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A study of the shelf-life of critical culture media

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Summary

The shelf-life of a culture medium is the maximum period of validity for optimum preparation and preservation. Apart from the composition of the medium, the factors that influence shelf-life are sterilisation method, preservation and packaging procedures, storage temperature and exposure to light. The shelf-life of a culture medium is defined by evaluating its basic chemico-physical characteristics so as to obtain the correct growth and characterisation of a specific microorganism. This research was conducted from March to September 2003 on 12 'critical' culture media, i.e. media that had a coded shelf-life of not more than thirty days. Each medium was produced in three separate batches, with a total of 5 940 samples. The purpose of the study was to define a longer period of validity than that coded for each medium by evaluating weight reduction, pH, fertility and sterility. The shelf-life observed for each medium was longer than those coded. The new shelf-life takes into account both the operational needs of complex organisational structures and the efficiency of the medium, depending on its chemico-physical characteristics and storage and preservation methods.

Keywords

Culture media, Ecometric techniques, Quality control, Shelf-life.

Introduction

Culture media are nutrient substrates of varying degrees of complexity which are formulated for the cultivation of microorganisms. They must meet the nutritional requirements of micro-organisms which require sources of nitrogen, carbon, hydrogen, oxygen, sulphur, phosphorus, sodium, potassium, magnesium, iron and manganese in order to live and multiply.

The principal components of culture media are divided into the following classes: peptones, carbohydrates, indicators, selective agents, solidifying agents, enrichments, chromogenic and fluorogenic enzyme substrates (1). When each component is included in the formulation of a culture medium, it performs a precise function which, in addition to promoting the growth of certain microorganisms in preference to others, influences the period for which the medium remains effective.

The validity or 'shelf-life' of a culture medium means the maximum time during which a medium retains all the chemico-physical characteristics essential for the correct growth and characterisation of a specific microorganism.

The factors that most affect the shelf-life of a culture medium, apart from its composition, are the sterilisation method (autoclaving, filtration) and preservation procedures (packaging, storage temperature and exposure to light).

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The purpose of this study was to assign to each selected culture medium a longer shelf-life than the one currently coded by the Culture Medium Production Department of the *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale'* (IZSA&M) in Teramo, Italy. No bibliographic data relating to studies conducted by other authors on this topic are available. The reference legislation and the manuals produced by distributors of culture media, which occasionally indicate a shelf-life, often indicate such a short shelf-life that it is incompatible with the operational needs of complex organisational structures.

The culture media studied were selected from among those most frequently used by the departments of the headquarters and diagnostic sections of the IZSA&M. All had a coded shelf-life of not more than thirty days.

The culture media examined were *Campylobacter* blood free agar (Karmali) (8, 9), *Campylobacter* selective medium (Skirrow) (8, 9), *Bacillus cereus* agar (MYP agar) (7), Hayflick agar (11), Hayflick broth (11), *Pseudomonas* CN agar base (3), lactose 2,3,5-triphenyltetrazoliumchloride (TTC) agar with Tergitol (4), Aesculin bile azide agar (5), Slanetz Bartley agar containing TTC (5), Thiaucourt broth (2, 13, 14), Thiaucourt agar (2, 13, 14) and XLD (xylose lysine deoxycholate) medium (6).

Materials and methods

Production of the media

The survey was conducted from March to September 2003. A detailed description of production is only given for media for which a comprehensive formulation is not available on the market. For each medium tested, three distinct batches were produced according to the procedures presented in Table I.

Shelf-life parameters and observation times of the media tested

The shelf-life of the culture media to which this study relates was established by evaluating weight reduction (for agar media), pH, sterility and fertility. The verification of the parameters was conducted after the coded expiry dates, from 30 April 2003 (T_1). Subsequent checks were performed on 15 May (T_2), 27 May (T_3), 3 June (T_4), 10 June (T_5), 17 June (T_6), 26 June (T_7), 9 July (T_8), 16 July (T_9), 28 July (T_{10}), 26 August (T_{11}) and 10 September (T_{12}).

Labelling, weighing, packaging and storage

Each plate and/or test tube from each batch of culture media produced was identified with an adhesive label stating the code of the medium, the number of the production batch and the serial number of each item within the production batch. Each plate of agar medium was weighed with an analytical balance (Sartorius). For each batch of culture media, groups of nine plates (for the agar media) and six test tubes (for the liquid media) were formed with shrink film. After packaging, the media were stored immediately in suitable containers in cold storage at +5°C ± 3°C.

Sampling

Agar media

For each batch of each agar medium tested, a sampling unit of nine plates was used in accordance with the relevant internal operating procedure (10). Consequently, the sample tested on each occasion for each agar medium consisted of 27 plates.

Broth

For each batch of culture broth, a sampling unit of six test tubes was used in accordance with the relevant internal operating procedure (10). Consequently, the sample tested on each occasion for each broth consisted of 18 test tubes.

Weight reduction check

At each check point (T_1-T_{12}) for each agar medium, the sample of 27 plates, consisting of the sampling unit of 9 plates for each of the 3 batches produced, was weighed with an analytical balance (Sartorius) to evaluate the percentage weight reduction.

pH check

At each check point (T_1-T_{12}) for each culture medium, the pH value for each of the three batches produced was measured with a pH-metro (Mettler-Toledo), calibrated to pH 7 and pH 4, as described below.

Agar media

The pH value was taken on two of the nine plates that formed the sampling unit for each batch (previously subjected to the weight reduction check). Consequently, the sample checked on each occasion for each agar medium consisted of 6 plates.

Broth

Here, the pH value was read on two of the six test tubes constituting the sampling unit for each batch. Consequently, the sample checked on each occasion for each broth consisted of 6 test tubes.

Sterility

At each check point (T_1-T_{12}) for each culture medium, the sterility of each of the three batches produced was evaluated as described below.

Agar media

Two plates were incubated at 21°C ± 1°C for 48 h and two additional plates at 36°C ± 1°C for 48 h (of the nine plates constituting the sampling unit for each batch already subjected to the weight reduction check). The sample checked on each occasion for each agar medium consequently consisted of 12 plates.

Broth

One test tube was incubated at 21°C ± 1°C for 48 h and another at 36°C ± 1°C for 48 h of the six test tubes constituting the sampling unit for each batch. The sample checked on each occasion for each broth consequently consisted of 6 test tubes.

Fertility

At each check point (T_1-T_{12}) the fertility of each of the three batches produced was evaluated for each culture medium observing the growth, after suitable incubation, of the control strains (Table II) seeded according to the procedure described below.

Agar media

For media produced in 90 mm-diameter plates, namely: C157, C158, C205 and C328, fertility was evaluated using the ecometric technique (12).

For the mycoplasma media produced in 60 mm-diameter plates, namely: C252 and C324, the fertility evaluation was conducted by seeding a drop of culture broth of the control strain suitable for the type of medium, and checking growth after suitable incubation.

For the other media produced in 60 mm-diameter plates, namely: C273, C280, C281 and C283, the fertility evaluation was conducted by simple streak plate seeding of the control strains, because the small size of the plates did not enable the correct application of the ecometric technique (overlap of bacterial growth).

Three of the nine plates that made up the sampling unit for each batch (already subjected to the weight reduction check) were used for each agar medium. The sample checked for each agar medium on each occasion was therefore 9 plates.

Mycoplasma culture broths (C253 and C323)

Seeding was performed using three drops of culture broth of the control strain suitable for the type of medium, from two of the six test tubes constituting the sampling unit and, after suitable incubation, the culture broth was seeded on a suitable selective medium to check its growth.

The sample checked on each occasion for each broth consequently consisted of 6 test tubes.

Table I
Testing of twelve culture media to compare shelf-life

Medium	Internal code	Base medium and procedure	Batches produced	Petri dishes/ test tubes
Campylobacter blood free agar (Karmali)	C157	Biolife, Milan, Italy Karmali antibiotic supplement (Biolife)	484 486 487	180 Ø90 mm Petri dishes
Campylobacter selective medium (Skirrow)	C158	Blood agar base medium N° 2 (Oxoid, Basingstoke, UK) *Campylobacter* growth supplement (Oxoid) *Campylobacter* Skirrow supplement (Oxoid) Laked horse blood (IZSA&M, Teramo)	611 613 624	180 Ø90 mm Petri dishes
Pseudomonas CN agar base	C273	Base medium (Oxoid) 1% (v/v) glycerol(Carlo Erba) *Pseudomonas* CN supplement (Oxoid)	524 525 526	180 Ø60 mm Petri dishes
Lactose TTC agar with tergitol	C280	Dehydrated base medium (Merck, Darmstadt, Germany), 0.05% TTC solution BDH, Poole, UK)	621 622 631	180 Ø60 mm Petri dishes
Aesculin bile azide agar	C281	Complete dehydrated medium(Biolife)	588 606 607	162 Ø60 mm Petri dishes
Slanetz Bartley agar with TTC	C283	Dehydrated base medium (Biolife) 1% TTC solution (BDH, Poole, UK)	572 573 574	180 Ø60 mm Petri dishes
XLD agar	C328	Complete dehydrated medium (Oxoid)	574 587 598	162 Ø90 mm Petri dishes
Bacillus cereus agar (MYP agar)	C205	For each batch: 40 g peptone bacteriological (Oxoid) 4 g meat extract (Biolife) 40 g sodium chloride (Carlo Erba) 40 g D-mannitol (Carlo Erba) 0.1 g red phenol (Sigma) 60 g agar bacteriological (Biolife) dissolved by heating in 4 l deionised water. After correcting the pH (7.2 ± 0.2), the base medium was autoclaved at 121°C ± 1°C for 15 min ± 1 min, cooled in a water bath at 50°C ± 2°C and supplemented with 40 ml of a10^6 IU/100 ml solution of polymyxin B sulphate (Sigma) and 400 ml egg emulsion (Biolife)	472 475 476	200 Ø90 mm Petri dishes
Hayflick agar	C252	For each batch: 70 g PPLO agar (Difco) 20 g tryptose (Oxoid) 2 g glucose (Riedel-de-Haën) dissolved by heating in 1 400 ml deionised water. After correcting the pH(7.8 ± 0.2), the base medium was autoclaved at 115°C ± 1°C for 30 min ± 1 min, cooled in a water bath at 50°C ± 2°C and supplemented with 280 ml of inactivated equine serum (IZSA&M) and 560 000 IU of penicillin G (Sigma)	438 446 455	108 Ø90 mm Petri dishes

Table I (continued)

Medium	Internal code	Base medium and procedure	Batches produced	Petri dishes/ test tubes
Hayflick broth	C253	For each batch: 21 g PPLO broth (Difco) 10 g tryptose (Oxoid) 1 g glucose (Riedel-de-Haën) 10 g yeast extract (Panreac) dissolved by heating in 700 ml of deionised water. After correcting the pH (7.8 ± 0.2), the base medium was sterilised by filtration with a 0.22 µm sterile (Millipore)filter and supplemented with 140 ml of inactivated equine serum (IZSA&M) and 280 000 IU penicillin G (Sigma)	439 447 456	140 test tubes each containing 5 ml
Thiaucourt broth	C323	For each batch: 21 g PPLO broth (Difco) was dissolved by heating in 600 ml of deionised water After correcting the pH (7.8 ± 0.2), the base medium was autoclaved at 121°C ± 1°C per 1 5 min ± 1 min, cooled in water bath at 50°C ± 2°C, supplemented with: 10 ml of a 1% (w/v) thallium acetate solution(Alpha Aesar Johnson Matthey) sterilised by filtration with an 0.22 µm sterile filter(Millipore), 10 ml of a 1% (w/v) ampicillin solution (Sigma sterilised by filtration with an 0.22 µm sterile filter(Millipore), 100 ml of a solution of 4% (w/v) sodium pyruvate (Baker) and 1% (w/v) glucose (Riedel-de-Haën) sterilised by filtration with an 0.22 µm sterile filter (Millipore), 200 ml inactivated equine serum (IZSA&M)and 100 ml of 25% (w/v) fresh yeast extract(IZSA&M) sterilised by filtration, after various filtration with decreasing diameters using a 0.22 µm sterile filter (Millipore)	437 445 457	200 test tubes each containing 5 ml
Thiaucourt agar	C324	For each batch: 70 g PPLO agar (Difco) was dissolved by heating in1 200 ml of deionised water After correcting the pH (7.8 ± 0.2), the base medium was autoclaved at 121°C ± 1°C for 15 min ± 1 min, cooled in a water bath at 50°C ± 2°C, supplemented with: 20 ml of a 1% (w/v)thallium acetate solution (Alpha Aesar Johnson Matthey) sterilised by filtration with an 0.22 µm sterile filter (Millipore), 20 ml of a 1% (w/v) ampicillin solution (Sigma) sterilised by filtration with a 0.22 µm sterile filter (Millipore) 200 ml of a solution of 4% (w/v) sodium pyruvate (Baker) and 1% (w/v) glucose (Riedel-de-Haën) sterilised by filtration with an 0.22 µm sterile filter (Millipore), 400 ml inactivated equine serum (IZSA&M) and 200 ml of 25% (w/v) fresh yeast extract (IZSA&M) sterilised by filtration, after various filtrations with decreasing diameters, using a 0.22 µm sterile filter (Millipore)	436 444 454	108 Ø60 mm Petri dishes

TTC 2,3,5-triphenyltetrazoliumchloride
XLD xylose lysine deoxycholate
MYP mannitol-egg yolk-polymyxin
PPLO pleuropneumonia-like organism

Table II
Control strains, method, incubation conditions and expected results of twelve 'critical' culture media

Medium	Control strains	Method	Incubation	Expected result
C157	*Campylobacter jejuni* ATCC 33291 *Escherichia coli* ATCC 25922	E	42°C x 48 h in microaerophilia	*C. jejuni:* growth ≥70% *E. coli*: growth 0%
C158	*Campylobacter jejuni* ATCC 33291 *Escherichia coli* ATCC 25922	E	42°C x 48 h in microaerophilia	*C. jejuni*: growth ≥70% *E. coli*: growth 0%
C205	*Bacillus cereus* 66/10	E	37°C x 24 h aerobiosis	*B. cereus*: growth ≥70%
C252	*Mycoplasma agalactiae* (PG2)	S	37°C x 48-72 h 10% CO_2	Growth
C253	*Mycoplasma agalactiae* (PG2)	S	37°C x 48-72 h 10% CO_2	Growth
C 273	*Pseudomonas aeruginosa* ATCC 27853	S	37°C x 24 h aerobiosis	Growth
C280	*Escherichia coli* ATCC 25922	S	37°C x 24 h aerobiosis	Growth
C281	*Streptococcus faecalis* ATCC 29212	S	37°C x 24 h aerobiosis	Growth
C283	*Streptococcus faecalis* ATCC 29212	S	36°C x 44 h aerobiosis	Growth
C323	*Mycoplasma agalactiae* (PG2)	S	37°C x 48-72 h 10% CO_2	Growth
C324	*Mycoplasma agalactiae* (PG2)	S	37°C x 48-72 h 10% CO_2	Growth
C328	*Salmonella* Enteritidis ATCC 31194 *Escherichia coli* ATCC 25922	E	37°C x 24 h aerobiosis	*S.* Enteritidis: growth ≥70% *E. coli*: growth 0%

E ecometric technique
S seeding

Acceptance criteria

Weight reduction

The weight reduction value over time of the samples from the batches of each culture medium had to be less than or equal to 5%.

pH

The pH value of the samples of each batch of each culture medium had to fall within the tolerance range typical of that medium.

Sterility

The samples of each batch of each culture medium had to be sterile after incubation at 21°C ± 1°C and 36°C ± 1°C for 48 h in conditions of aerobiosis.

Fertility

The samples of each batch of each culture medium had to be fertile after suitable incubation with the positive control strains. For the media in which the ecometric technique was applicable (C157,

C158, C205 and C328), the growth of the positive control strains, incubated under suitable conditions, had to be greater than or equal to 70% (D5, C5, B4 etc.), whereas there had to be an absence of growth of the negative control strains, when used (C157, C158 and C328), i.e. 0% (Table II).

Non-conformity with any of these parameters by a single sampling unit representing one of the three batches of each culture medium gave rise to an evaluation of non-conformity for the remaining sample of the medium.

Results

Weight reduction

Throughout the observation period, the plates of culture media C205, C252, C273, C280, C281, C283 and C324 never presented a mean percentage weight reduction exceeding 5% at any of the observation points (Fig. 1).

However, this value was exceeded for C157 between the 9th and 10th checks (–5.30% at T_{10}), for C158 between the 10th and 11th checks (–5.68% at T_{11}), and for C328 between the 11th and 12th observations (–6.73% at T_{12}) (Fig. 1).

pH

All the batches of the following culture media tested presented variations in pH value which always remained within the preset tolerance limits: C205, C252, C253, C280, C281, C283, C323 and C324.

However, the remaining culture media exceeded the established tolerance limits on the 12th check: in particular, at T_{12}, C157 presented a mean value for the three batches of pH 7.115 (lower tolerance limit: pH 7.2); at T_{12}, C158 presented a mean pH value of 7.105, i.e. 0.095 units above the lower tolerance limit; at the 12th check, C273 presented a mean pH of 6.587, i.e. below the lower tolerance limit

Table III
Fertility values (30 April-10 September 2003)
(values below the acceptable limit are shown in bold)

Batch	Fertility (expressed in % with ecometric technique)											
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	T_{11}	T_{12}
C157												
484	95	75	80	80	80	80	80	80	70	90	70	70
486	95	75	80	80	80	80	80	80	70	90	70	**40**
487	95	75	80	80	80	80	80	80	70	90	70	**45**
C158												
611	95	75	80	80	80	80	80	80	70	90	70	**40**
613	95	75	80	80	80	80	80	80	70	90	70	70
624	95	75	80	80	80	80	80	80	70	90	70	70
C205												
472	95	80	95	80	80	80	80	90	90	90	80	**40**
475	95	80	95	80	80	80	80	90	90	90	80	80
476	95	80	95	80	80	80	80	90	90	90	80	80
C328												
587	100	100	100	100	90	95	100	80	70	70	70	**45**
598	100	100	100	100	90	95	100	80	70	70	70	**45**
608	100	100	100	100	90	95	100	80	70	70	70	70

T_1 30 April
T_2 5 May
T_3 27 May
T_4 3 June
T_5 10 June
T_6 17 June
T_7 26 June
T_8 9 July
T_9 16 July
T_{10} 28 July
T_{11} 26 August
T_{12} 10 September

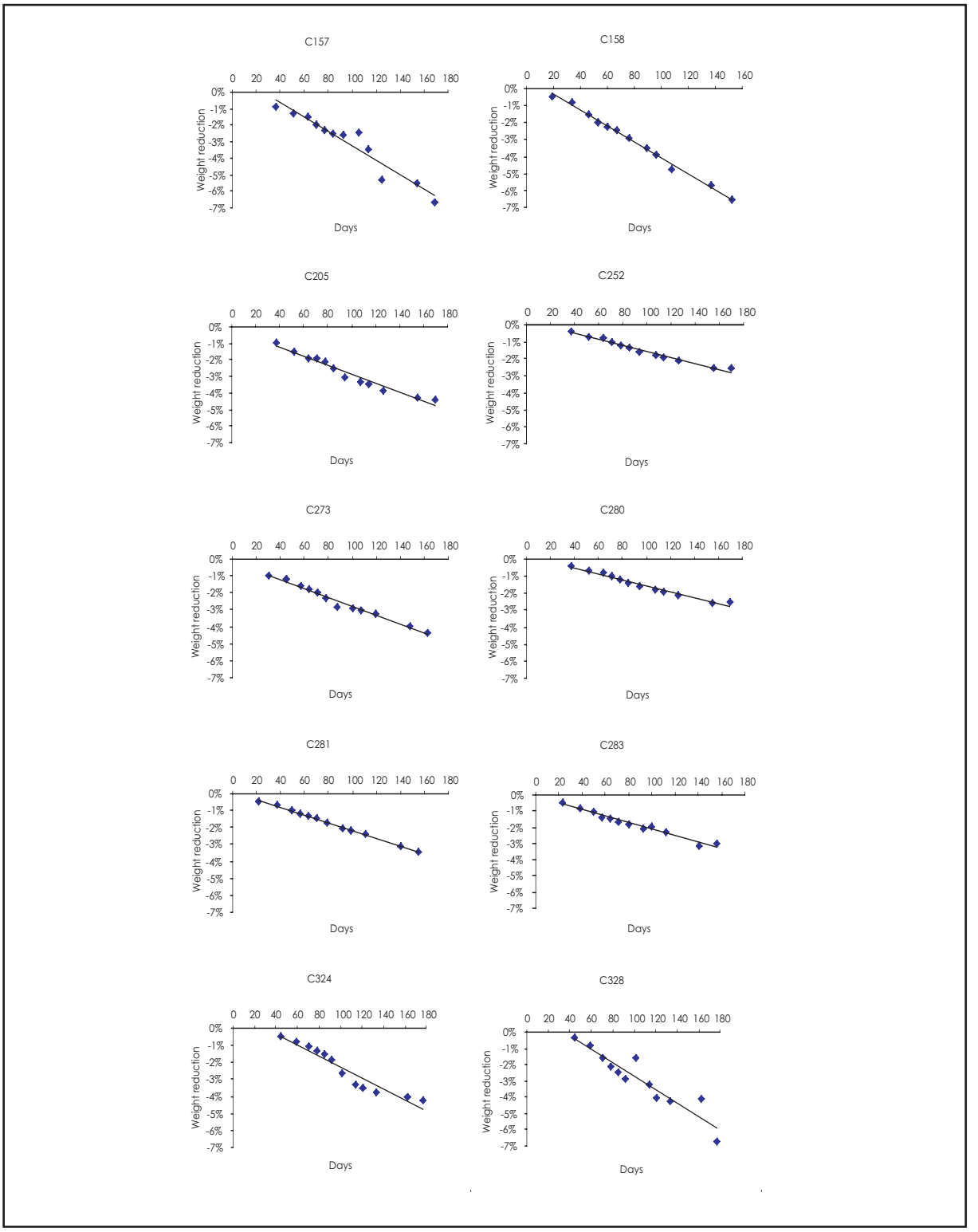

Figure 1
Weight reduction of media in Petri dishes

of 0.313 units; at T_{12}, C328 also presented a mean pH value below the lower tolerance limit, i.e. 0.072 units (Fig. 2).

Sterility

All batches of culture medium were sterile at both control points (21°C ± 1°C and 37°C ± 1°C) throughout the observation period.

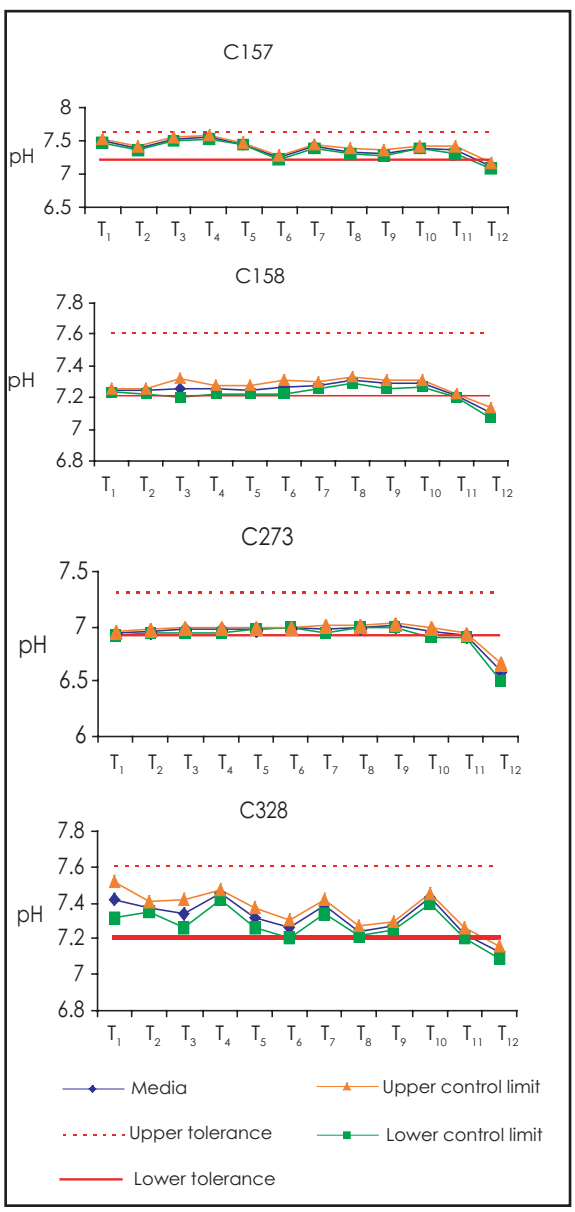

Figure 2
Media with pH trend falling outside the tolerance limits

Fertility

Batches C280, C281 and C283 were fertile throughout the observation period. In regard to the other culture media tested, however, one or two of the three batches examined at the 12th control proved infertile or presented a percentage fertility value (ecometric seeding) below the preset limit (Table III).

Discussion

An analysis of the parameters selected for the shelf-life check (weight reduction, pH, sterility and fertility) demonstrated periods of validity in excess of the coded and assigned shelf-life on the 9th, 10th, 11th and 12th checks for all twelve media, as shown in Table IV.

A considerable difference was observed between the currently coded shelf-life and that obtained experimentally.

Table IV
Comparisons of analytical shelf-life

Medium	Shelf-life (days)		
	Coded	Observed	Assigned
C157	3	113	90
C158	5	108	90
C205	2	155	120
C252	30	155	120
C253	30	162	120
C273	30	148	90
C280	10	148	90
C281	14	155	90
C283	14	156	90
C323	30	162	120
C324	30	162	120
C328	5	162	90

The number of days defined as the 'observed shelf-life', refers to the observation time at which all four of the selected parameters chosen fell within the established ranges.

The date of completion of the research (T_{12}) was established after demonstrating that the pH value was beyond the tolerance limits for some media, or that one or more of the three batches examined was infertile or presented a percentage fertility value below the preset limit.

On the basis of the shelf-life limits observed for each culture medium, it was considered appropriate to assign a new shelf-life below the said limits (Table IV) that corresponded to at least two other favourable checks for each batch which, on the basis of a negative cumulative binomial distribution, corresponds to over 95% probability of detecting the presence of at least one non-conforming sample for a prevalence of 40% (Fig. 3).

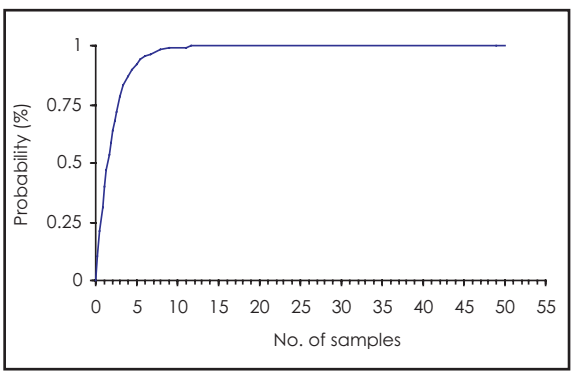

Figure 3
Cumulative curve of the probability of detecting a non-conforming sample in a population with a prevalence of 40%

The choice of the new dates is appropriate to meet the operational requirements of a complex organisational structure and to guarantee the maintenance of the basic chemico-physical characteristics essential for the correct growth and characterisation of a specific microorganism. Moreover, this reduction in time between the observed shelf-life and the assigned shelf-life represents an additional safety factor for the purpose of ensuring the efficiency of the medium, against the errors that can occur during storage and/or in the preservation of the medium.

Official American Type Culture Collection (ATCC) control strains were used for the fertility tests performed on the media used in the survey.

It would be of value to conduct further studies to compare the percentages of recovery (growth) of the ATCC strains most commonly used, which have now adapted to growth on culture media, with those of the corresponding wild strains.

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