# SUMMARY OF LA LETTRE DE CECALAIT, N° 40 (1ST quarter 2002)

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## Evaluation of the Bactocount IBC

*(based upon the CECALAIT phase I evaluation report)*

Bactocount IBC is an automatic analyser for the determination of bacteria countS in raw milk, developed by BENTLEY Instruments (USA) and marketed in France by its french subsidiary, Bentley Instruments sarl. It works using the flow cytometry principle, with an epifluorescence microscopy detection, after chemical, heat and ultrasonic treatment. CECALAIT has recently evaluated its analytical characteristics (phase I assay)

### APPARATUS

It is run by a micro-computer for analyses and calibration. The sample is automatically taken and transferred into a well of the incubation wheel (with an incubation reagent). The reagent consists of a fluorochrome alcaline buffered solution, proteolytic enzyme and reaction catalyst, in order to solubilize and disperse protein, fat and somatic cells and to stain bacterial nucleic acids. The mixture is then incubated 8 mn at 50°C and part of it is injected into a hydrodynamically focused fluid stream. The bacterial cells flow one by one through a laser beam, which excites the stained cells, detected by an epifluorescence microscope objective. The fluorescent impulses of stained bacteria are discriminated, amplified by a photomultiplier, counted and converted into Individual Bacterial Cell (IBC)/ ml. A laboratory performed calibration allows the conversion of IBC / ml into CFU / ml.

### EXPERIMENTAL

Using non-heated samples, the following characteristics were evaluated :

- stability
- carry-over effect
- linearity
- determination of the detection limit
- repeatability
- accuracy
- influence of milk composition

The evaluation was performed according to IDF standards 100B, 128A, 135B, 161A and to AFNOR NF V 03-110 standard.

#### ① STABILITY

The stability was evaluated by the duplicate automatic analysis of milk sets, every 15 mn for half a day (about 20 measurement cycles), according to the actual working conditions of a milk payment laboratory.

Repeatability and reproducibility calculations were performed according to IDF 135 B.

The results of the test, performed as usual with 3 counting levels and 2 different methods of milk sample preservation showed a stability defect of the analyser, exclusively at the lower counting levels. BENTLEY Ins attributed it to the initially selected counting threshold. Therefore, they modified this setting for the further tests. Two additionnal tests were performed then :

- pre-test using two herd milk samples : a rich one and a poor one, stored between 0 and 2 °C
- full test using a set of 6 duplicate milk samples, ie a poor one, a medium one and a rich one, stored between 0 and 2°C, with and without addition of the azidiol preservative (0.33%).

The new results showed a relative geometric standard deviation of reproducibility ($GRSD_R$) from 6 to about 15 %. It seems dependent on the contamination level, but independent of the method of preservation. However, at the lower contamination levels (poor milk samples), these values (about 6%) are close to the observed repeatability values (mean standard deviation of repeatability Sr=0.023 log). The mean standard deviation of reproducibility ($S_R$) is 0.043.

#### ② CARRY-OVER

The carry-over effect was evaluated by the duplicate automatic analysis of two milk sets (rich and poor), 20 times, in the following sequence : RICH MILK – RICH MILK – POOR MILK - POOR MILK.

The test was performed using 3 different levels, with individual milks or reconstituted milks (by a mixture of microfiltration retentate and filtrate). The instrument settings had been performed by BENTLEY Ins (contamination coefficient set at 0).

The carry-over effect (Tc %) was estimated with following equation :

$$Tc\% = \left[ \frac{\sum (POOR1) - \sum (POOR2)}{\sum (RICH2) - \sum (POOR2)} \right] \times 100$$

Under these conditions, the carry-over of the Bactocount IBC was around 0.0 to 0.05 % whatever the average contamination level of the sample. These values comply with the maximum limit of 1% usually allowed in routine methods for determination of milk composition, used for milk payment purposes. In the same context, this limit may also be used for bacterial enumeration.

### ③ EVALUATION OF LINEARITY

Linearity was evaluated by triplicate automatic analysis, following increasing, then decreasing levels, of a series of milks with bacterial contamination evenly distributed over the whole area of counting.

Two types of milk matrices were used :

- Milk enriched by low temperature (4 à 8 °C) incubation for 24 to 72 hours.
- recombined milk made with a mixture of microfiltration retentate, microfiltration filtrate and cream.

Whatever matrix (natural or recombined milk), the results showed a minor linearity defect of the instrument on the whole range tested. The manufacturer should apply a 3-order polynomial to the bulk signal to correct this defect. (mean linear Sy,x : 0.031 log ; 3-order polynomial Syx : 0.016 log). Having thus obtained a satisfactory linearity on the whole counting area, the users could then calibrate the instrument using a simple linear equation.

### ④ LIMIT OF DETECTION

The detection limit of a method is the lowest value for which the user of the method can be sure that it is different from 0.

Whatever the mathematical calculation model used in order to determine the lower limit of detection, the instrumental signal was significantly different from the blank signal at a counting level as low as $0.33.10^3$ CFU / ml.

However, the instrument's detection threshold corresponds to the precision required for routine purposes.

### ⑤ EVALUATION OF REPEATABILITY

The repeatability was evaluated by automatic analysis of :

- 822 (820 finally kept) herd milk samples, analysed according to IDF standard 128 (the sample racks are analysed twice in rapid succession)
- 410 (409 finally kept) samples selected among the 822 samples taken for evaluation of the accuracy, analysed in duplicate in rapid succession (repeatability setting of the instrument).

The results were first obtained in $10^3$ IBC / ml (there is a precalibration of the instrument to convert IBC values into CFU equivalent values : slope : 0.5). They were then transformed into Log $CFU_{Bactocount\ IBC}$ / ml and later into Log CFU / ml equivalent values using the calibration equations obtained in the course of the accuracy evaluation.

Indeed, two calibration equations had been defined according to the instrument's settings. The first tests had been performed using the initially selected counting threshold, which was later modified after the non-satisfactory first stability tests. This initial threshold, called threshold 1, corresponded to following settings : WTH = 29.3 ; HTH = 0.36*.

However, all values obtained during CECALAIT's tests were calculated again later by BENTLEY Ins, using a new threshold. This new setting had been defined in order to improve the instrument's precision when it was used in the LIAL Franche-Comté laboratory, which was one of the laboratories that performed the "phase II" evaluation. This new threshold, called threshold 2, corresponded to following settings : WTH = 0.0 ; HTH = 0.40

So, the calibration equations used are :

- with Threshold 1 : Y = 0.8291 x APP + 0.5406
- with Threshold 2 : Y = 1.0463 x APP - 0.624

where Y is the Log CFU/ml value, given by the reference method and X the $CFU_{Bactocount\ IBC}$ given by the instrument

** WTH : corresponding to the width of the peak*
*HTH : corresponding to the height of the peak.*

However,there was not enough time left to perform all the tests again. So in repeatability tests, only the tests in duplicate in rapid succession were repeated.

Tables 1 to 3 show the results. The levels correspond to french milk payment levels. See keys for tables 1 to 3 in La Lettre de CECALAIT, pages 2-3

The three tables show that the standard deviation of repeatability Sr is about 0.0445 log, *ie* the relative geometric standard deviation of repeatability is 10.8%. This value remains far below the limit recommended for milk payment purposes for bacterial counting (Sr = 0.15 log).

### ⑥ EVALUATION OF ACCURACY

Accuracy was estimated by using the residual standard deviation (Sy,x), where Y is the value given by the reference method (Log CFU / ml) and X the "CFU equivalent" given by Bactocount IBC (Log $CFU_{Bactocount\ IBC}$/ ml).

The samples giving plates with too high a bacterial count, or with contaminating or spreading colonies, were discarded.

## ↳ Procedure

In order to obtain a representative population, with evenly distributed bacterial contamination, 410 herd milks were selected according to the results of a duplicate analysis (IDF 128) using the Bactocount IBC. They were selected among the 822 samples taken (on 9 different days, from march to june 2001) by two different interprofessional laboratories (LIAL FC -Laboratoire Interprofessionnel d'Analyses Laitières de Franche-Comté- and LDA 39 -Laboratoire Départemental d'Analyses du Jura-.

Milks were kept at 0-2 °C for 2 to 4 hours prior to analysis. Instrumental analyses were performed in consecutive duplicate, followed by a duplicate analysis using the reference method (IDF 100 B).

The analyses were performed on 9 non-consecutive days over a 4 month period, where the instrument's setting was kept unchanged. Each analitycal series came from the same bulk-milk tank (24 to 48 h of tank storage), taken in duplicate at the farm and transported following the usual way for milk payment samples.

However, three samples with abnormally high repeatability values were not included in the statistical treatment for accuracy.

### ↳ Results

As for the evaluation of repeatability, the tests had been performed using threshold 1. But, in the same way, the values were also recalculated using threshold 2 (see ⑤).

The results shown here were obtained using the best setting, ie threshold 2.

A simple linear regression was applied. It was calculated (on log transformed values) on the population of 371 milk samples (mean bacterial level : 41000 CFU/ml) and gave the following relation above 2000 (lower detection limit claimed by the manufacturer):

Threshold 2 (WTH = 0.0 ; HTH = 0.40)

**Log (Reference) = 1.0463 x Log ($CFU_{Bactocount\ IBC}$ / ml) - 0.6240**

with mean bias to the reference values = + 0.392 & Sy,x = 0.388

The estimation of precision obtained was :

**± 1.96 x 0.388 ie ± 0.760 Log CFU / ml**

That means that the upper and lower limits of the 0.95 confidence interval for Y are

**Log Y + 0.760 and Log Y - 0.760**

However, considering only the range 10 000 to 1 000 000 CFU/ml, the residual standard deviation became : 0.335 Log. The estimation of precision then became :

**± 1.96 x 0.335 soit ± 0.657 Log CFU / ml**

That means that the upper and lower limits of the 0.95 confidence interval for Y are

**Log Y + 0.657 and Log Y - 0.657**

The intrument's precision improved when the lower level samples were discarded (below 10000 CFU / ml). This may be related to the observed linearity defect (cf ③), affecting the lower values more.

These performances were obtained by using a simple linear regression for studying the accuracy. Considering the observed minor linearity defect, they could certainly be improved, provided that the bulk instrument signal is linear over the whole measuring range.

#### ⑦ EVALUATION OF THE INFLUENCE OF MILK COMPOSITION

Furher tests were conducted in order to study a possible influence of the composition of the milk samples on the accuracy performance. There was no significant reduction of the residual variance. The bacterial enumeration performed by Bactocount IBC thus seems insensitive to the fat, protein or somatic cell content of milks.

## CONCLUSION

The Bactocount IBC -at the time, a prototype, without temperature stabilization -, was evaluated (phase I) at CECALAIT, seeking an agreement for milk payment purposes. Stability, carry-over and repeatability have been found satisfactory.

Concerning linearity and accuracy, which are closely related, it was concluded that the linearity had to be adjusted in order to have a better idea of the accuracy performance of the instrument.

At the end of this phase, the manufacturer commited itself to setting the necessary changes in the instrument, especially regarding linearity.

That had to be done. Indeed, the instrument, since then, was used under routine conditions, in two interprofessionnal laboratories (LIAL FC and LDA39) for four months, for the assays corresponding to the phase II of the evaluation procedure for the agreement for milk payment purposes. This study in the two labs gave the following repeatabiliy and accuracy results :

➢ **REPEATABILITY (ALL LEVELS)**

see table page 4 of la Lettre de CECALAIT

➢ **ACCURACY** (simple determination by Bactocount)

↳ LDA 39

(426 samples, mean bacterial level 49000 CFU/ml)

**Log (Reference) = 0.7309 x Log (IBC/ ml) + 1.18**

with Sy,x = 0.283 log

The estimation of precision is then :

**± 1.96 x 0.283 ie ± 0.5547 Log UFC / ml**

↳ LIAL FC

(498 samples, mean bacterial level 20000 UFC/ml)

**Log (Reference) = 0.6422 x Log (IBC/ ml) + 1.405**

with Sy,x = 0.309 log

The estimation of precision is then :

**± 1.96 x 0.309 ie ± 0.605 Log UFC / ml**

However, considering only levels higher than 10 000 CFU/ml, the results become :

**Log (Reference) = 0.4867 x Log (IBC/ ml) + 2.286**

with Sy,x = 0.264 log

The estimation of precision is then :

**± 1.96 x 0.264 ie ± 0.5174 Log UFC / ml**

The new tests, performed during the phase II of the evaluation of the instrument, finally validated the manufacturer's changes, especially regarding linearity and accuracy. Therefore, the instrument's calibration can be performed by a simple linear regression. Moreover, accuracy appeared better in phase II than in phase I.

Considering the whole set of results, the CST (Commission Scientifique et Technique) concluded that the analytical performances of the instrument comply with the requirements of milk payment purposes. Bactocount IBC of Bentley Instruments can now officially be used for milk payment purposes (see French Official Journal, 3rd January 2002).

The list of abbreviations and bibliographic references are in « La Lettre de CECALAIT »