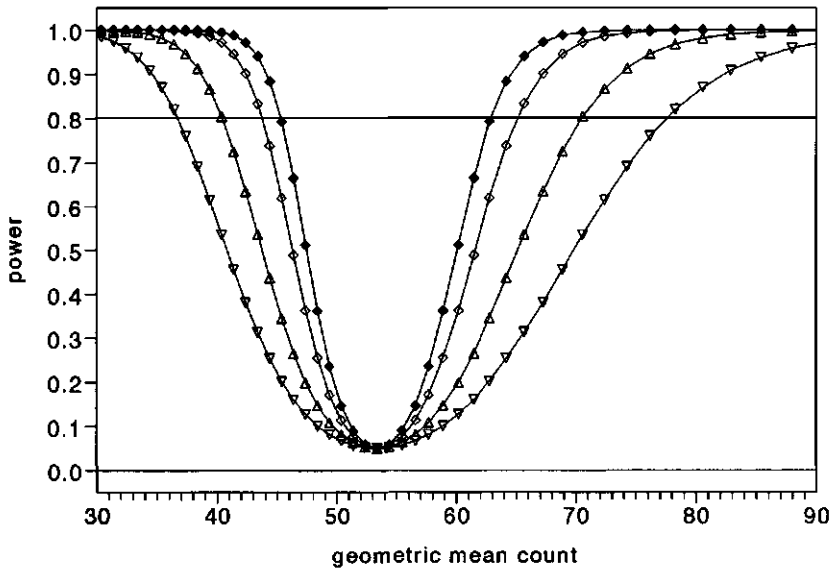


Figure 4. Relationship between the power, the geometric mean count and the number of capsules (I = 1 ∇; I = 2 Δ; I = 3 ◊; I = 4 ♦) examined, using two replicates per capsule for the *Bacillus cereus* CRM 528 enumerated on MEYP agar after 24 h incubation at 30 °C (certified value 53.4).

Table 3 presents, for the same conditions of examination, the lower and upper value for the observed geometric mean values for a power of $\geq$ 0.8 for various combinations of numbers of capsules and replicates. The values presented are rounded up or down in the same way as for the values presented in an user table.

As can be seen from Table 3 the effect of examining each capsule in duplicate is most pronounced when small numbers of capsules are examined. The effect of the number of replicates is demonstrated in Figure 5 for the examination of one and five capsules. From these figures it can be seen that the effect of more than two replicates is relatively small especially using a higher number of capsules.

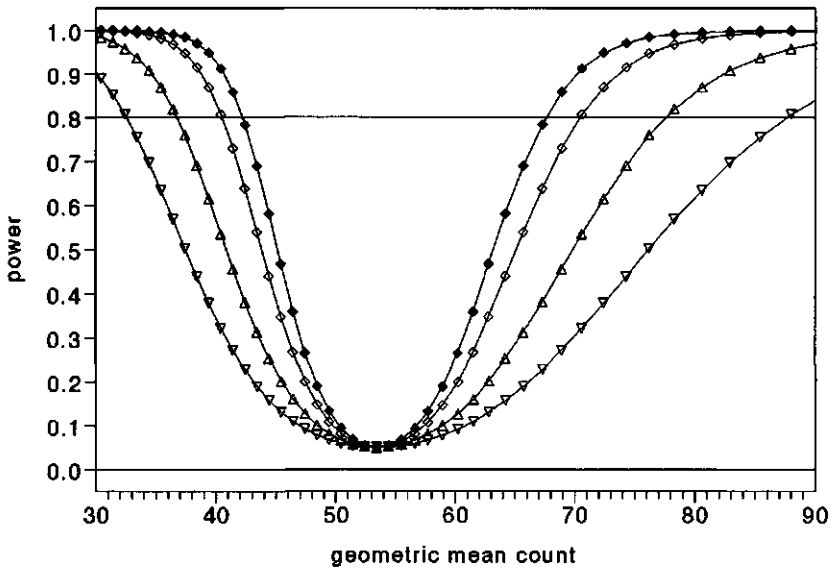
Based on the user tables and the results of the power analysis Van Dommelen (1995) recommended the number of capsules that should be examined. For the *B. cereus* CRM examination of five capsules in duplicate is recommended. A larger number of capsules will lead to only a small improvement in detecting a difference from the certified value. The difference in limits between the user table (see chapter 4) and Table 3 are small using the recommended number of capsules. This means that when a laboratory finds a geometric mean value in an experiment which is, according to the user table, significantly different from the certified value then, according to Table 3, it shows that a systematic difference between the true laboratory mean and the certified value exists. Where the true laboratory mean is only slightly different from the certified value the chance of detecting such a small systematic

difference is minimal, meaning that the results found in an experiment will, in most cases not be significantly different from the certified value.

Table 3. Lower and upper geometric mean values at a power of at least 0.8 in relation to the number of capsules and replicates examined using the *Bacillus cereus* CRM on MEYP agar after 24 h incubation at 30 °C (certified value 53.4).

Number of capsules examined	Number of replicates examined	Lower geometric mean	Upper geometric mean
1	1	32	88
	2	36	78
2	1	37	77
	2	40	71
3	1	39	73
	2	42	68
4	1	40	70
	2	43	67
5	1	41	69
	2	43	66
6	1	42	68
	2	44	65
7	1	42	67
	2	44	64
8	1	43	66
	2	44	64
9	1	43	66
	2	45	64
10	1	44	65
	2	45	63

The recommendation made by van Dommelen (1995) was based on the usual practice in laboratories of examining only 1 or 2 replicates per sample. As the price of CRMs is very high, alternative combinations of less capsules and more replicates per capsule may be beneficial. Using the variance components for the *B. cereus* CRM ($\hat{\sigma}^2_L$ = 0.00028; $\hat{\sigma}^2_{cap}$ = 0.00039 and $\hat{\sigma}^2_{sub}$ = 0.00413) the variance for combinations of numbers of capsules and replicates can be calculated. An alternative with a comparable variance for the examination of five capsules and two replicates per capsule (variance components 0.00077) would be three capsules and four replicates per capsule (variance component 0.00075). However for 3 capsules/4 replicates more plates are needed. It is therefore for the laboratory to decide the optimal combination of capsules and replicates.



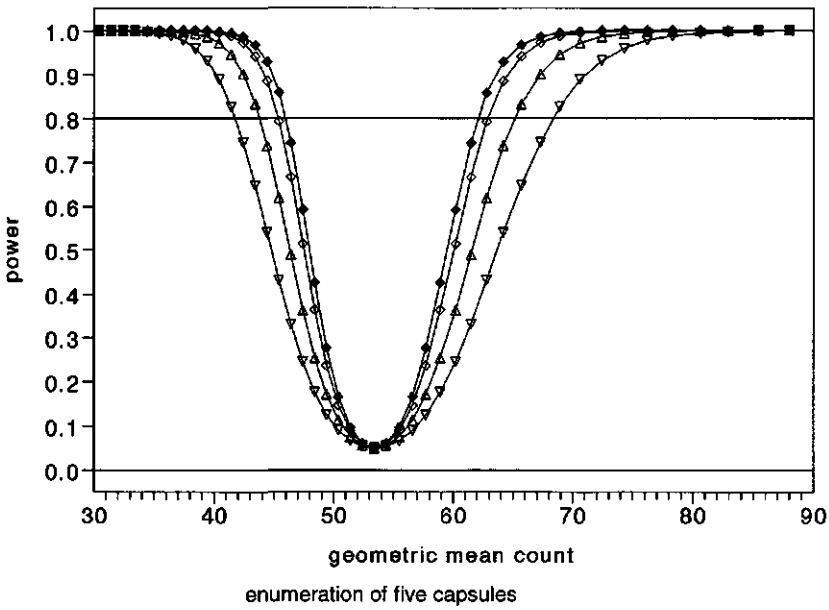


Figure 5. Relationship between the power, the geometric mean and the number of replicates (J = 1 ∇; J = 2 Δ; J = 3 ◊; J = 4 ♦) for the *Bacillus cereus* CRM enumerated on MEYP agar after 24 h incubation at 30 °C (certified value 53.4).

Non certified reference materials

The level of contamination determined for each batch of RM is based on the examination of 20 capsules, in duplicate, on a single day, using the same batch of medium, and in one laboratory. The geometric mean count and its 95 % confidence interval are calculated from the results obtained with these 20 capsules. The interval is then calculated using $\log_{10}$ transformed counts and the following formula:

$$LL = \hat{\mu} - t_{1-\alpha/2,l-1} \bullet \hat{\sigma}_x \quad \text{and} \quad UL = \hat{\mu} + t_{1-\alpha/2,l-1} \bullet \hat{\sigma}_x$$

where: l = number of capsules examined.
LL = lower limit.
UL = upper limit.

$t_{1-\alpha/2,l-1}$ = Student t-value at (1 - α/2) fractile and l-1 degrees of freedom.

$\hat{\sigma}_x$ = standard deviation of ($\log_{10}$ transformed) counts.

$\hat{\mu}$ = mean of ($\log_{10}$ transformed) counts.

Before these limits can be use as a reference value additional information on the variance between laboratories is needed. This can be obtained from the certification study on a similar RM. The different variance components (for laboratories, capsules and replicates) are presented in the certification report. The variance for the laboratory component is added to the other variance component to calculate the 95 % confidence interval as follows:

$$\hat{\mu} \pm t_{1-\alpha/2,L-1} \bullet \sqrt{\sigma_x^2 + \sigma_L^2}$$

The adjusted interval now represents the interval within which the results of other laboratories should fall.

A similar procedure can be followed when precision data are available. The laboratory component is the difference between the reproducibility (R) and repeatability (r) values. The variance component for laboratories is calculated by dividing the R and r values by 2.8. The values are then squared and finally the lowest value is subtracted from the highest. The laboratory variance component obtained can be included in the formula above. No precision data are published to date in international standards, but a current project financed by the European Commission is determining the precision of a number of ISO standard methods for the European standardisation organisation (CEN).

Qualitative reference materials

Certified reference materials

As with the quantitative CRM user tables also have been constructed for the qualitative CRM for the small numbers of capsules that are normally examined in practice. Instead of an upper and lower limit of the geometric mean, for qualitative CRMs, the user tables must give the minimum number of capsules that must be found positive for the target organism. The calculations are based on the certified fraction of capsules in which the target organism could not be detected. The certified fraction is based on the number of negatives found in the certification study using a specified detection method. The total number of capsules

examined in the certification study was high (each of the 10 - 12 laboratories examined *ca* 45 capsules) and the number of negatives was small. This means that only a small number of capsules will not contain the target organism and will therefore result in the expected negative detection rate. Using the binomial distribution the chances of finding a certain number of negative (or positive) capsules out of a given number of capsules examined can be calculated using the certified fraction of negative capsules (p_{neg}) in the formula presented below:

$$p_y = \left(\frac{I}{y}\right) \bullet (p_{neg})^{I-y} \bullet (1 - p_{neg})^{y},$$

$$\left(\frac{I}{y}\right) = \frac{I!}{y! \bullet (I-y)!} \qquad \text{and} \qquad I! = I \bullet (I-1) \bullet (I-2) \bullet \ldots \bullet 1$$

where: p_y = chance of finding "y" positive isolations.
p_{neg} = certified fraction of negative capsules.
y = number of positive isolations.
I = number of capsules examined.
I - y = number of negative isolations.

Using these formulae the chances for a specific event ("y" positive isolations out of "I" capsules examined) is calculated. To obtain the 95 % confidence limit for the number of capsules that must be found positive the individual chances of obtaining "I", "I-1", "I-2" etc. positive isolations (thus 0, 1, 2 etc. negative isolations) are added together until the cumulated chance is equal to or higher than 95 %. Using these calculations user tables are prepared in which the minimum number of positive isolations are presented in relation to the number of capsules examined and the certified fraction of negatives.

Here again the power of the analysis is also of importance. The number of capsules that should be examined will depend on the maximum acceptable difference between the certified fraction of capsules in which the target organism could not be detected (p_{neg}) and the fraction of capsules in which the target organism could not be detected by the laboratory examining the capsules (p_{lab}). Figure 6 presents the power of analysis in relation to the number of capsules examined and the value for p_{lab}, using the certified value (p_{neg} = 0.012) for the *Listeria monocytogenes* CRM 595 (In 't Veld *et al.*, 1996).

As for the quantitative CRM a power of 0.8 is used as a minimum for the qualitative CRM. Figure 6 shows that the more capsules that are examined the smaller the detectable difference between p_{neg} and p_{lab}. However, the difference between the examination of 20 and 30 capsules is very small. This is a result of the relationship between the number of capsules examined and the number of capsules expected to be found positive for the target organism. Using the formulas for the binomial distribution presented earlier, the number of negatives expected in relation to the number of capsules examined can be calculated. For a p_{neg} of 0.012 and an α of 5 %, no negatives are expected when 1 to 4 capsules are examined. One negative can be expected when 5 to 29 capsules are examined and two negatives when 30 to 68 capsules are examined. The changes in the expected number of positives and their effect on the power of analysis can be seen in Figure 7, the sudden drop in the curve represents the power of analysis.

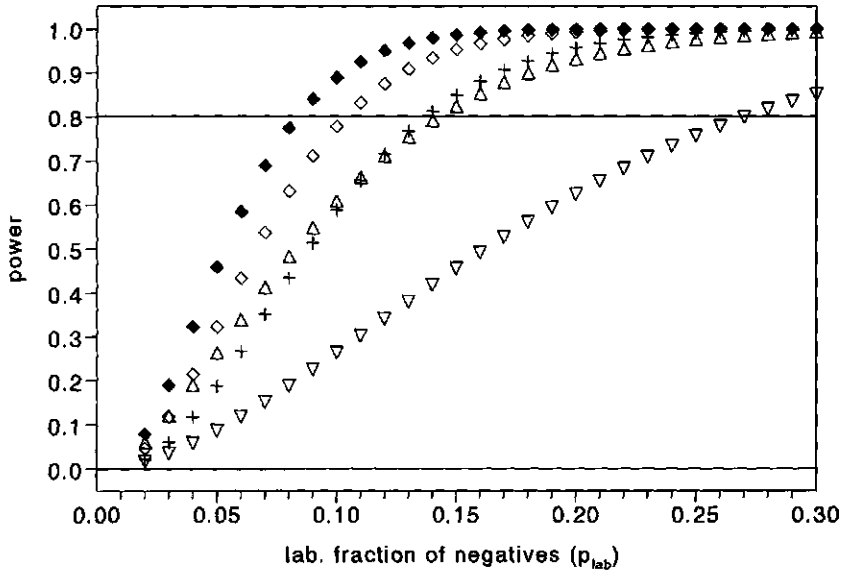


Figure 6. Relationship between the power, the number of capsules examined (I = 10 ∇; I = 20 Δ; I = 30 +; I = 40 ◊; I = 50 ♦) and the fraction of negatives found by the laboratory (p_{lab}) for a certified fraction of negatives (p_{neg}) of 0.012 for the *Listeria monocytogenes* CRM 595.

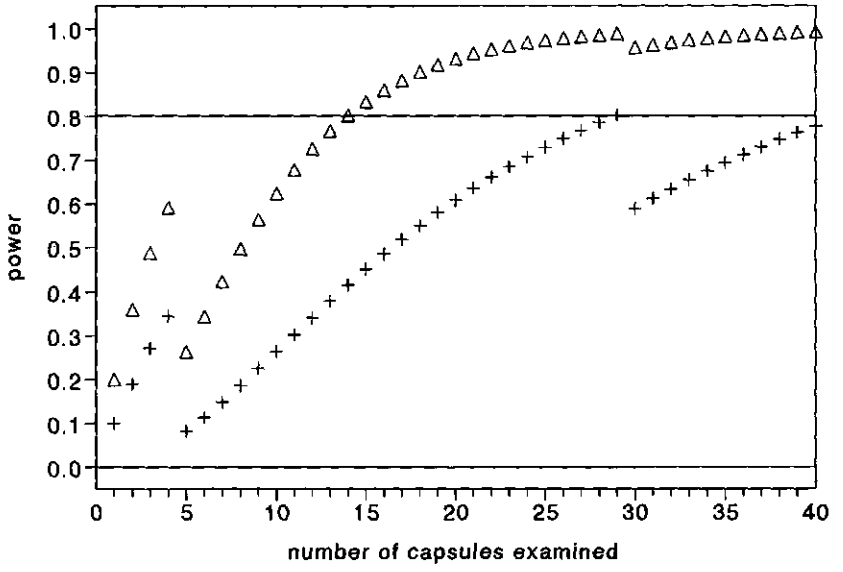


Figure 7. Relationship between the power and the number of capsules examined for two values of p_{lab} (Δ = 0.20 and + = 0.10) using the *Listeria monocytogenes* CRM 595 (p_{neg} 0.012).

Once a laboratory has decided which fraction of negatives is acceptable in relation to the certified fraction of negatives (or which difference between p_{neg} and p_{lab}) it is easy to calculate the required (and therefore recommended) number of capsules that must be examined for a power of ≥ 0.8. Subsequently the minimum number of capsules that must be found positive is calculated using $\alpha = 0.05$. Such a user-table was prepared for the *L. monocytogenes* CRM (In 't Veld *et al.*, 1996) and is presented in Table 4.

Table 4. Recommended number of capsules to be examined and minimum number of capsules to be found positive in order to detect a certain laboratory fraction of negatives (p_{lab}) with a power of ≥ 0.80, using the *Listeria monocytogenes* CRM 595 and a certified fraction of negatives of 0.012.

p_{lab}	**Recommended number of capsules to be examined**	**Minimum number of capsules to be found positive for *L. monocytogenes***
3 %	378	370
4 %	197	192
5 %	110	107
6 %	91	88
7 %	60	58
8 %	53	51
9 %	47	45
10 %	29	28
11 %	27	26
12 %	24	23
13 %	23	22
14 %	21	20
15 %	19	18

Non certified reference materials

The percentage of negative capsules (not containing the target organism) and the 95 % confidence upper limit, as presented with each batch of RMs, are based on the enumeration of 50 capsules in a single laboratory at one time according to a specific protocol. This protocol was specially developed for the enumeration of low numbers of organisms in a single capsule and is thus different from the procedure employed by the user of the RM. The theoretical percentage of negative capsules is calculated based on the average level of contamination and the assumption of a Poisson distribution. It is also tested by the T_2 test

(see chapter 1) to determine whether the counts conform to a Poisson distribution. When the variation between counts is significantly larger than would be expected for a Poisson distribution the homogeneity factor ($T_2/(I-1)$) is included in the calculation of the fraction of negatives. The formula for calculation of the upper limit for the fraction of negatives (including the homogeneity factor) is presented below.

$$\exp - \left(\frac{\hat{\mu} + t_{0.95,49} \cdot \sqrt{\hat{\mu} \cdot \left(\frac{T_2}{I-1} \right)}}{\sqrt{I}} \right)$$

where: $t_{0.95,49}$ = Student t-test value with $\alpha = 0.05$ and 49 degrees of freedom.
$\hat{\mu}$ = mean of counts.
I = number of capsules examined.
T_2 = Cochran's dispersion test statistic.

The number of capsules that should be found positive out of a given number examined can easily be calculated from the fraction of negatives (or the upper limit for this fraction) indicated with the batch of RM. It can be done in the same way as for the user tables with the certified RM using the formula for binomial distribution.

The advantage of the enumeration procedure for the qualitative RM is that both the level and homogeneity of the batch can be checked. Using a detection procedure only the percentage of capsules not containing the target organism can be determined and a large number of samples must be examined in order to state the fraction of negatives with a precision similar to that of the enumeration procedure. The reconstitution procedures for enumeration and for detection of qualitative RMs are different and might lead to a difference between the detection results found in practice and those obtained by the special procedure for the enumeration of the capsules. Fortunately, only a small difference was observed between enumeration and detection results in the certification studies of the *Salmonella* (see chapter 3) and *L. monocytogenes* RMs (In 't Veld *et al.*, 1996). In both studies a somewhat higher fraction of negatives was found using the detection procedures. Thus for qualitative RMs the enumeration procedure is preferred in order to determine the fraction of negatives of a batch as the costs for the checks will be much lower and the difference between the enumeration and detection procedures is small in relation to the use of the RM.

DISCUSSION

Use of RMs on a routine basis

Quantitative RMs

Two types of control chart (Shewhart and Cusum) have been presented as tools to monitor the quality of the quantitative methods routinely carried out in a laboratory. The Shewhart chart, which is already in use in laboratories in The Netherlands, works very well with a stable RM. The control limits can be calculated from the first 20 ($\log_{10}$ transformed)

counts, although not all laboratories use $\log_{10}$ transformation of counts for Shewhart charts (Mulder and Strikwerda, 1996). Figure 2a/b presents two types of Cusum chart that both lead to conclusions about the control of the measurement process that are similar to those from a Shewhart chart. The fact that Cusum charts are more sensitive for the detection of small shifts in the level of contamination (trend) is best demonstrated by the chart prepared according to the ISO/TR 7871 document (Anon., 1997). The V-mask in Figure 2a is placed in a position where this trend is most obvious, although according to the ISO/TR 7871 there was actually no trend as none of the observations exceeded the lines of the V-mask. This possible trend is not indicated as clearly in the other Cusum chart (Figure 2b). The advantage of the combined S^+/S^- charts is that they are much easier to interpret as fixed action limits are defined.

For RMs that are not fully stable Shewhart and Cusum charts as such do not function well (see Figure 3). Kalman's filtering, however, gives an opportunity to apply the basic principles of Shewhart control charts using data obtained from such RMs. Detection of out of control situations from Shewhart charts both with and without Kalman's filtering led to similar conclusions (when used on data sets where no decrease in the level of contamination occurred). However, this procedure requires further testing and with application to more data sets to optimise its use. A disadvantage of Kalman's filtering is that it becomes impossible to detect small trends. Some trends might be caused by other factors related to the laboratory rather than due to the instability of the RM. Therefore, use of Shewhart charts with Kalman's filtering must be limited to instances where the instability of the material is proven and not to compensate for the occurrence of changes in the level of contamination. Kalman's filtering is preferred to a linear regression model because the rate of decrease in numbers of organisms in the RMs is not always constant. Stability tests carried out with both RMs (see chapter 5) and also data from the literature (LiCari and Potter, 1970) indicate that the rate of decrease is not always constant.

Qualitative RMs

Detection of out of control situations by the routine use of qualitative RMs is not as sensitive as it is with quantitative RMs. The recommendation to examine only a single capsule on each occasion will mean that only large deviations in performance will be detected with a reasonable probability. However, because of costs and labour, it is not feasible to increase the number of capsules examined each time. Thus it is of importance (even more than with the quantitative RMs) and strongly recommended that several capsules are examined at regular intervals as described for the occasional use of CRMs. The frequency of this type of testing is difficult to define but, for example, a change in the batch of dehydrated medium used would be a good reason to increase the number of capsules examined.

Use of RMs on an occasional basis

Quantitative RMs and CRMs

The variance components (for laboratory, capsule and replicates) for a CRM will determine the magnitude of the difference between the true laboratory mean and the

certified value that can be detected. Table 5 presents the variance components for the quantitative CRMs produced so far.

Table 5. Variance components (on $\log_{10}$ scale) for quantitative CRMs.

CRM containing	Medium used for enumeration	Variance component		
		laboratories ($\hat{\sigma}^2_L$)	capsules ($\hat{\sigma}^2_{cap}$)	replicates ($\hat{\sigma}^2_{sub}$)
Enterococcus faecium (CRM 506)	KFA	0.0128[a]	0.0032[a]	0.0126[a]
	m-EA	0.0069[a]	0.0064[a]	0.0095[a]
Enterobacter cloacae (CRM 527)	LSA	0.0086	0.0028	0.0073
Bacillus cereus (CRM 528)	MEYP	0.00028	0.00039	0.00413
	PEMBA	0.00052	0.00126	0.00303
Escherichia coli (CRM 594)	T7A (30/37)	0.0073	0.0027	0.0051
	LSA (30/37)	0.0055	0.0030	0.0193

a = variance components on $\log_e$ scale

For the *B. cereus* CRM these variance components are small. For example, examination of five capsules in duplicate of this CRM will detect a difference of 22 % between the certified value and the true geometric mean of the laboratory (for a lower and upper geometric mean value of 43.7 and 65.3 respectively and a certified value of 53.4). In comparison the variance components for the *Enterobacter cloacae* CRM (Mooijman *et al.*, 1995) are much higher and will lead to a much wider range for the upper and lower geometric mean value than with the *B. cereus* CRM. The *E. cloacae* CRM has a certified value of 34 on Lauryl Sulphate Agar (LSA) and the corresponding lower and upper geometric mean values are 16.7 and 70.3 respectively (examination of five capsule in duplicate) which corresponds to a difference of 104 % between the true laboratory mean and the certified value. This difference in variance components cannot solely be due to a difference in the homogeneity of the CRMs, but is also related to differences in methodology and the physiological state of the organisms in the CRM. The levels of the selective agents of the LSA agar might be critical for the recovery of sublethally injured organisms from the CRM and/or the preparation of the agar is critical.

When a laboratory uses a non certified RM a confidence interval can be calculated that includes the variation between laboratories. Between laboratory variance data can be obtained from the certification study of a similar RM or precision data included in international standards. However, is it permissible to use the laboratory variance component of a CRM, which is determined for a specific batch, for batches of non-certified RMs? Differences between batches of RMs are related to the level of contamination and the homogeneity. The variation caused by these factors are included in the within laboratory variance (variance of capsules and replicates) and thus will be determined for each batch of RM. The between laboratories variance component can therefore be regarded as independent of the batch of CRM and so the laboratory variance component of a CRM is

applicable to batches of RM. The confidence limits calculated for an RM are based on the examination of 20 capsules in duplicate, a number not likely to be examined in a laboratory. In order to estimate the confidence limits for smaller numbers of capsules examined the lower and upper limit presented on page 125 must be adjusted by multiplying the term $\hat{\sigma}_x$ by the square root of the number of capsules examined divided by 20. For example, when four capsules instead of 20 are examined the term $\hat{\sigma}_x$ must be multiplied by a factor of $\sqrt{5}$.

Qualitative RMs and CRMs

From Table 4 it is clear that, using qualitative CRMs, it is very expensive to demonstrate that the performance of a laboratory (p_{lab}) is close to the certified fraction of negatives. In practice examination of 20 - 30 capsules at one time is considered feasible. This means that a p_{lab} of 10 % or more can be detected with a high probability (power $\geq$ 0.8). Here again the drop in the power curve greatly affects the number of capsules that must be examined. This effect can be eliminated in the ideal situation where a CRM has a p_{neg} of 0 %, in this situation all capsules examined should be positive. A negative detection result proves that a laboratory has a p_{lab} of > 0%. But how high is this p_{lab} value likely to be? A user table similar to Table 4 can be constructed for a p_{neg} of 0 %, the results are presented in Table 6 which shows the minimum value for p_{lab} that can be detected in relation to the number of capsules examined. If a CRM has a p_{neg} = 0 % the number of capsules to be examined is reduced for a certain value of p_{lab}. However, it is still difficult to demonstrate small differences between p_{neg} and p_{lab}. Examination of 20 - 30 capsules will only reveal a p_{lab} of $\geq$ 6 %.

Table 6. Relationship between the laboratory fraction of negatives (p_{lab}) and the number of capsules to be examined for a power of $\geq$ 0.8 and a p_{neg} of 0 %.

p_{lab}	**Recommended number of capsules to be examined**
1 %	161
2 %	80
3 %	53
4 %	40
5 %	32
6 %	27
7 %	23
8 %	20
9 %	18
10 %	16

RMs with a p_{neg} of 0 % can be prepared by raising the average level of contamination of the batch. However, this has its limitations as the higher the level the easier it will be to detect the target organism. Raising the average level of the batch to a mean of 8 or 9 cfp/capsule reduces the chance of obtaining a capsule without the target organism to three and one respectively out of 10,000. The batches of CRM produced so far consist of 5,000 - 10,000 capsules.

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Appendix A: Kalman's filter model

Sometimes the value (i.e. the level of contamination) of RMs is subject to a gradual change. Often this change is a slow decrease in the level of contamination as a result of prolonged storage. The change has no effect on the use of a control chart as long as it is taken into account. Assuming a fixed value for the RM an out of control situation would be reached some time after such change has set in. Appropriate quality control would consist of a comparison of a current observation with its expectation, where the expectation must take into account the gradual change in the value that has taken place up to the time of observation.

The Kalman's filter model (KF) is eminently suited for this purpose as it attempts to estimate the "state" (here: the true value) of the process based on all prior observations. The essence of the KF is that there exists, at any sequential observation k, a "state" x_k that slowly changes according to the formula:

$$x_{(k+1)} = a \bullet x_k + w_k$$

However, the state is only observed with error as y_k according to the following formula:

$$y_k = x_k + v_k$$

The variables w_k and v_k are zero mean random variables with variances var(w_k) and var(v_k), respectively, a is a constant.

Standard formulae for estimating the state (value) at point k are available. Essentially they are weighted means of the estimated state at point $(k-1)$ and the observation at k, y_k. If the process has no built-in drift (i.e. $a = 1$) then the best predictor for the next observation $(k+1)$ is simply the state at point k. This would provide a suitable starting point for control, as this "best predictor" can be compared with the actual observation $y_{(k+1)}$. If the observation differs "too much" from its predictor, this is a signal that somewhere an error has occurred.

The problem encountered when applying this model in laboratory practice is finding suitable values for var(w_k) and var(v_k) as these are not themselves estimated by the KF. If the observations are counts, then it seems logical - as a first approximation - to assume a Poisson distribution. Root transforming those Poisson counts would then automatically give a value for var(v_k) of 0.25 (Rao, 1973). A suitable value for var(w_k) has to be found empirically by using training sets of similar data. For the example in Figure 3 var(w_k) was set at 0.01 as this gave the smoothest control limits and followed the changes in the level well.

Often, however, the counts are not truly Poisson distributed, but are subject to extra-Poisson variation (overdispersion), it therefore seems appropriate to take this into account by specifying var(v_k) = 0.25•odf$_k$, where odf$_k$ is the overdispersion factor at observation k. It is then possible to estimate the overdispersion factor from the data. At the start of the process no overdispersion is expected, so odf$_k$ = 1. The control limits for a Shewhart chart at point $x_{(k+1)}$ are subsequently calculated as:

$$x_k \pm 2 \bullet \sqrt{\text{var}(v_k) \bullet odf_k} \qquad \text{and} \qquad x_k \pm 3 \bullet \sqrt{\text{var}(v_k) \bullet odf_k}$$

for the warning limits and the actions limits respectively.

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The design and reproduction quality of reference materials.

ABSTRACT

During the development and evaluation of the RMs market research was undertaken to collate the opinions of potential customers on the characteristics of the product developed. During this market research representatives of both inspection and production laboratories (all with some knowledge of the product) were interviewed. From this research a number of requirements for the reference materials became apparent and these requirements were compared to the developed product. Most of the requirements (Juran's customer's needs) could be or already were included in the product, but some were difficult or even impossible to include at the moment. The requirement for a simple and short pre-treatment for quantitative RMs was particularly evident. Based on the results of this market research production of the RMs was set up at the SVM with the help of the RIVM. The increase in sales of the RMs over the first years of commercial production (*ca* 10 % per year) was much less than predicted by the market research. To obtain information on the organisation of quality assurance programme in laboratories and the role of RMs in these programmes a second series of interviews was organised. From these interviews it became apparent that the routine use of RMs is important. This was not clear at the time of the original market research. The quantitative RMs were used in conjunction with control charts. It was also apparent that the level of contamination of the qualitative RMs should be increased to eliminate the risk of finding capsules without the target organism. In order to increase the sale of the RMs more information on how the RMs can be used in quality assurance of routine microbiological examinations should be made available to laboratories in Europe, in particular to those which are accredited.

INTRODUCTION

This chapter will focus on the quality of the reference materials from the viewpoint of potential users of RMs and CRMs and is based on interviews held with such users. The quality of a product depends on two factors, the design quality and the reproduction quality. The design quality indicates how far the designed or developed product conforms to the wishes and/or expectations of the target group or part of the market for which the product is intended. The reproduction quality indicates to what extent the product of continuous manufacture conforms to the product as designed (Van den Berg, 1993).

The reference materials in their present form (gelatin capsules containing artificially contaminated spray dried milk) were first described by Beckers and co-workers (1985). The intended use of these first RMs, containing *Salmonella typhimurium*, was to check the performance of the ISO standard method for the detection of *Salmonella*. Other materials were developed from 1986 onwards under contract with the former Bureau Communautaire de Reference (BCR) (now known as Standards, Measurement and Testing (SM&T)) of the European Commission. BCR/SM&T is involved in the development and certification of RMs for a range of measurements, but at that time was not covering food and water microbiology. The aim of the BCR/SM&T is to promote mutual recognition of analytical measurements by increasing confidence in results. The BCR also lays down basic requirements for RMs (see chapter 1). Each of the materials developed for food and water microbiology was evaluated in a collaborative study and the results discussed with the participants. Once several materials were successfully evaluated, market research among potential users of both RMs and CRMs within laboratories in the EU was undertaken to compare the requirements of these users with the RMs as developed and to determine the need for both RMs and CRMs (Van Oostwaard and Rauwerdink, 1993). CRMs were considered as a special batch of RMs because of the additional data provided and thus the increased price. During the market research interviews estimates of the prices of RMs and CRMs were presented. As a result of the findings of this research the production of non certified RMs was taken up by the Foundation for the Advancement of Public Health and Environmental Protection (SVM) and the certification process for RMs was initiated (see also chapters 3 and 4). In 1998 a second series of interviews was held to update the information obtained in the first series. Four laboratories that use the reference materials were selected for these interviews.

DESIGN QUALITY OF THE REFERENCE MATERIALS

Identification of customers

The identification of customers is the first step in the Juran quality planning roadmap (Van den Berg, 1993). Juran is one of the main experts in the field of production quality in recent years. At the start of the BCR-project in 1986 no future customers, as such, were identified, the aim at that time was to determine whether or not stable and homogeneous microbiological RMs could be developed. It was, more or less, taken for granted that laboratories operating in the field of microbiological examination of water and food were the future customers for RMs. Laboratories from food inspection services, research institutes and universities were involved in the collaborative studies organised to evaluate the

materials intended for use in food microbiology. At first no laboratories from the food industry were involved. For the evaluation of RMs intended for use in water microbiology laboratories from water production, water inspection, research institutes and universities were involved. During market research laboratory managers and/or quality managers were interviewed by a marketing expert from a consulting agency (BMT, Utrecht, The Netherlands) and an expert (food or water microbiologist) from the National Institute of Public Health and the Environment (RIVM). A differentiation was made between food or water inspection (governmental) laboratories and production (industrial) laboratories. The laboratories selected were regarded as leading laboratories in their particular country and were familiar with all or some of the RMs developed. Central research laboratories of food producers were also interviewed as they can influence the use of RMs throughout their company laboratories. All the inspection laboratories interviewed were also involved in the collaborative studies organised to evaluate the RMs. In total 22 laboratories were interviewed, four from food production, six from food inspection, four from water production, five from water inspection and three were involved in both water and food inspection. These laboratories were located in nine different countries throughout Europe (France, Germany, Greece, Spain, Sweden, Switzerland, The Netherlands, Portugal and the United Kingdom). The disadvantage of choosing laboratories that were familiar with the RMs was that they may have been biased, however, there was an advantage in that they are able to evaluate the need for this particular material. Based on the results of the market research it was concluded that all laboratories interviewed were intending to use RMs and thus could be regarded as potential customers.

Identification of customer needs

After the groups of customers were identified their specific needs had to be identified (second step in the Juran quality planning roadmap). At the time knowledge of what RMs were and how they could be used was not widespread among microbiology laboratories. The driving force behind the use of RMs was the introduction of or working with a quality assurance system and accreditation of the laboratory. Accreditation was more important to inspection laboratories than to production laboratories, although most laboratories had at that time no quality assurance system based on the EN 45001 standard (Anon., 1991). Most of the requirements of customers could not be related to a specific group of users. These requirements are referred to as general requirements and were identified in the two series of interviews and also during discussions with participants in the collaborative studies for the evaluation of the RMs. The general requirements could be summarised as follows:

- ease of use (no or only a short pre-treatment needed and the pre-treatment procedure not requiring specific apparatus etc.).
- availability of a variety of products covering the range of organisms (pathogens and non-pathogens) routinely tested for in the laboratory.
- combinations of various organisms in a single RM (so the same RM could be used to test various methods).
- clear instructions on use and interpretation of data.
- easy storage conditions.
- able to be used in combination with routine samples (spiking of samples).

- low in price.
- known stability and homogeneity.
- security of supply.

From the first series of interviews it became apparent that the materials would be used mainly in collaborative studies and for method development/evaluation. The most important requirement of RMs for use in collaborative studies is the stability of the material, as this will guarantee that all laboratories participating in the study are testing identical samples. The advantage of using RMs for method development/evaluation is that the samples will be inoculated with a well defined number of organisms and with well defined stress conditions.

The idea of using RMs in first line quality control (i.e. quality assurance of routine microbiological examinations) by routine production and inspection laboratories caused some hesitation due to the cost of RMs and, more important, the cost and time needed for the additional tests required. Provision of information on the advantages of the use of RMs in first line QC may overcome this hesitation. Most laboratories just beginning to use RMs in first line QC had similar considerations at first, but this attitude changed after some time to a more positive appraisal of the use of RMs.

Comparison of customer needs with RMs as developed

After the second step in the Juran quality planning roadmap a number of steps were included to translate the customers' needs into a final product. The characteristics of the RMs were generally regarded as satisfactory. However, the requirements for RMs in general needed to be compared to the RMs developed as those interviewed were already familiar with the material at the time of the market research. Table 1 presents an overview of the priorities in requirements for RMs. Extension of the range of RMs available was regarded as more important than improvement in the characteristics of the materials. The main item where the requirements of the user did not meet the characteristics of the RMs was in relation to the pre-treatment of the RMs (ease of use).

Pre-treatment of RMs

A distinction must be made between the RMs for qualitative tests and those for quantitative tests. For qualitative tests, especially with the *Salmonella* RMs, no pre-treatment is usually necessary as the gelatin capsule can be added directly to the enrichment broth which is subsequently incubated at 37 °C. When the incubation temperature is lower (for example, 30 °C as used in the first enrichment step for the detection of *Listeria monocytogenes* according to the ISO standard (Anon., 1997)) pre-warming of the (pre)enrichment broth at 37 °C is necessary for good and rapid dissolution of the gelatin capsule. After pre-warming and addition of a capsule the broth can be incubated at the desired temperature.

For the quantitative RMs a more time consuming pre-treatment is necessary. The capsules have to be reconstituted before inoculation of the plates or, as is common in water microbiology, passing through the membrane filter. The water related laboratories clearly expressed their requirement for a shorter pre-treatment of the RMs as they mainly use quantitative membrane filtration methods. The reconstitution procedure takes 30 - 40

minutes in addition to the time needed (*ca* 15 minutes) for pre-warming the tubes containing the peptone saline solution. In addition the incubation temperature for reconstitution (38.5 °C) is not commonly used in the laboratory and thus a separate waterbath is needed. A reconstitution temperature of 37 °C would be more acceptable to the user but this results in a poorer dissolution of the capsule.

Table 1. Requirements and priorities for RMs by food production and food inspection laboratories based on the results of the market research.

Micro-organism	Qualitative/quantitative test	Priority[a]	RM available
Salmonella	Qualitative	I	yes
Listeria monocytogenes	Qualitative	II	yes
	Quantitative	III	yes
Escherichia coli	Qualitative	I/II	no
	Quantitative	I	yes
Staphylococcus aureus	Qualitative	II	no
	Quantitative	I/II	no
Bacillus cereus	Qualitative	II	no
	Quantitative	I	yes
Clostridium perfringens	Qualitative	II/III	no
	Quantitative	I	yes
Yersinia enterocolitica	Quantitative	II/III	no
Campylobacter	Quantitative	II	no
L.D.-*Streptococci*	Qualitative	II	no
Vibrio	Quantitative	III	no
Total count (30 °C)	Quantitative	I	yes[b]
Total count (55 °C)	Quantitative	III	no
Yeasts	Quantitative	II	no
Moulds	Quantitative	II	no

a priority I = high priority (needed by most of those interviewed).
priority II = reasonably high priority.
priority III = low priority (only occasionally needed).
b RM containing *E. faecium* (available for water microbiology) is recommended for this purpose.

Wider test tubes (diameter 26 mm) than normal should also be used for optimal reconstitution. All these factors (time and temperature for reconstitution and size of the tubes) are a result of the use of gelatin capsules as containers for the contaminated milk powder. The advantage of using gelatin capsules as containers is twofold. Firstly, it is easy to fill equal amounts of milk powder into each capsule. Pharmacists use the standard deviation of the weight divided by the average weight of the capsule as a measure of the variation in weight of filled capsules. Their criterion for variation in weight is a maximum of 3 %. In practice values of less than 2 % were obtained, for example with the *Bacillus cereus* CRM 595 (In 't Veld *et al.*, 1995). The second advantage is that controlled, or even better, slow, reconstitution prevents osmotic shock of the micro-organisms in the milk powder. The negative effect of osmotic shock has been demonstrated for RMs containing *Salmonella* (Beckers *et al.*, 1985) and *Listeria monocytogenes* (see chapter 1). In particular for the *Salmonella* RM osmotic shock had a large effect on recovery of the organism.

Range of available RMs

During the market research a list was made of the requirements and priorities for RMs (see Table 1). The availability of RMs was limited in comparison to the requirement of the potential customers. The desired range of test organism covered almost all the pathogenic bacteria found in foods as well as a number of general plate count methods. For a number of organisms both quantitative and qualitative materials were required. Comparison of these requirements with the RMs available demonstrated that most of the priority I RMs were already available.

The need for both a quantitative and qualitative RM for *Escherichia coli* for food examination was recognised as this was one of the materials that has been evaluated in a collaborative study (In 't Veld and van Dommelen, 1994). However, the stability of this material was not acceptable as determined by an enumeration method for *E. coli*, based on the ISO 6391 (Anon., 1988) involving a four hour resuscitation step at 37 °C. Also this RM was difficult to use for the enumeration of *Enterobacteriaceae* or coliforms as the Violet Red Bile Glucose agar (VRBG) or Violet Red Bile Lactose agar (VRBL) respectively would yield only a few organisms per plate due to the high selectivity of these media without a prior resuscitation step. For water microbiology satisfactory stability results were obtained using Lauryl Sulphate Agar (Anon., 1990). Incubation of this agar involved a four h resuscitation step at 30 °C. The results were acceptable for certification of this material (Mooijman *et al.*, 1996a).

A specific RM for total counts at 30 °C was not developed as the main interest in the development of RMs for food microbiology was for pathogens. However, the RM containing *Enterococcus faecium* is suitable for this purpose. This RM has been evaluated, and also certified, for total counts at 37 °C in water microbiology (Mooijman *et al.*, 1994) and therefore can be used for the total count method at 30 °C in food microbiology.

The *Listeria monocytogenes* RM is the only RM not given priority I. This material was developed as a reaction to the interest in this organism in the 1980's due to some large foodborne outbreaks of listeriosis. Among the RMs given priority II and III are a number of organisms for which the current production process (spray drying of bacterial culture in milk) does not give satisfactory results (for example *Campylobacter* and *Vibrio* strains); these

organisms are too sensitive to the high drying temperatures and/or the low water activity of the milk powder.

In addition to the availability of the RMs containing specific micro-organisms gelatin capsules containing sterile milk powder are also available in capsules of the same colour combination as those containing the target micro-organism. These samples are used as negative control samples mainly in collaborative studies. In practice false positive results have been obtained in all collaborative studies involving qualitative tests. The requirement for negative control samples was not evaluated in the market research.

Production of combinations of several micro-organisms in a single RM is possible but is much more difficult than single organism RMs. The following items have to be considered before such an RM can be produced:

- stability of the various organisms in the RM (the period for use of the RM will be determined by the least stable organism).
- adjustment of the level and homogeneity of a single organism.
- cost of production compared to RMs containing a single organism (the first mixing steps for each organism should be separate and the level and homogeneity would need to be tested individually).

Instructions for use and interpretation of data

Each RM is supplied with information on the batch and also the procedure to follow to determine the level of contamination or the percentage of negative capsules, the reconstitution procedure, routine and incidental use (see also chapter 6), interpretation of results and the producer.

Storage conditions for RMs

For all RMs (and also for CRMs) a storage temperature of -20 °C ± 5 °C is prescribed in the instructions for use of the materials. For most types of organisms this temperature is essential in relation to the specified stability of the material. For certain RMs and CRMs (those containing spores of *B. cereus* and *C. perfringens*) higher storage temperatures (for example 5 °C or 22 °C) will have little or no effect on the specified stability, however storage at -20 °C is still recommended in order to avoid confusion. Most users of RMs do not have a problem in providing storage at -20 °C as most have freezers for a variety of purposes.

Price of RMs

The laboratories interviewed did not find the price of RMs too high (55 DFL per set of 10 capsules (*ca* 25 ECU)). The price is low compared to the cost of examination of an RM, especially when methods for pathogens are used.

Stability and homogeneity of RMs

Most of the RMs produced so far have been prepared from the same HCMP that was used in collaborative studies for the evaluation of the RMs, for certification and for determination of the stability of the material. For some RMs (for example *Listeria monocytogenes* or *Enterobacter cloacae*) it has been proven and for many others it is assumed that a new batch of RM has similar (or even better) stability than previous batches of RMs prepared from the same HCMP. The RMs currently available have a stability period of at least one year after production (the time at which the level of contamination of the batch

was determined) at a storage temperature of -20 °C. Stability at higher temperatures is such that the transport conditions (RMs are cooled by ice packs during transport if necessary) will have no considerable influence on the level of contamination.

From the market research study it became clear that the requirement of the user for a stable product was such that the longer the stability period the better. The period specified at present is regarded as very good, considering the materials contains living organisms.

Limits are set for the homogeneity of the material during production. The target for homogeneity is a Poisson distribution, overdispersion is accepted as long as the variation in the counts (for quantitative and qualitative RMs) does not exceed twice that of an Poisson distribution. This limit is acceptable to users. In general the variation in counts observed by the users of RMs is smaller than they expected.

Security of supply

The batches of RMs for the collaborative studies were originally produced by the RIVM within the frame-work of the contract with the BCR/SM&T. Such batches were also supplied to interested laboratories but the numbers produced were limited and a regular supply could not be guaranteed. A secure supply was explicitly demanded by industry, but could not be guaranteed by the RIVM as this did not fit with the core tasks and the financial/economical structure of the Institute. Therefore a secure supply was arranged by placing the production of the RMs with the SVM as the organisation was already responsible for the production and marketing of vaccines.

REPRODUCTION QUALITY OF THE REFERENCE MATERIALS

Reproduction of reference materials

Before setting up production and marketing by SVM the following items were considered in order to assure the reproduction quality of the RMs:

- implementation of the existing production technique and training of production staff.
- quality control of production (and sale).
- management of production and stock.
- preparation of instructions for use.
- provision of information on the new product for the sales department staff.
- promotion of the product.

Figure 1 presents a general flow diagram of the production and control process as implemented at SVM.

Implementation of the existing production technique and training of production staff

The existing production technique could be used as the size of the batches of RMs at the start of production (3,000 - 5,000 capsules) was comparable to the size of batches produced for certification of the materials. The production staff of SVM were trained in the mixing of batches (use of pestle & mortar and mixing apparatus) and filling of gelatin capsules.

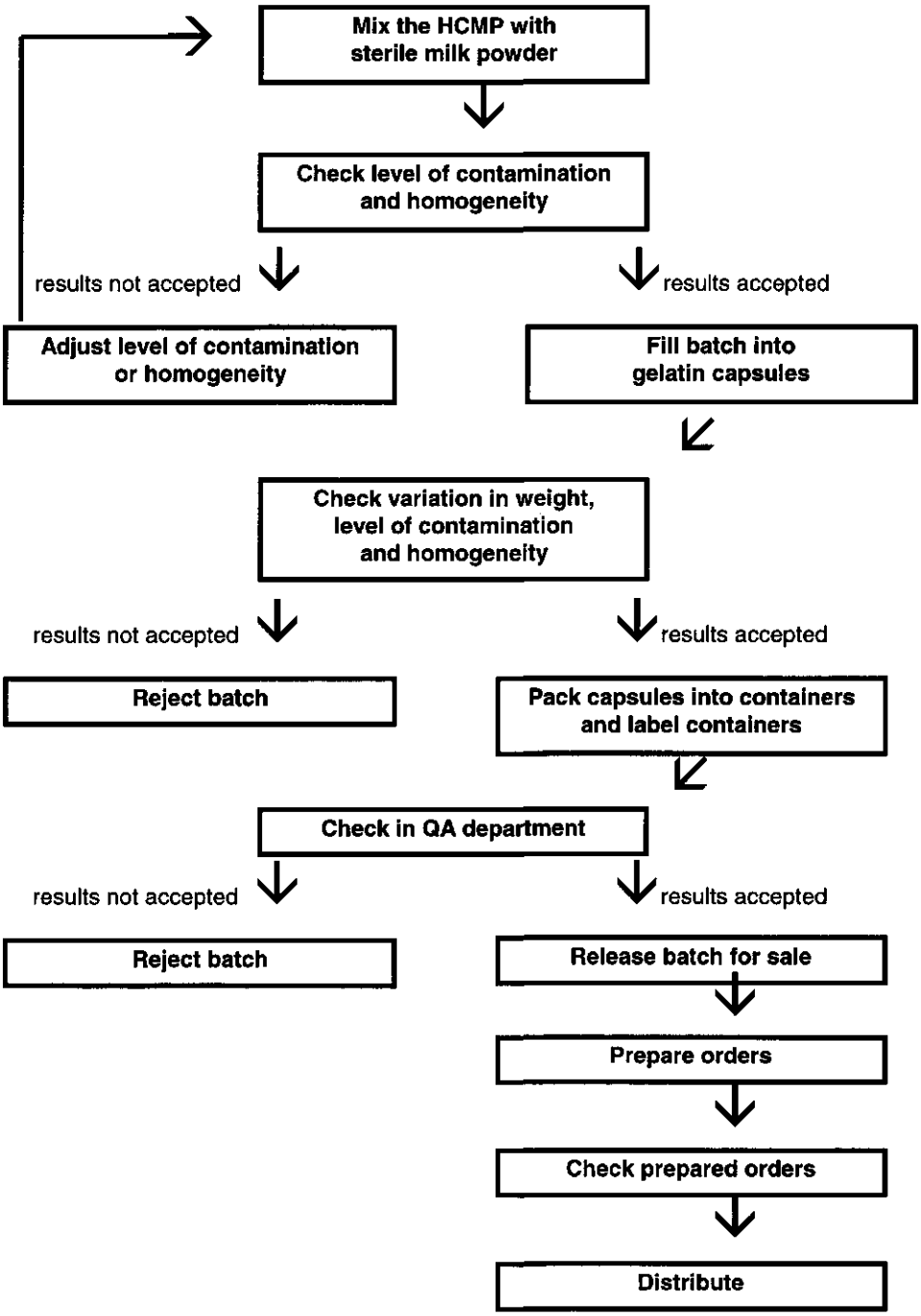

Figure 1. Flow diagram of the production process for RMs at SVM.

Quality control of production

A protocol for the preparation of a new batch of each type of RM was prepared which specified the amount of HCMP and sterile milk powder to be mixed and the steps necessary for mixing the powders in order to obtain the desired level of contamination. In general batches of 1 - 2 kg mixed powder are prepared. The first stage in the preparation of a batch takes the form of a control loop in which the batch is mixed, the level and homogeneity are assessed and the test results are compared to predefined criteria. When the test results do not meet the criteria, the batch is remixed until the test results do conform to the criteria. After each mixing procedure two samples of the mixed powder are taken, gelatin capsules are filled and two sets of 10 capsules are examined in duplicate (two sets of 25 capsules for the qualitative RMs) for their level of contamination and homogeneity. Based on these results the mean level of contamination and the homogeneity are calculated for quantitative RMs as presented in chapter 1. Criteria for the level of contamination and homogeneity were formulated based on previous experience. For the qualitative RMs the fraction of negatives is calculated based on the level of contamination and homogeneity of the batch. The results are checked and when the batch conforms to the criteria set the batch is filled into gelatin capsules. The variation in weight of the filled capsules is checked, the criterion to be met is a value of less than 0.03 for the standard deviation divided by average weight. For this purpose one capsule out of every set of 60 filled (the number of capsules that can be filled at the same time) is used. A further 20 capsules (or 50 capsules for quantitative RMs) are checked done by an independent accredited laboratory for level of contamination and homogeneity. The results are checked using the criteria applied for the earlier check(s). The gelatin capsules are then packed in sets of 10 capsules per container. Each container is labelled with information on the level of contamination of the test material, including, for quantitative RMs, the 95 % confidence limits or, for qualitative RMs, the fraction of negatives including a 95 % upper confidence limit. Before the batch is finally released for sale the QA department of SVM checks the results laid down in the test reports.

Management of production and stock

The time taken to produce a batch of RMs depends on a number of factors of which the most important is the number of microbiological examinations needed to check the level of contamination and homogeneity. For each batch it is expected that the level of contamination and/or homogeneity will need to be adjusted 2 to 3 times before the prescribed criteria are met. Once the entire batch is filled into gelatin capsules an external check is made which takes a further 2 - 3 weeks. The entire procedure leads to a minimum production time (from the start of mixing to the availability of the batch for sale) of four weeks. In practice production times of 2 - 3 months are assumed. This will mean that for proper management of production an inventory of stock and expected rate of sale are essential. Another point that has to be considered in production planning is the expiry date of the batches. Normally batches are sold for up to three months before expiry date. This means that the start of production of a new batch must be *ca* six months before the expiry date of the current batch.

In order to reduce production time the number of mixings (and therefore checks) should be kept to a minimum. Although mixing schemes are set up based on previous experience a single mixing step is normally not sufficient. This is due to a combination of the following

factors: non-homogeneity of the HCMPs, variation in counts due to the method of examination, the number of samples used and the criteria for acceptance of a batch. For the better selling RMs more powder is mixed than is needed for the production of one batch of filled capsules. The powder left over after filling the required number of capsules for the batch is stored for production of the next batch. This procedure saves both the time and cost of mixing and checking the level of contamination after each mixing. This previously mixed powder will be checked for the level of contamination before it is used for filling the next batch of capsules.

Preparation of instructions for use

With each order instructions for use of the particular type of RMs are supplied. The information in the instructions for use has been described earlier in this chapter. A draft of the instructions for use were sent to a number of potential users for their opinion on the information supplied. The final instructions were prepared taking into account their comments.

Information on the new product for the sales department staff

SVM is already selling a range of products but including mainly vaccines. The RMs are quite different to the products already sold. The sales department will be the first point of contact for questions or remarks on the materials and their use. To enable the sales staff to handle simple questions from the user a short training course on the general aspects of the RMs was given. To provide additional support in the marketing of the RMs the microbiologists involved in the development of the materials are available for advice and assistance for example in relation to more detailed questions on the RMs and quality assurance aspects.

Promotion of the product

The product was promoted in three ways:

1. by the production of two leaflets, one containing general information on the RMs including a list of those available and their prices, the other presenting information on the quality assurance of examination by both occasional and regular use of the qualitative and quantitative RMs and CRMs.
2. by advertising in water- and food industry magazines aiming to supply information on the availability of the RMs to the laboratory manager. Advertisements however, only appeared in Dutch magazines as the price for the adverts was very high and it was considered not to be cost effective. Although the EU financially supported the development of the materials no funds were available for promotion of the product. The reason for this is that the EU cannot recommend or support the use of a specific commercial product.
3. by presenting scientific information and/or data by the microbiologists involved in the development of the RMs at symposia and congresses. This information includes the development and evaluation of the materials and also the use of RMs in quality control.

EXPECTED AND REALISED DEMAND FOR RMs

Based on the market research an estimate of the immediate need for RMs and the expected increase in demand was made. The immediate demand for RMs was estimated at 20,000 - 30,000 capsules per year. No differentiation was made between the demand for the various types of RM. It was expected that after an initial demand of 20,000 to 30,000 capsules per year there would be an increase to 100,000 - 200,000 capsules per year within 1 - 2 years. This predicted strong increase in demand did, however, not happen. A gradual but steady increase was observed starting from *ca* 17,000 capsules sold in 1994 (1 year after production at SVM started) and increasing to *ca* 30,000 in 1998 (prediction based on the results of the first half of 1998). The *Salmonella* RM accounted for more than 40 % of the total demand. This is due to the fact that *Salmonella* is the pathogen most frequently tested for by food producers and this qualitative RM is easy to use. Possible explanations for the difference between the expected and realised sales and ways in which sales might be stimulated, include:

- The use of RMs on a national and international scale.
  Although no clear difference in the expected use between countries was observed in the market research the use of RMs is mainly limited to laboratories in The Netherlands (more than 50 % of the RMs sold in 1995 and 1996). Information on the availability and use of the RMs should be focused on other European countries in order to increase sales. Accreditation bodies could stimulate the use of RMs in laboratories when they stipulate the use of control samples having a defined number of organisms.
- Availability of alternative RMs.
  At the time of the market research no alternative RMs were available. At the present time a number of alternatives exist although some are still at the experimental stage. The differences between the RMs developed and these alternatives (in terms of cost, application etc.) should be made clear to potential users.
- Availability of proficiency testing schemes.
  In addition proficiency testing schemes were not well developed at the time of the market research. Currently a range of such schemes are available, all having a large number of participants. Proficiency testing schemes have an advantage compared to the use of RMs in that they offer a comparison between different laboratories and can offer standardised samples that differ in the level of contamination for the target and background organisms in each distribution. However, the disadvantage of such schemes is the frequency of use, which is low compared to the possibilities for the routine use of RMs.

Both the alternative RMs and proficiency testing schemes are used for quality assurance by laboratories and thus can affect the demand for RMs produced by SVM. A short overview of these alternatives is presented below. A second series of interviews was organised to obtain information on how laboratories had organised the quality assurance of their microbiological examinations and the role and need for RMs.

Alternative reference materials

Two alternative freeze dried RMs, containing a mixture of micro-organisms are available. The first is produced by the National Food Administration in Uppsala, Sweden, as an off-shoot of their proficiency testing programme. The various micro-organisms are present at different levels and the mixture covers a range of food microbiological methods, including pathogens and general plate counts (Peterz and Steneryd, 1993). For each organism the level is specified (determined according to a specified Nordic Committee on Food Analysis (NMKL) method) and the variation between samples is based on the variation in results obtained by the National Food Administration and also adjusted for the variation between laboratories participating in the proficiency tests. Thus laboratories should obtain results within a specified 95 % confidence interval (the range differing from 0.5 to 1.2 $\log_{10}$ units depending on the organism). The stability period for these samples is 12 months when stored at -20 °C. The samples are reconstituted in 50 ml BPw and should then be used immediately. The price for a set of ten samples is *ca* 120 ECU (*ca* 250 DFL). Compared to the RMs developed by the RIVM/SM&T these Swedish RMs although they cover a wider range of organisms and are easy to use are more expensive and have a shorter shelf life.

The second alternative is the Quanti-Cult$^{PLUS\ ™}$ which contains pre-quantified (10 - 100 cfp) micro-organisms per vial. The organisms (a limited number of ATCC strains) are dried in a cap which is placed on a tube containing the rehydration fluid. Reconstitution is performed at 35 - 37 °C for ten minutes after which the contents of the vial are applied to the medium to be evaluated. Ten tests can be performed from one vial, but they must be started within 30 minutes of rehydration. The certificate supplied with the product only gives information on the strain and batch not the number of organisms present nor any variation within the batch. The stability period of the vials is specified as 6 - 10 months (depending on the organism) when stored at 5 °C. The price for a set of ten vials is 700 ECU (*ca* 1500 DFL). Compared to the RMs developed by RIVM/SM&T these RMs are expensive, although easy to use, have a limited stability and a less accurate quantification of the number of organisms present.

Two further types of RM are currently in an experimental stage. The first of these is the so called "Lenticules" developed by the Public Health Laboratory in Newcastle, U.K. These Lenticules are being evaluated in project called "Microbath" financed by the European Commission (SM&T) and also in the PHLS External Quality Assessment Scheme for water microbiology. A wide range of these RMs, containing both water and food related organisms are currently being tested for homogeneity and stability. The production process for the Lenticules is based on drying the organisms in a protective matrix at ambient temperature using silica gel as the drying agent. Small droplets of the bacteria suspended in the matrix when dried form the Lenticules which are lentil shaped. Discussion on the production possibilities of these RMs have started recently. The lenticules have the advantages that they are easy to reconstitute (a Lenticule is left for 10 minutes in a specified quantity of PS at room temperature and subsequently mixed) and are dried at ambient temperatures which eliminates any heat injury to the cells. When stability and homogeneity and price of the lenticules are satisfactory they will become real competitors to the RMs developed by the RIVM/SM&T.

The second experimental type of RM has been developed by the Dutch Food Inspectorate in Groningen. The production process is a simplification of that described by Schijven and co-workers (1994). The organism is cultured and diluted to the desired concentration in sterile whole milk. This suspension is then distributed into small vials and frozen at -20 °C (Mulder and Strikwerda, 1996). The material containing *S. aureus* proved to be stable for one year when stored at -20 °C. These RMs are used mainly in first line QC by laboratories of the Food Inspectorate in The Netherlands. Although some of these materials have proved to be stable for a year when stored at -20 °C the disadvantage is that they have to be shipped frozen which will limit their application in other laboratories.

Proficiency testing schemes

Proficiency testing schemes are used by participating laboratories to demonstrate their performance in comparison to others. The existing schemes provide simulated food or water samples at regular intervals, normally every 1 or 2 months. Details of a number of the schemes available are presented below:

- The PHLS Quality Assessment Schemes.

Various schemes (e.g. food and water microbiology) exist, all organised from the Public Health Laboratory Service in the United Kingdom. The food microbiology scheme started in 1991 and is run by the Food Hygiene Laboratory at the Central Public Health Laboratory in London. The samples, containing a mixture of organisms, are freeze dried. Within this scheme there are currently five sub-schemes each aiming at different groups of laboratories testing a wide range of or more specific samples. The standard scheme is intended for official control laboratories examining samples for a prescribed range of tests including pathogens. The extended scheme is more directed towards public health laboratories examining food samples for a wider range of organisms and including samples associated with outbreaks of food poisoning. For both of these schemes there are six distributions per year, each distribution containing 2 - 4 samples. Scores (2, 1 or 0) are allocated to the participants' results depending on whether the results are fully, partially or not correct. Costs are 1100 - 1250 ECU (2350 - 2700 DFL) per distribution year, excluding additional shipment costs for non UK members (*ca* 150 ECU).

- The Nordic Proficiency testing scheme.

This scheme is organised by the National Food Administration in Uppsala, Sweden and has been operating since the beginning of the 1980s. The samples are similar to those of the PHLS EQA scheme but are intended only for general food microbiology, they do not simulate food samples involved in outbreaks as with the PHLS EQA scheme. The method of evaluating results also differs from that of the PHLS. Participants' results are tested to determine whether they are outliers in relation to those of other laboratories by using *z*-scores. No scores are allocated to the laboratories' performance. The scheme is intended for laboratories in the Nordic countries, but is open to any laboratory. Three distributions are made per year each consisting of 4 or 5 samples covering a range of organisms to be tested. The costs are *ca* 625 ECU (*ca* 1300 DFL) per year.

- Food Examination Performance Assessment Scheme (FEPAS).

This scheme is operated from the Ministry of Agriculture, Fisheries and Food (MAFF) Central Science Laboratory in Norwich, UK. The samples consist of artificially contaminated freeze dried minced beef or chicken. Originally it was only available to UK laboratories but since July 1997 it has been available to laboratories in other countries. The samples contain a mixture of organisms, target and natural background flora. The target organisms include pathogenic, indicator and spoilage bacteria. Five distributions are planned per year each consisting of three samples giving a total of 15 different samples with different target organisms. The analysis of the data is performed according to the International Harmonised Protocol for the Proficiency Testing of (Chemical) Analytical Laboratories as far as it is applicable to microbiological data. The costs depend on the number of samples requested per year. For all 15 samples the costs will be *ca* 2200 ECU (*ca* 4600 DFL). For countries outside the UK additional shipping costs are added.

- Quality Management (QM).

This scheme is operated by a commercial company that produces samples intended for the food industry. It also produces samples specifically aimed at the dairy and brewing industries. Samples consist of milk powder or oat meal, they contain a mixture of organisms and are available for both pathogen and general plate count testing. The organisms are spray dried and reference is made to the evaluation of spray dried RMs by the BCR/SM&T. The frequency and number of samples to be examined are determined by the participant. Participants' results are reported as a *z*-score, with a reference value based on the median value of the study. Costs are *ca* 160 ECU (*ca* 335 DFL) per sample but can be lower depending on the frequency and number of samples examined.

The second series of interviews

For this second series of interviews four laboratories, two central laboratories from the food industry and two food inspection laboratories were selected. All had been familiar with the use of RMs for a number of years. The inspection laboratories were both accredited by the Dutch Board for Accreditation (formerly Sterlab). The industrial laboratories used a company quality assurance system and intended to become accredited in the future. RMs were used by all four laboratories for first line quality control. Three laboratories used the RMs produced by SVM, the fourth RMs prepared by themselves. The frequency of use was with every series of examinations. Reducing the frequency of use was not a point for discussion, the point of view of these laboratories was, that the performance of each series of examinations should be proven particularly in relation to possible disputes between production and inspection. The results obtained with the RMs were generally used to produce a type of control chart. Apart from the use of RMs for first line quality control, they were also used in method validation studies. This use of RMs for first line quality control is confirmed by other Dutch laboratories but differs from the conclusions of the original market research. At that time RMs were mainly used in collaborative studies and for method development/validation. The use of RMs in collaborative studies is likely to decrease in the future as the organisers of such studies are seeking samples that are prepared according to their specific requirements. The providers of proficiency testing schemes are more flexible in

producing samples that are more specific than the RMs produced by SVM. CRMs were either not regarded to be of great use (except by one of the inspection laboratories) or it was felt that insufficient information was available on the difference between RMs and CRMs.

The level of contamination or fraction of negatives presented for each batch of RMs produced by SVM were regarded as sufficiently accurate to be used by each laboratory. They felt there was no need to improve the confidence in these values as this would lead to a higher price. For industry the cost of quality control is critical, but the costs involved are mainly those for the additional examination and not the cost of an RM. The cost of examining RMs on a regular basis is too high for laboratories directly linked to a production site in industry. In these laboratories RMs are used less frequently (e.g. once a week) or not at all. For all laboratories, but especially for those linked to a production site, participation in proficiency tests is important in addition to or instead of the use of RMs. All four laboratories participated in various internally and externally organised proficiency tests.

The range of RMs made by SVM could not cover the entire range required by these laboratories. For one laboratory this was one of the reason(s) why they started to produce their own RMs. There is also a perceived need for RMs containing strains that can easily be distinguished from normal food isolates, for example to be able to trace false positive detection results. They also agreed that for the qualitative RMs the level of contamination should be increased so that the fraction of negative capsules is zero. A fraction of negatives of *ca* 1 % is regarded as too high. The opinion of the four laboratories on the reconstitution procedure for the quantitative RMs differed, although all of them had comments on the application of the procedure in the laboratory. It was not possible to obtain an opinion on the alternative RMs, except for the one laboratory that produced its own, as the other laboratories were not familiar with these alternatives. The laboratory that produced its own RMs felt that it was too early to compare them to the RMs produced by SVM as they were still in an experimental stage.

REQUIREMENTS AND POSSIBILITIES FOR IMPROVEMENT IN THE DESIGN QUALITY

From the data obtained from both series of interviews the following points that could improve the design quality of the materials were further considered:

- pre-treatment of the quantitative RMs.
- increase in the level of contamination of the qualitative RMs.
- use of strains with a marker.
- extension of the range of RMs.

Pre-treatment of the quantitative RMs

The need to change the procedure for pre-treatment of the quantitative RMs became apparent in the original market research and was confirmed during the second series of interviews. Based on the findings of the market research additional research was initiated to develop an alternative reconstitution procedure. For RMs containing organisms that are not affected by osmotic shock, for example those with spores of *B. cereus*, opening of the gelatin capsules and direct reconstitution in peptone saline solution is possible. However, this procedure leads to a larger variation in results when compared to those obtained with

the normal reconstitution procedure. This is due to the difficulties in transferring the powder quantitatively into the test tube containing peptone saline solution (see also chapter 1). The direct use of contaminated milk powder without capsules was also examined by Mooijman and co-workers (1996 b) using a strain of *Enterobacter cloacae*. Various parameters in the reconstitution procedure were varied such as temperature, the amount of peptone saline solution used and the reconstitution time. After reconstitution one ml volumes were membrane filtered. Almost all alternative procedures showed a poorer homogeneity (larger variation in counts) and in most cases an effect of osmotic shock was apparent (the number of micro-organisms recovered was lower than with the "reference" reconstitution procedure).

A possible procedure for RMs with organisms that are not affected by osmotic shock (*Bacillus cereus* and *Clostridium perfringens*) could be to raise the level of contamination of the capsule tenfold (to *ca* 5 x 10^4 cfp/capsule) and reconstitute the capsules in 100 ml of peptone saline using a stomacher. After reconstitution 0.1 ml is then plated onto the medium of choice. Another advantage of this approach is that reconstitution can be combined with the inoculation of 10 grams of a food sample at a level of 5 x 10^3 per gram of food. For other organisms that are only slightly affected by osmotic shock (for example *Enterococcus faecium*) it will be necessary to determine the effect of the osmotic shock and also whether this approach would lead to reproducible results.

Raising the level of contamination of the RMs has limits in relation to the amount of HCMP needed. For organisms showing no or only limited (less than one $\log_{10}$ unit) die off during the spray drying process problems are not expected. However, with many other organisms a reduction of 2 - 3 $\log_{10}$ units is not uncommon. For these organisms increasing the level of contamination of the RM would pose a problem, as the level of contamination of the HCMP is too low to give the higher final contamination level in the capsule.

Increase in the level of contamination of the qualitative RMs

For the qualitative RMs the level of contamination should be increased so that the fraction of negative capsules is zero. There should be no doubt whether a failure of a laboratory to isolate the target organism is due to an analytical error or to a capsule not containing the organism. The target contamination level for production of new batches of RMs is between 5 and 6 cfp/capsule. This corresponds to a maximum fraction of negatives of 1.6 %, taking into account a value for $T_2/(I - 1)$ of 2. Increasing the target level of contamination to between 8 and 9 cfp/capsule could lead to an expected maximum fraction of negatives of 0.1 %. A further increase is not recommended, as the level of contamination should be as close as possible to the theoretical detection limit.

Use of strains with a marker

The strains used in an RM should be easy to distinguish from normal food or water isolates. The type of marker used should be simple so that each laboratory can easily demonstrate this feature. The need for marked organisms is to eliminate the possibility of a false positive result, this is particularly important with detection methods. For example to demonstrate that the strain isolated from a food sample is the same strain used for the production of the RM. Several types of markers can be used such as, for example, an

antibiotic resistance that is not commonly found in the specific type of organism or the incorporation of fluorescence or luminescence genes in the organism.

Extension of the range of RMs

From both series of interviews it was clear that the existing range of RMs is small compared to the requirements of different laboratories. The alternative RMs cover a wider range of organisms. This is likely to affect the demand for RMs produced by SVM in the future.

RECOMMENDATIONS

- The target group of RM users should be defined as inspection laboratories and central industrial laboratories.
- In general the larger laboratories have less difficulties with the specific requirements for reconstitution of the quantitative RMs. However, the reconstitution procedure for the quantitative RMs should be made more user friendly, to allow the market to be extended to the smaller routine laboratories. The existing and developing alternative RMs are more user friendly in this respect.
- The routine use of RMs (as first line quality control) gives an opportunity for laboratories to check the performance of methods at a much higher frequency than with proficiency testing. The routine use of RMs is common in The Netherlands but less so in other (European) countries. Efforts to extend the market for RMs should be directed towards the routine use of the materials. More information on the use of RMs in first line quality control should be made available to potential users. At the moment a project called Equase (financed by SM&T) is working on the introduction of quality assurance principles into laboratories undertaking microbiological examination of water samples. A similar project should be initiated targeting control laboratories testing food samples.

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INTRODUCTION

The development of the reference and certified reference materials described in this thesis is built upon a line of work that began in the early 1970's with the organisation of collaborative studies in order to standardise the detection method for *Salmonella*. For this particular purpose identical samples were needed for all the laboratories throughout Europe participating in these collaborative studies. In order to obtain sublethally injured organisms, a culture of *Salmonella* was suspended in milk and dried in Petri dishes at 43 °C (Edel and Kampelmacher, 1973). Subsequently the dried milk was ground, further diluted with milk powder and used as a separate inoculum for spiking test samples. Later, the *Salmonella* was suspended in milk, spray dried and, after dilution, filled into gelatin capsules (Beckers *et al.*, 1985). This principle is still in use for the production of RMs and CRMs today.

THE REQUIREMENTS OF REFERENCE MATERIALS

Three general requirements for reference materials can be formulated. Reference materials should be stable, homogeneous and represent the samples that are examined by a laboratory in practice as far as possible (representativity of the reference material).

Stability

From the viewpoint of the user of certified and non-certified RMs the level of contamination must be stable within defined limits over a period of at least one year at the recommended storage temperature (-20 °C). The stability of various RMs at storage temperature is presented in chapters 1, 3 and 4. All the materials described fulfilled the requirement for stable RMs as no significant decrease in the level of contamination was demonstrated. Most of the RMs tested were stable over periods much longer than a year, for example, the *Salmonella* RM was stable for more than six years. Sometimes a small but significant increase in the level of contamination was observed. This increase, however, was so small that it hardly affected the use of the RM in practice. It is difficult to explain this increase in the contamination level but one reason that might contribute to the effect is the experience of the technician in reconstituting the samples.

Apart from testing the stability at storage temperature the materials were also tested for their stability at higher temperatures in an attempt to simulate the conditions of shipment. The *B. cereus* RM proved to be very stable (up to four weeks at 37 °C) and was probably due to the use of spores. Other materials, such as the *Listeria* RM, were less stable thus cooling during shipment of these RMs is necessary.

Homogeneity

The theoretical distribution of micro-organisms in a sample is described by a Poisson distribution (Niemelä, 1983; Heisterkamp *et al.*, 1993). The T_1 and T_2 tests were used to determine whether the variation within a single RM (T_1 test) or between different RMs (T_2 test) was according to a Poisson distribution. In practice overdispersion was found between different RMs but not within reconstituted RMs. At the start of the project homogeneity was sometimes difficult to achieve. However, the homogeneity of different

batches of RMs was improved by the introduction of mixing by means of a mortar and pestle (see chapter 1). For production of an acceptable batch of RMs a limit for the value of $T_2/(I\text{-}1)$ of ≤ 2 was set. This limit was set based on empirical results obtained in practice when the HCMPs were mixed with sterile milk powder. The results from the collaborative and certification studies confirmed that this limit had little influence on the results obtained by laboratories. Table 5 in chapter 6 shows that for the quantitative CRMs the variance component due to capsules (homogeneity of the materials) is small compared to the total variation (sum of all variance components). Hence, it is concluded that the homogeneity of the RMs is acceptable.

Representativity

Evaluation of the effect of the production process

In the ideal situation an RM should be similar to the sample that is examined by a laboratory in practice. However, for almost all RMs and CRMs this is not possible to achieve as it would be impossible to fulfil the requirements for stability and homogeneity. An RM should also represent a sample or a physiological state of the organism that is critical for the method to be evaluated and thus the target organisms should have a certain degree of sublethal injury. However, too much injury to the target organisms would make it difficult to obtain a representative RM. In the spray drying process the organisms become injured due to the high temperatures used for drying (heat injury) and also to the drying process itself (osmotic injury). Chapter 1 describes how the production process for RMs influenced the behaviour of the quantitative *Bacillus cereus* RM and the qualitative *Listeria monocytogenes* RM. From the results presented it is concluded that the effect of the production process and storage conditions did not change the representativity of the RMs.

Both heat injury and osmotic shock were observed to have an effect on the *L. monocytogenes* RM. The osmotic shock effect emphasises the need for a standardised reconstitution procedure (use of the gelatin capsules). The heat injury effect makes the target organism more sensitive towards the selective agents used in both the enrichment broths and the isolation media. This effect is illustrated by the prolonged lag phase of the *L. monocytogenes* grown in Listeria enrichment broth (LEB) or half strength Fraser broth (½ FB) (see chapter 1, Table 10). With the *B. cereus* RM the spores present in the material required a prolonged time for germination, this was due to the production and storage conditions, but it did not affect the normal counting procedure for *B. cereus*. No heat shock was necessary to germinate the spores.

Evaluation in collaborative studies

In addition to the use of RMs in an individual laboratory, the materials were further evaluated in collaborative studies involving laboratories throughout Europe. The three studies organised to evaluate the *L. monocytogenes* RMs are presented in chapter 2. It was concluded that the RMs produced are suitable for testing laboratory performance of methods for the detection of *L. monocytogenes*.

The first study, in which *L. monocytogenes* was the only organism present in the RM, showed that most laboratories detected the expected number of *L. monocytogenes* positive

samples. The study demonstrated the ability of laboratories to detect low numbers of sublethally injured organisms. The next studies aimed to evaluate the selectivity of the methods by the addition of competitive micro-organisms to the enrichment broth containing the RM. In the second study competitive organisms were added in the form of a capsule containing a selection of organisms spray dried in milk and in the third study naturally contaminated food samples were added. In both studies a decrease in the expected number of positive isolations was observed, the greatest decrease being when naturally contaminated food samples were added. The effect of the addition of food depended on the food type and was not the same in all laboratories. The addition of RMs to food samples proved to be a sensitive tool to evaluate the performance of a laboratory in relation to the method and type of food added.

CERTIFICATION

The highest status that an RM can obtain is that of a certified RM. Several studies were undertaken to certify RMs that had been shown to fulfil the requirements of a reference material. The certification procedure for the qualitative *Salmonella* RM and the quantitative *B. cereus* RM are described in chapters 3 and 4 respectively. In these studies it was demonstrated that the materials developed met the high standard set by the BCR for certification of RMs. In total six materials, *Enterococcus faecium*, *Enterobacter cloacae* and *Escherichia coli* for water microbiology and *Salmonella typhimurium*, *Bacillus cereus* and *Listeria monocytogenes* for food microbiology, were certified over a period of a few years. To date these CRMs are the only microbiological CRMs available world-wide.

PRODUCTION OF HCMPs AND ALTERNATIVES

Two different spray dryers were used for the production of HCMPs over the study period. At first a Stork pilot plant spray dryer, located at the Agricultural University in Wageningen, The Netherlands, was used. Later a Niro mobil minor spray dryer was purchased for the production of HCMPs at the RIVM. The Niro is much smaller than the Stork spray dryer and milk powders with different properties were obtained with the two pieces of equipment. The HCMPs produced by the Niro dryer consisted of much finer particles, while the Stork HCMPs resembled more the milk powder that is available commercially. The Stork dryer is operated using pasteurised milk concentrated up to 450 $g.l^{-1}$ total solids while the Niro dryer uses sterile milk concentrated up to 240 $g.l^{-1}$ total solids. The HCMPs obtained using the Stork spray dryer contained spores (10^2 - 10^3 spores per gram HCMP) due to the fact that the milk used was only pasteurised and also unfiltered air was used for drying. The Stork dryer is operated at lower inlet and outlet temperatures and uses milk with a high total solid content, both factors that will favour the survival of micro-organisms (Dega *et al.*, 1972; Thompson *et al.*, 1978). Although the Stork dryer produces better HCMPs (in terms of survival of the organisms and mixing properties of the powder) it has the disadvantage that other organisms (mainly spores) will be present in the powder at relatively high numbers.

During the production of the highly contaminated milk powders (HCMPs) by spray drying the micro-organisms become sublethally injured (both heat injury and injury due to osmotic

shock). Some of the organisms become so injured that the requirement for stability cannot be obtained. For such organisms the extent of injury must be reduced. As injury due to osmotic shock cannot be avoided by drying, reduction in injury must be effected by a reduction in the injury due to heat. To eliminate the heat injury effect the organisms must be dried at lower temperatures. For this purpose fluid bed spray granulation was tested for the production of HCMPs as an alternative to spray drying (see chapter 5). The production of HCMPs by this method is much easier than by spray drying. However, the expected improvement in survival using spray granulation was not observed when compared to spray drying using low outlet temperatures (*ca* 70 °C), indicating that heat injury was not the main factor for survival. Homogeneity of the HCMPs produced by fluid bed spray granulation is more difficult to obtain than for spray drying. Attention must be paid to the process of spraying the contaminated milk onto the milk powder in order to obtain homogeneous HCMPs.

The spray drying process is not well suited for testing the effect of various culturing or drying conditions on the survival of the micro-organisms as the drying process is very laborious. This testing can be achieved more easily with spray granulation. In fact, the survival of *E. coli* through the drying process could be improved by addition of sucrose to the milk and the survival of *S. aureus* by the addition of sodium chloride (NaCl) during culturing. These results confirm the expectations based on laboratory studies, using a model drying system on silica gel (Janning *et al.*, 1994; Janning, 1995). Janning tested the effect of the addition of sucrose and trehalose to the milk used for suspending the bacteria before drying onto silica gel and also the effect of adding some compatible solutes to the BHI used as culture medium. To stimulate the uptake of these compounds during growth NaCl was added to the BHI to create an osmotic stress. In general, the additions improved the percentage survival and the stability of the dried organisms. The effect of the addition of high concentrations of sugar to the milk was the most pronounced. The effect of sucrose was better than that of trehalose but the best results were obtained with milk supplemented with 2M sucrose.

Before a definite recommendation can be given on the use of spray granulation instead of spray drying more information on the long term stability of the HCMPs produced by spray granulation is needed.

USE OF REFERENCE MATERIALS

The possible uses of RMs are presented in chapter 6. A distinction is made between occasional and routine use of RMs in quality assurance for both quantitative and qualitative RMs. In chapter 7 the actual use of RMs is analysed on the basis of the results of a market research (Van Oostwaard and Rauwerdink, 1993) and interviews with users of the RMs held in 1998. Over recent years quality assurance systems have been introduced into many laboratories and the majority of them are or intend to become accredited. The introduction of a quality assurance system stimulated participation of laboratories in proficiency testing schemes in order that they may demonstrate their performance in comparison to other laboratories. Participation in a proficiency testing scheme is a primary step when a quality assurance system is set up in a laboratory, and many such schemes have been set up in the

past 10 years with the number of participating laboratories continuing to increase. The use of an RM could be the next step. At the time of the market research the use of RMs for quality assurance of routine microbiological examination met with some resistance, but from the later interviews it became clear that this type of use is becoming more and more established. In general it is recommended that, for QA of routine examination, RMs should be used with every series of examinations (Lightfoot and Maier, 1998). However, when a measurement process has been proven to be under control then the frequency of use of RMs can be reduced. The less frequent examinations are thus used as a means of verifying that the measurement process is still under control. A guideline for the frequency of use is difficult to give as it will depend on many factors such as, for example, the consequence of not detecting an out of control situation.

Although the general requirements for laboratories to become accredited are laid down in an international standard (EN 45001, Anon., 1991) and more detailed information for microbiological laboratories is now available (e.g. Eurachem EAL-G18, Anon., 1996) the requirements with regard to the type of controls are not the same in different countries. For example the use of a quantitative control for a quantitative method is common in accredited laboratories in The Netherlands but not in most other countries. Thus, further harmonisation of requirements for accreditation in different countries is needed. The Commission of the EU intends to stimulate this harmonisation in their fifth framework programme. In addition, projects such as the Equase project (an EU SM&T project promoting the introduction of quality assurance in small laboratories) can stimulate harmonisation of requirements for accreditation.

DESIGN AND REPRODUCTION QUALITY OF RMs

The market research (Van Oostwaard and Rauwerdink, 1993) and the interviews held in 1998 (described in chapter 7) supplied information on the design quality of the product. A number of the customers' needs could not be met by the RMs developed. The main problem was the need for a simple and rapid reconstitution procedure for the quantitative RMs. Studies aimed at producing alternative reconstitution procedures did not give satisfactory results. An alternative reconstitution procedure is only possible for organisms that do not experience osmotic shock upon reconstitution of the milk powder.

The level of contamination of the qualitative RMs should be slightly increased to eliminate the fraction of capsules not containing the target organism. An increase in the level of contamination also has a positive effect on the power analysis for the CRMs as the drop in the power curves will disappear.

Comparisons between the RMs and alternatives indicate that the RMs developed are competitive with respect to homogeneity, stability and price. However, the alternative RMs are much easier to reconstitute than the gelatin capsules. The main application of the materials should be in first line QC. In addition the qualitative RMs can easily be used as a standard inoculum for samples in collaborative studies or method development/validation. The RMs cannot be regarded as a suitable alternative to proficiency testing as the samples examined change from test to test with respect to the type and number of strain(s) included and the level of contamination.

The production of RMs and CRMs is similar, both are produced in a comparable manner using the same HCMP. The main difference between an RM and CRM is the collaborative study organised to establish the certified values. The confidence in the values stated for each batch of RM can be increased when more laboratories are involved in the establishment of the values and/or the comparison of the batch of RMs with an existing batch of a CRM. Further standardisation and certification of the production process could be used to predict the level of contamination of batches of RMs. However, the confidence of users in the values stated for the current RMs is high and changes that lead to a higher price for the RMs are not desirable at present. The fact that the RMs have been developed by and produced in non commercial companies gives confidence that the level of contamination of the RMs is as stated.

CONCLUSIONS

- The RMs developed fulfil the basic requirements for reference materials (i.e. stability, homogeneity and representativity). They proved to be useful in evaluating laboratory performance to detect or enumerate micro-organisms.
- The qualitative RMs can be used easily to evaluate the influence of food matrices on the detection of the target organism.
- The increase in sale of the RMs is less than expected from the market research, however, an average increase in sale of 10 % per year was realised between 1995 and 1998.
- Compared to the alternatives the developed RMs are most suited to situations where stability and homogeneity are crucial, as for example, in quality assurance of routine microbiological examinations. The use of RMs in this respect is increasing as confirmed by the increase in sales of RMs.
- Six RMs have been successfully certified by the Standards, Measurements and Testing programme (SM&T) of the European Commission. The sale of CRMs is low compared to the RMs as they are used only occasionally (i.e. to determine the accuracy of laboratories or new methods).

RECOMMENDATIONS

- The level of contamination of the qualitative RM should be increased in order to eliminate the chance of some capsules not containing any target organisms.
- The reconstitution procedure for the quantitative RMs should be improved as this hampers the application of these RMs.
- Laboratories, especially outside The Netherlands, must be informed about the existence and application of RMs for quality assurance of (routine) microbiological analysis.

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Since 1986 the National Institute of Public Health and the Environment (RIVM) has worked on the development and evaluation of microbiological reference materials (RMs) with support from the European Communities Bureau of Reference (BCR), now called Standards Measurement and Testing (SM&T). The RMs are the result of efforts, which was started many years ago, to develop standardised samples that could be used in collaborative studies and in particular for the evaluation of methods for the detection of *Salmonella*. This work ultimately led to the development of capsules filled with artificially contaminated milk powder. The objectives of this thesis were to evaluate the possibility of producing RMs that fulfil the general requirements for such materials i.e. stability, homogeneity and representativity, to produce certified RMs (CRMs) and to set up (commercial) production of RMs.

The basic process for preparation of the RMs is the spray drying of bacteria suspended in milk with subsequent mixing of the contaminated milk powder (called highly contaminated milk powder; HCMP) with sterile milk powder until the desired level of contamination is achieved. The mixed powder is then filled into gelatin capsules. Various RMs were developed using this process, for example, the RMs for *Bacillus cereus* and *Listeria monocytogenes* as described in chapter 1. The *B. cereus* RM is an RM containing *ca* 10^4 colony forming particles (cfp) per capsule and is intended for the evaluation of enumeration (quantitative) methods (they are also referred to as quantitative RMs). This RM is first reconstituted in 10 ml peptone saline solution after which 0.1 ml is plated onto an agar plate for enumeration of the organism. The *L. monocytogenes* RM is an RM containing only *ca* 5 cfp per capsule and is intended for the evaluation of detection (qualitative) methods (also called qualitative RMs). This RM is added, whole, to a (pre-warmed) enrichment broth.

Both RMs were used to characterise the behaviour of the material in relation to the general requirements for RMs. They were stable at storage temperature (-20 °C) over a period of more than 96 weeks. The *B. cereus* RM was stable for at least four weeks at higher temperatures (tested up to 37 °C). The *L. monocytogenes* RM showed a significant decrease in the level of contamination when stored for four weeks at 5 °C or higher, indicating the need for cooling during transport of this material. Both RMs could be produced homogeneously, meaning that the variation between the number of cfp was less than twice the value expected from a Poisson distribution. The *L. monocytogenes* HCMP 2-2 could not be diluted homogeneously to a level of five cfp per capsule due to the high level of contamination of this HCMP. In general, mixing using a mortar and pestle improved homogeneity of the final mixed powders. The representativity of the *B. cereus* RM was tested by examining the effects of osmotic shock and heat injury and of heat shock, storage time and lysozyme on spore germination. The parameters examined for the *L. monocytogenes* RM included the effects of osmotic shock, heat injury, pre-warming of enrichment broth and incubation time on recovery of the organism. For the *B. cereus* RM no effect of the various parameters tested could be observed, but with the *L. monocytogenes* RM there was an effect due to both heat injury and osmotic shock. As a result of heat shock (tested by comparing direct selective enrichment and non selective pre-enrichment) 40 - 50 % less positive isolations were observed with the direct selective enrichment. The effect of osmotic shock (tested by comparing free milk powder and the powder filled in gelatin capsules) led to *ca* 10 % less positive isolations using

the free milk powder. The growth rate of *Listeria* using RMs was faster than with a heat treated culture of the same strain (*ca* 2 $\log_{10}$ units difference after 31 h incubation at 30 °C).

As a part of the evaluation of the RMs a number of collaborative studies were organised. Chapter 2 describes three international collaborative studies using the *L. monocytogenes* RM. This RM was tested both with and without competitive micro-organisms. The competitors were added either as a capsule containing a mixture of strains or as a food sample. The food samples were tested in combination with RMs at two contamination levels (*ca* 5 cfp per capsule and *ca* 100 cfp per capsule). Based on the known level of contamination of the RMs the number of positive isolations expected was calculated and compared to the results obtained by the laboratories. Most found the expected number of *L. monocytogenes* isolations when no competitors were present. However, the addition of a capsule containing competitive micro-organisms (*ca* 3 x 10^4 cfp per capsule) reduced the rate of positive isolations from 97 % to 80 %. In the presence of food samples the positive rate decreased even further. No relationship between the effect of the competitive micro-organisms and the type of food product and/or the detection method used was observed. The RMs proved to be useful in evaluating the performance of a detection method in the presence of competitive micro-organisms.

Once the stability, homogeneity and representativity of an RM are proven it can be used to produce a CRM. The procedure necessary for obtaining a qualitative CRM for *Salmonella typhimurium* is described in chapter 3 together with the results of the collaborative study organised to establish the certified values. The batch was certified based on the results from 9 or 10 laboratories for the number of *Salmonella* cfp in a single capsule (mean value 5.9) and the fraction of capsules in which no *Salmonella* could be detected (2.7 % using the ISO detection method). The certification of the quantitative *B. cereus* RM is described in chapter 4. For certification, the number of cfp on two media, MEYP (incubated at 30 °C) and on PEMBA (incubated at 37 °C) was determined. The certified geometric mean value on MEYP after 24 h incubation was 53.4 cfp per 0.1 ml reconstituted capsule solution (95 % confidence interval 51.7 - 55.2) and on PEMBA 55.0 cfp per 0.1 ml (95 % confidence interval 52.8 - 57.4), both sets of figures are based on the results from 11 laboratories. The certificate for both CRMs gives the mean expected value and the 95 % confidence limits. From these results user tables are constructed presenting the 95 % confidence limits for the number of capsules (and replicates per capsule in the case of quantitative CRMs) likely to be examined in practice.

Chapter 5 describes the evaluation of an alternative method for the preparation of HCMPs by means of fluid bed spray granulation. This method was chosen as this drying procedure (spraying of concentrated milk containing the micro-organism onto sterile milk powder held fluidised by means of air) does not require drying temperatures as high as those necessary for spray drying. A number of different micro-organisms (*Bacillus cereus, Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella enteritidis* and *Staphylococcus aureus*) were dried using this procedure and the resulting materials tested for homogeneity and stability when stored at -20 °C and 22 °C. The strain of *C. jejuni* did not survive the drying procedure, no organisms could be recovered from the milk powder the day after the drying. The homogeneity of the various HCMPs

produced varied widely. The time used for spraying the milk onto the powder was a critical factor in obtaining homogeneous HCMPs. An initial decrease in numbers of organisms was found comparable to that obtained with spray dried material. This indicates that the drying temperature is not the only factor determining survival of organisms through the drying process. The stability at 22 °C showed, for most strains, a non linear decrease and the results obtained were not as good as those for silica gel or spray dried material. Satisfactory results were obtained with stability tests at -20 °C, but more information is needed on long term stability. Production of HCMP by spray granulation is less laborious than by spray drying and the spray granulation process is better suited for testing the influence of culturing and drying conditions on the survival of micro-organisms.

Chapter 6 describes the use of RMs and CRMs. A distinction is made between the use of both quantitative and qualitative RMs for quality assurance of routine and occasional examinations. Control charts such as the Shewhart chart can be constructed for the routine use of quantitative RMs. The control limits for the Shewhart chart are calculated using log-transformed counts and can be back-transformed to produce a chart for counts on the original scale. Each data point on the chart is the result from a single enumeration of one capsule. It is possible to adapt the method of calculating the control limits for RMs that are not fully stable by using the principle of Kalman's filtering. For routine use of qualitative RMs it is recommended that a single capsule is examined on each test occasion. The results from a series of tests can be compared to the expected number of positive isolations as calculated for the occasional use of RMs. Certified RMs are used only occasionally and are mainly intended for the determination of trueness in a laboratory. Results of power analyses are presented to demonstrate the minimum difference that can be detected between the certified value and the true laboratory mean (for quantitative CRMs) or the fraction of negatives (for qualitative CRMs). For the qualitative RM and CRM a fraction of negatives of 0 % is needed in order to minimise the number of capsules necessary for evaluation of laboratory performance. Additional information is required on the variation between laboratories for occasional use of non-certified RMs in order to interpret results. The laboratory variance component of a CRM can be used to adjust the confidence limits of an RM determined by a single laboratory.

During the development and evaluation of the RMs market research was undertaken to obtain the opinions of potential customers on the characteristics of the product. In this market research, reported in 1993, both inspection and production laboratories (all with some knowledge of the materials) were interviewed. The results of this market research are presented in chapter 7. A number of requirements for the materials became apparent and these were compared with the product developed. Most of the requirements could be or already were included in the product, but some would be difficult or even impossible to incorporate. The specific requirement for a simple short pre-treatment for the quantitative RMs was evident and has not been resolved. On the basis of the results of the market research production of the materials was set up at the SVM with the assistance of RIVM. The actual sale of RMs is gradually increasing but the increase is much slower than was predicted by the market research. In order to obtain information on the organisation of quality assurance in laboratories and the role played by RMs, a second series of interviews

was organised in 1998. It became apparent from these interviews that the use of RMs in quality assurance of routine microbiological examination is important; this was not as apparent at the time of the original market research. Some laboratories used a type of control chart for evaluation of results from the use of quantitative RMs over a period of time. It was also agreed that the level of contamination of the qualitative RMs needs to be increased to eliminate the chance of finding capsules that do not contain the target organism. To increase the sale of the RMs more information on their use in QA should be made available to laboratories throughout Europe, especially to those that are accredited and also for routine examinations. A comparison between the RMs developed and alternatives from other sources indicates that they are competitive with respect to homogeneity, stability and price. However, as far as the quantitative RMs are concerned, the alternative RMs are much easier to reconstitute than the gelatin capsules.

Based on the studies undertaken it can be concluded that RMs and CRMs can be produced, that fulfil the accepted requirements for such materials. Market research indicated that there is a need for this kind of material and, therefore, the production of RMs was set up at SVM. Although sales of the RMs are less than expected they are increasing. The RMs developed are competitive with existing alternative RMs and are useful for quality assurance purposes.

List of available reference materials (non-certified and certified).

reference materials (non-certified) (available from SVM, Bilthoven, The Netherlands)

- *Bacillus cereus* RM (5,000 cfp per capsule)
- *Clostridium perfringens* RM (5,000 cfp per capsule)
- *Enterobacter cloacae* RM (500 cfp per capsule)
- *Enterococcus faecium* RM (500 cfp per capsule)
- *Escherichia coli* RM (500 cfp per capsule)
- *Listeria monocytogenes* RM (5 cfp per capsule)
- *Listeria monocytogenes* RM (5,000 cfp per capsule)
- *Salmonella panama* RM (5 cfp per capsule)
- *Staphylococcus warneri* RM (500 cfp per capsule)

certified reference materials (available from EC, IRMM, Geel, Belgium)

- *Enterococcus faecium* CRM 506
- *Salmonella typhimurium* CRM 507
- *Enterobacter cloacae* CRM 527
- *Bacillus cereus* CRM 528
- *Escherichia coli* CRM 594
- *Listeria monocytogenes* CRM 595

Sinds 1986 heeft het Rijksinstituut voor Volksgezondheid en het Milieu (RIVM) gewerkt aan de ontwikkeling en de evaluatie van microbiologische referentiematerialen (RM's) met ondersteuning van het "Communities Bureau of Reference" (BCR), tegenwoordig "Standards Measurement and Testing" (SM&T) genaamd. De RM's zijn het resultaat van de inspanningen, die jaren geleden waren opgestart, om gestandaardiseerde monsters te ontwikkelen die gebruikt konden worden in ringonderzoeken en in het bijzonder voor de evaluatie van methoden voor de detectie van *Salmonella*. Dit werk leidde uiteindelijk tot de ontwikkeling van capsules gevuld met kunstmatig besmet melkpoeder. De doelstellingen van het werk beschreven in dit proefschrift waren het evalueren van de mogelijkheid om RM's te ontwikkelen die voldoen aan de algemene eisen voor zulk soort materialen (d.w.z. stabiliteit, homogeniteit en representativiteit), het produceren van gecertificeerde RM's (CRM's) en het opzetten van de (commerciële) productie van RM's.

Het basis proces voor de bereiding van RM's is het sproeidrogen van bacteriën gesuspendeerd in melk en vervolgens het mengen van het besmette melkpoeder (zogenaamd hoog besmet melkpoeder; HCMP) met steriel melkpoeder tot het gewenste besmettingsniveau. Het gemengde poeder wordt dan afgevuld in gelatine capsules. Diverse RM's zijn volgens dit proces ontwikkeld, zoals bijvoorbeeld het RM voor *Bacillus cereus* en *Listeria monocytogenes* beschreven in hoofdstuk 1. Het *B. cereus* RM is een RM dat circa 10^4 kolonie vormende deeltjes (cfp) per capsule bevat en is bedoeld voor de evaluatie van telmethoden (kwantitatieve methoden, ook wel gerefereerd als kwantitatieve RM's). Dit RM wordt voor gebruik eerst gereconstitueerd in 10 ml pepton zout oplossing waarna 0,1 ml uitgespateld wordt op een agarplaat om het organisme te kunnen tellen. Het *L. monocytogenes* RM is een RM dat maar circa 5 cfp per capsule bevat en is bedoeld voor de evaluatie van detectie (kwalitatieve) methoden (ook wel kwalitatieve RM's genoemd). Dit RM wordt voor gebruik in z'n geheel toegevoegd aan een (voorverwarmd) ophopingsmedium.

Beide RM's werden gebruikt om hun gedrag te karakteriseren in relatie tot de algemene eisen voor RM's. Beide RM's waren stabiel over een periode van minimaal 96 weken bij opslagtemperatuur (-20 °C). Het *B. cereus* RM was stabiel voor minimaal 4 weken bij hogere temperaturen (getest tot maximaal 37 °C). Het *L. monocytogenes* RM gaf een significante daling van het besmettingsniveau te zien bij opslag gedurende 4 weken bij 5 °C of hoger, waarmee de noodzaak werd aangegeven voor koeling tijdens transport van dit materiaal. Beide RM's konden homogeen worden geproduceerd, dat wil zeggen dat de variatie tussen de aantallen cfp minder was dan tweemaal de waarde zoals die van een Poisson verdeling wordt verwacht. Het *L. monocytogenes* HCMP 2-2 kon niet homogeen worden verdund tot een niveau van 5 cfp per capsule als gevolg van het hoge besmettingsniveau van dit HCMP. In het algemeen werd de homogeniteit van het uiteindelijke mengsel verbeterd door te mengen met behulp van een mortier en vijzel. De representativiteit van het *B. cereus* RM werd getest door het onderzoeken van het effect van osmotische shock en hitte beschadiging en van hitte shock, opslagtijd en lysozym op ontkieming van de sporen. De parameters onderzocht met het *L. monocytogenes* RM omvatte het effect van osmotisch shock, hitte beschadiging, voorverwarming van ophopingsmedia en incubatie tijd op het terugvinden van het organisme. Met het *B. cereus* RM kon geen effect van de diverse parameters worden waargenomen, maar met het *L. monocytogenes* RM was er een effect als gevolg van hitte beschadiging en

osmotische shock. Het resultaat als gevolg van de hitte shock (getest door vergelijking van direct selectief ophopen en niet selectieve voorophoping) was dat 40 – 50 % minder positieve isolaties werden waargenomen met het direct selectief ophopen. Het effect van osmotische shock (getest door vergelijking van los melkpoeder met poeder gevuld in gelatine capsules) leidde tot circa 10 % minder positieve isolaties met het gebruik van het losse melkpoeder. De groeisnelheid van *Listeria* vanuit het RM was hoger dan met een hitte behandelde cultuur van dezelfde stam (circa 2 $\log_{10}$ eenheden verschil na 31 uur incubatie bij 30 °C).

Als onderdeel van de evaluatie van de RM's werd een aantal ringonderzoeken georganiseerd. Hoofdstuk 2 beschrijft een drietal internationale ringonderzoeken met het *L. monocytogenes* RM. Dit RM werd getest met en zonder competerende micro-organismen. De competerende micro-organismen werden toegevoegd als een capsule die een mengsel van stammen bevatte of als een monster van een levensmiddel. De levensmiddelenmonsters werden getest in combinatie met RM's op twee niveaus (circa 5 cfp per capsule en circa 100 cfp per capsule). Gebaseerd op het bekende besmettingsniveau van de RM's werd het verwachte aantal positieve isolaties berekend en vergeleken met de verkregen resultaten van de laboratoria. De meesten vonden het verwachte aantal *L. monocytogenes* isolaties wanneer er geen competerende micro-organismen aanwezig waren. Echter, de toevoeging van een capsule die competerende micro-organismen bevatte (circa 3 x 10^4 cfp per capsule) reduceerde het aantal positieve isolaties van 97 % tot 80 %. In de aanwezigheid van levensmiddelenmonsters werd het gevonden aantal positieven nog verder verlaagd. Er werd geen relatie waargenomen tussen het effect van de competerende micro-organismen en het soort levensmiddel en/of de gebruikte detectiemethode. De RM's bewezen nuttig te zijn voor het evalueren van de prestatie van een detectiemethode in de aanwezigheid van competerende micro-organismen.

Toen eenmaal de stabiliteit, homogeniteit en representativiteit van een RM was bewezen kon het gebruikt worden voor de productie van een CRM. De noodzakelijke procedure om een kwalitatief CRM voor *Salmonella* te verkrijgen is beschreven in hoofdstuk 3 tezamen met de resultaten van het ringonderzoek dat werd georganiseerd om de gecertificeerde waarden vast te stellen. De batch werd gecertificeerd, op basis van de resultaten van 9 of 10 laboratoria, voor het aantal *Salmonella* cfp in één capsule (gemiddelde waarde 5.9) en de fractie capsules waarin geen *Salmonella* kon worden aangetoond (2.7 % volgens de ISO detectie methode). De certificering van het kwantitatieve *B. cereus* RM is beschreven in hoofdstuk 4. Voor certificatie werd het aantal cfp op twee media, MEYP (geïncubeerd bij 30 °C) en PEMBA (geïncubeerd bij 37 °C) vastgesteld. Het gecertificeerde geometrisch gemiddelde op MEYP na 24 uur incubatie was 53,4 cfp per 0.1 ml van de gereconstitueerde capsule oplossing (95 % betrouwbaarheidsinterval 51,7 – 55,2) en op PEMBA 55,0 cfp per 0,1 ml (95 % betrouwbaarheidsinterval 52,8 – 57,4), beide data sets zijn gebaseerd op de resultaten van 11 laboratoria. Het certificaat voor beide CRM's geeft het verwachte gemiddelde en de 95 % betrouwbaarheidsgrenzen. Gebaseerd op deze resultaten zijn gebruikerstabellen gemaakt die de 95 % betrouwbaarheidsgrenzen geven voor het aantal capsules (en herhalingen per capsules in het geval van een kwantitatief CRM) dat in praktijk waarschijnlijk zal worden onderzocht.

Hoofdstuk 5 beschrijft de evaluatie van een alternatieve methode voor de bereiding van HCMP's door middel van "fluid bed spray granulation". Deze methode was gekozen omdat deze droogprocedure (geconcentreerde melk dat micro-organismen bevat wordt verneveld op steriel melkpoeder dat met behulp van lucht in werveling wordt gehouden) niet zulke hoge droogtemperaturen nodig heeft als sproeidrogen. Een aantal verschillende micro-organismen (*Bacillus cereus, Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella enteritidis en Staphylococcus aureus*) werd gedroogd volgens deze procedure. De hieruit verkregen materialen werden getest op homogeniteit en stabiliteit bij opslag bij 22 °C en -20 °C. De *C. jejuni* stam overleefde het droogproces niet, er konden, één dag na het drogen, geen organismen meer in het melkpoeder worden teruggevonden. De homogeniteit van de diverse geproduceerde HCMP's varieerde sterk. De tijd die nodig was om de melk op het poeder te vernevelen was een kritische factor voor het verkrijgen van homogene HCMP's. De gevonden initiële afname van het aantal organismen was vergelijkbaar met die verkregen met sproeidrogen. Dit duidt erop dat de droogtemperatuur niet de enige factor is die de overleving van de organismen in het droogproces bepaalt. De stabiliteit bij 22 °C toonde, voor de meeste stammen, een niet lineaire afname aan en de verkregen resultaten waren niet zo goed als die met silicagel of gesproeidroogd materiaal. Stabiliteit bij -20 °C gaf bevredigende resultaten, maar meer informatie over de stabiliteit op de lange termijn is nodig. Productie van HCMP met "spray granulation" is minder arbeidsintensief dan met sproeidrogen en het "spray granulation" proces is beter geschikt om de invloed van kweek en droog condities op de overleving van de micro-organismen te testen.

Hoofdstuk 6 beschrijft het gebruik van RM's en CRM's. Een onderscheid wordt gemaakt tussen het gebruik van zowel kwantitatieve als de kwalitatieve RM's voor de kwaliteitsborging van routinematig en incidenteel onderzoek. Voor het routinematig gebruik van de kwantitatieve RM's kunnen controlekaarten, zoals de Shewhart controlekaart, worden geconstrueerd. De controlegrenzen van de Shewhart kaart worden berekend op de log-getransformeerde tellingen en kunnen worden teruggetransformeerd om een kaart te produceren voor tellingen op de originele schaal. Elk data punt in de kaart is het resultaat van het onderzoek van één capsule in enkelvoud geteld. Voor RM's die niet volledig stabiel zijn is het mogelijk om de manier voor het berekenen van de controlegrenzen aan te passen door gebruik te maken van het principe van de Kalman's filtering. Voor het routinematig gebruik van kwalitatieve RM's wordt aanbevolen om één capsule te onderzoeken voor elke test gelegenheid. De resultaten van een serie van testen kunnen worden vergeleken met verwachte aantal positieve isolaties zoals berekend voor het incidenteel gebruik van RM's. Gecertificeerde RM's worden alleen incidenteel gebruikt en zijn voornamelijk bedoeld voor het vaststellen van de "ware waarde" (trueness) in een laboratorium. De resultaten van "power analyses" worden gepresenteerd om het minimale verschil dat kan worden aangetoond tussen de gecertificeerde waarde en het werkelijke laboratorium gemiddelde (voor kwantitatieve CRM's) of de fractie negatieven (voor kwalitatieve CRM's) aan te tonen. Voor de kwalitatieve RM's en CRM's is een theoretische fractie negatieven van 0 % nodig om het aantal capsules dat noodzakelijk is voor de evaluatie van de laboratorium prestaties te minimaliseren. Aanvullende informatie over de variatie tussen laboratoria is nodig om, bij

het incidentele gebruik van niet gecertificeerde RM's, de resultaten te kunnen interpreteren. De laboratorium variantie component van een CRM kan gebruikt worden om de, in één laboratorium bepaalde, betrouwbaarheidsgrenzen van een RM aan te passen.

Gedurende de ontwikkeling en evaluatie van de RM's werd een marktonderzoek gehouden om de mening van potentiële klanten over de eigenschappen van het product, te peilen. In dit marktonderzoek, dat in 1993 werd gerapporteerd, werden zowel controle en productie laboratoria (allen met enige kennis van de materialen) geïnterviewd. De resultaten van dit marktonderzoek worden gepresenteerd in hoofdstuk 7. Een aantal behoeften voor de materialen werd duidelijk en deze werden vergeleken met het ontwikkelde product. De meeste van de behoeften konden of waren reeds opgenomen in het product, maar voor enkele zou het moeilijk of zelfs onmogelijk zijn om dit erin op te nemen. De specifieke behoefte aan een simpele kortdurende voorbehandeling voor de kwantitatieve RM's was duidelijk en kon nog niet verbeterd worden. Op basis van de resultaten van het marktonderzoek werd de productie van de materialen opgezet bij SVM met assistentie van het RIVM. De werkelijke verkoop van de RM's neemt geleidelijk toe maar de toename is veel langzamer dan was voorspeld uit het marktonderzoek. Ten einde informatie te verkrijgen over de organisatie van kwaliteitsborging in laboratoria en de rol die RM's hierin spelen werd een tweede serie van interviews georganiseerd in 1998. Uit deze interviews werd duidelijk dat het gebruik van RM's voor de kwaliteitsborging van routinematige microbiologisch onderzoek belangrijk is; dit was nog niet zo duidelijk ten tijde van het originele marktonderzoek. Enkele laboratoria gebruikten een soort controlekaart voor de evaluatie van resultaten over een bepaalde periode verkregen met de kwantitatieve RM's. Men was het er ook over eens dat het besmettingsniveau van de kwalitatieve RM's verhoogd moest worden om de kans, op het vinden van capsules die het doel organisme net bevatten, uit te sluiten. Om de verkoop van de RM's te verhogen zou meer informatie over het gebruik van RM's in kwaliteitsborging beschikbaar gemaakt moeten worden aan laboratoria in Europa, met name aan diegenen die geaccrediteerd zijn en tevens routinematig onderzoek uitvoeren. Een vergelijking van de ontwikkelde RM's met alternatieven van andere producenten geeft aan dat de ontwikkelde RM's concurrerend zijn wat betreft homogeniteit, stabiliteit en prijs. Echter, voor zover het de kwantitatieve RMs betreft, zijn de alternatieve RM's veel gemakkelijker te reconstitueren dan de gelatine capsules.

Gebaseerd op het uitgevoerde onderzoek kan worden geconcludeerd dat RM's en CRM's kunnen worden geproduceerd die voldoen aan de algemeen aanvaarde eisen aan zulke materialen. Marktonderzoek toonde aan dat er een behoefte is aan dit soort materialen en daarom werd de productie van de RM's opgezet bij SVM. Ondanks dat de verkoop van de RM's lager is dan verwacht, neemt ze wel toe. De ontwikkelde RM's zijn concurrerend met de bestaande alternatieve RM's en zijn nuttig voor kwaliteitsborgingsdoeleinden.

Lijst van beschikbare (gecertificeerde) referentie materialen.

referentie materialen (verkrijgbaar bij SVM, Bilthoven, The Netherlands)

- *Bacillus cereus* RM (5.000 cfp per capsule)
- *Clostridium perfringens* RM (5.000 cfp per capsule)
- *Enterobacter cloacae* RM (500 cfp per capsule)
- *Enterococcus faecium* RM (500 cfp per capsule)
- *Escherichia coli* RM (500 cfp per capsule)
- *Listeria monocytogenes* RM (5 cfp per capsule)
- *Listeria monocytogenes* RM (5.000 cfp per capsule)
- *Salmonella panama* RM (5 cfp per capsule)
- *Staphylococcus warneri* RM (500 cfp per capsule)

gecertificeerde referentie materialen (beschikbaar bij EC, IRMM, Geel, Belgium)

- *Enterococcus faecium* CRM 506
- *Salmonella typhimurium* CRM 507
- *Enterobacter cloacae* CRM 527
- *Bacillus cereus* CRM 528
- *Escherichia coli* CRM 594
- *Listeria monocytogenes* CRM 595

Zoals met zovele proefschriften is ook dit proefschrift niet zonder hulp van velen tot stand gekomen. Bij deze wil ik hen die een bijdrage hebben geleverd bedanken, echter in een volgorde die niet de volgorde van belangrijkheid van de bijdrage weerspiegelt.

Diane Roberts, a special word of thanks to you for your valuable comments and suggestions for improvement of the thesis, your input in the discussion on the collaborative studies organised over the past 11 years and your enjoyable lunch/diner conversations. Eddy Maier and Ben Griepink for their enthusiasm for the project on the development of microbiological reference materials and as inspiring chairmen. I have learned a lot from you both. The (more than 40 different) participants in the collaborative studies organised are thanked for their input in the discussions and their willingness to participate in the sometimes very laborious studies.

Het werk had niet tot stand kunnen komen zonder al het laboratoriumwerk dat in de projecten is uitgevoerd. In volgorde van tijd waren dit: Wilma Ritmeester, Jannie Hobers en Marleen de Winter. Wilma, dank je voor je voortdurende (ook nadat je niet meer op het project werkte) adviezen/commentaar op de opzet en uitvoering van het werk en je kritische houding ten opzichte van kwaliteitszaken in het algemeen. Marleen, jij hebt het het langste volgehouden op het project; ook jij had een waardevolle kritische houding ten aanzien van de opzet en uitvoering van het werk. Ook is er een bijdrage geleverd door mensen die niet direct bij het project betrokken waren. Behalve hun bijdrage aan het laboratoriumwerk waren er ook de opmerkingen en discussies over de aanpak en uitvoering van het werk. In dit verband wil ik Pop Soentoro, John Dufrenne en Ellen Delfgou-van Asch bedanken. Kirsten Mooijman wilde ik bedanken voor samenwerking over de vele jaren waarin we vergelijkbaar werk hebben uitgevoerd, de perikelen van het projectmedewerkerschap hebben mogen ervaren en je commentaar op delen van het proefschrift. Mary Manavakis, je pakte het werk snel op en hebt gedurende de laatste 2 jaar van het project een grote hoeveelheid werk op je genomen. Arie Havelaar bedankt voor je inzet als projectleider en alles wat hiermee samenhangt en voor het accepteren van het co-promotorschap aan het einde van de "rit". Andre Henken bedankt voor je gedetailleerde commentaar op het concept proefschrift. Serve Notermans wilde ik bedanken voor zijn inbreng bij het schrijven van mijn (eerste) publicaties, Harry Beckers voor het feit dat ik altijd bij je terecht kon voor vragen zowel in mijn begintijd op het RIVM als later toen je bij Nestle ging werken. Een reeks van wiskundigen/(bio)statistici (Janneke Hoekstra, Noor van Strijp-Lockefeer, Judith van Dommelen, Siem Hiesterkamp en Nico Nagelkerke) wilde ik bedanken voor hun inzet en vermogen zich in te leven in de wereld van de microbiologische analyses. Met name Nico voor zijn bijdrage in de laatste fase van het schrijven. Jullie hebben mij het nut van statistiek doen inzien en mijn desinteresse weten om te buigen. Verder wilde ik nog een aantal LWL/MGB'ers noemen die betrokken zijn geweest bij het project en/of het schrijven van het proefschrift. Elise Nijkamp, Reina van der Heide, Arjen van de Giessen, Yves Moriamez, Birgit Janning, Henri Kamphuis en Monique de Nijs. Ernst Rozendaal voor het maken van de opnames voor de kaft van het proefschrift.

Arthur Rauwerdink en Ton van Oostwaard wilde ik bedanken voor de gezellige en leerzame tijd van het marktonderzoek. Nadine Brentlin, Harry beckers, Hans van Dijk, Ellen de Ree, Annemarie Mulder en Klaas Strikwerda voor hun bijdrage aan de (tweede serie)

interviews. Leden van de taakgroep kwaliteit van de KMK, met name Annemarie Mulder en Petra Koenraad, voor de samenwerking en het gebruik van de SRM gegevens. De taakgroep ringonderzoeken van de KMK en de voorloper hiervan voor de mogelijkheid om de RMs te kunnen gebruiken bij de interne ringonderzoeken. Rijkelt Beumer wilde ik bedanken voor zijn bijdrage aan een aantal droog experimenten. Jan Stapersma voor het ter beschikking stellen van de verkoop gegevens, Maaike Keizer en Lisette Keulen voor hun overzichten van de afnemers van de RM's.

Marcus van de Berg wilde ik bijzonder bedanken voor het aannemen van het promotorschap, zijn waardevolle en verhelderende kijk op kwaliteitszaken en zijn geduld bij het tot stand komen van dit proefschrift.

Als laatste wilde ik Selma bedanken voor haar steun op de lange weg naar het voltooien van het proefschrift.

Paul Hubertus in 't Veld werd op 19 december 1960 geboren in Rotterdam. In 1979 behaalde hij het VWO-diploma aan de Openbare Scholengemeenschap Walburg in Zwijndrecht. Daarna vervolgde hij zijn opleiding aan de Landbouwuniversiteit Wageningen. In 1987 studeerde hij af in de Levensmiddelentechnologie met als afstudeervakken Levensmiddelenchemie en Levensmiddelenmicrobiologie.

Vanaf 1987 is hij werkzaam bij het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) te Bilthoven, op het Laboratorium voor Water en Levensmiddelenmicrobiologie (LWL) dat na een reorganisatie in 1996 onderdeel is geworden van het Microbiologisch Laboratorium voor Gezondheidsbescherming (MGB). Gedurende de periode 1987 - 1995 heeft hij gewerkt aan de ontwikkeling en de evaluatie van microbiologische referentiematerialen voor de levensmiddelenmicrobiologie, een project gefinancieerd door de Europese Commissie. Huidige werkzaamheden liggen op het gebied van Communautair Referentielaboratorium voor *Salmonella*, validatie van microbiologische methoden ten behoeve van de Europese standaardisatie organisatie (CEN) en de risico analyse van voedselpathogenen (met name entero-toxine productie van *Bacillus cereus*).

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