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NF VALIDATION
Validation of alternative analytical methods
Application in food microbiology

Summary report

EN ISO validation study of the Listeria Precis™ method for the enumeration of *Listeria monocytogenes* in food products and environmental samples

Quantitative method

This report includes 38 pages, with 4 appendixes.
Only copies including the totality of this report are authorised.

Version 1
November 19, 2014

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which must be returned to ADRIA Développement or destroyed internally.

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The modifications are highlighted.

Quality assurance documents related to this study can be consulted upon request from Oxoid, part of Thermo Fisher Scientific.

The technical protocol and the result interpretation were realised according to the EN ISO 16140 and the AFNOR technical rules.

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○ **Expert Laboratory**:	**ADRIA Développement** ZA Creac'h Gwen F-29196 QUIMPER Cedex
○ **Studied method**:	**Listeria Precis™ method for enumeration of *Listeria monocytogenes***
○ **Validation standard**:	NF EN ISO 16140 (October 2003): Food microbiology – Protocol for the validation of alternative methods
○ **Standard method**:	ISO 11290-2 (1999) & ISO 11290-2/A1 (2004): Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 2: enumeration of *Listeria monocytogenes* in foods
○ **Scope**:	**All human food products and environmental samples**
○ **Certification organism:**	AFNOR Certification

1 INTRODUCTION

1.1 Dates of the validation studies

The Listeria Precis™ method for enumeration of *Listeria monocytogenes* was validated on September 15, 2006 (certificate number UNI 03/05 – 09/06) for food products and environmental samples.

In 2007, an extension study was obtained for a new confirmation test: OBIS MONO.

The alternative method was renewed in 2010 and 2014.

1.2 Protocol and principle of the alternative method

The Listeria Precis™ method is based on a specific chromogenic media for *Listeria monocytogenes* enumeration.

The protocol is described in **Appendix 1**.

1.3 Standard method ◆

The reference method is the ISO 11290-2 (1998) & ISO 11290-2/A1 (2004): Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 2: enumeration of *Listeria monocytogenes* in foods (See **Appendix 2**).

◆ Analysis performed according to the COFRAC accreditation

2 VALIDATION STUDY RESULTS

2.1 Initial validation (realised by ASEPT, 2006)

2.1.1 Method Comparison Study

2.1.1.1 Linearity

Linearity is the ability of the method when used with a given matrix to give results that are in proportion to the amount of analyte present in the sample, that is an increase in analyte corresponds to a linear or proportional increase in results.

- ***Food matrices and protocols***

Six matrix/strain pairs were analysed, with five contamination levels and two replicates. At least, 50 analyses were performed by the alternative and the standard methods.

The contamination levels, the samples and the inoculated strains are presented in the table below:

Table 1

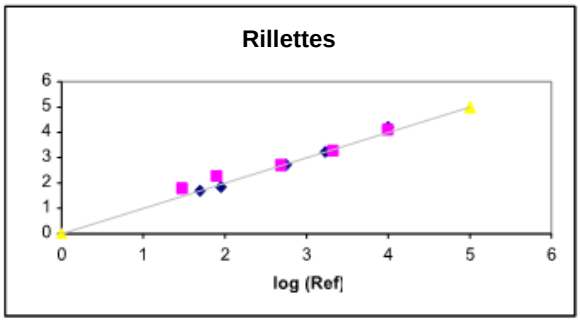
Samples	Strains	Contamination levels (CFU/g)
Rillettes	*Listeria monocytogenes* 4e	10 – 50 50 – 100 100 – 500 500 – 1 000 1 000 – 10 000
Raw milk	*Listeria monocytogenes* 1/2a	
Lettuce	*Listeria monocytogenes* 1/2a	
Smoked salmon	*Listeria monocytogenes* 1/2b	
Raw egg product	*Listeria monocytogenes* 1/2b	
Process water	*Listeria monocytogenes* 1/2a	

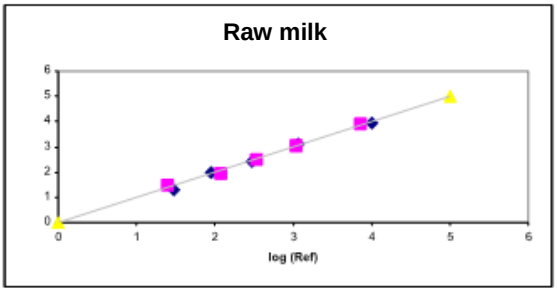
The samples were individually inoculated and were analysed in duplicate by the alternative and the reference methods.

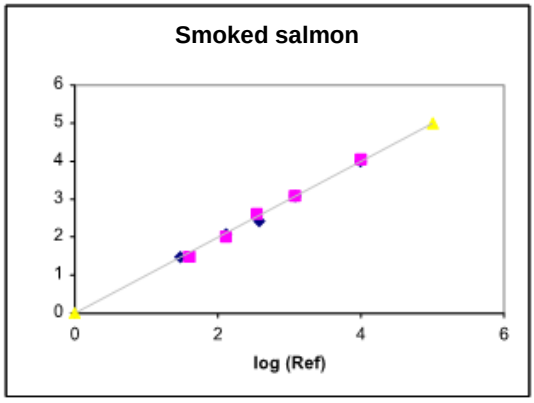
- ***Results***

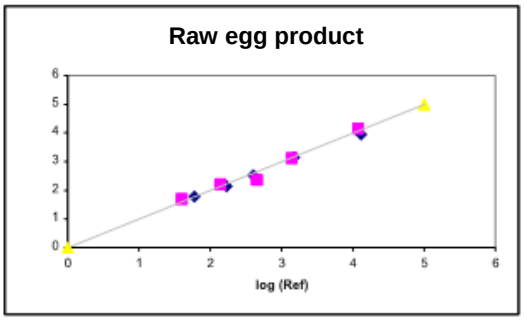
The bi-dimensional graphs are shown figure 1.

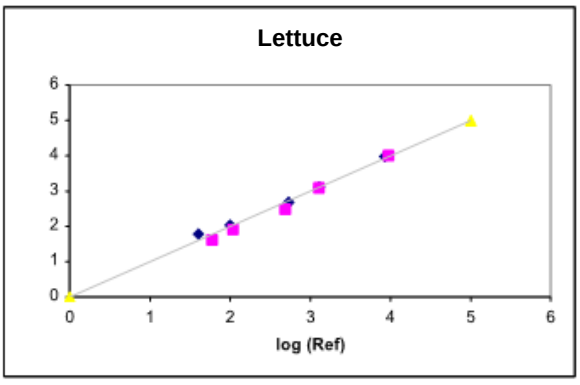
Figure 1 – Bi-dimensional graphs

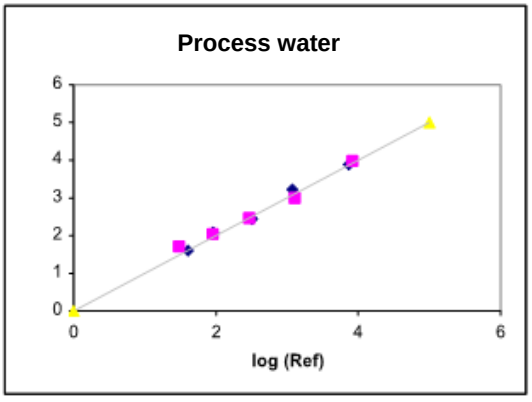












Statistical interpretations

Statistical interpretation results are provided in Table 2.

Table 2 – Statistical interpretations

Matrix	R	Selected regression	Rob.F	Critical value	P%	Correlation coefficient	Regression equation*
Rillettes	1.80	GMFR	6.09	5.41	4	0.9960	log (Alt) = 0.9887 log (Ref) + 0.1141
Lettuce	1.08	GMFR	0.00	5.41	100	0.9992	Log (Alt) = 1.0081 log (Ref) - 0.0257
Raw milk	1.02	GMFR	0.00	5.41	100	0.9997	Log (Alt) = 1.0188 log (Ref) - 0.0737
Smoked salmon	10.48	OLS1	1.806	5.41	26.3	0.9984	Log (Alt) = 1.0406 log (Ref) - 0.1418
Raw egg product	1.30	GMFR	5.48	5.41	4.9	0.9958	Log (Alt) = 0.9775 log (Ref) - 0.0161
Process water	1.84	GMFR	0.079	5.41	96.9	0.9985	Log (Alt) = 0.9680 log (Ref) + 0.1317

GMFR = orthogonal linear regression

OLS1 = ordinary least-square linear regression

x – axis = reference method

y – axis = alternative method

OLS1 = ordinary least-square linear regression

x axis = alternative method

y axis = reference method

Statistical Interpretation:

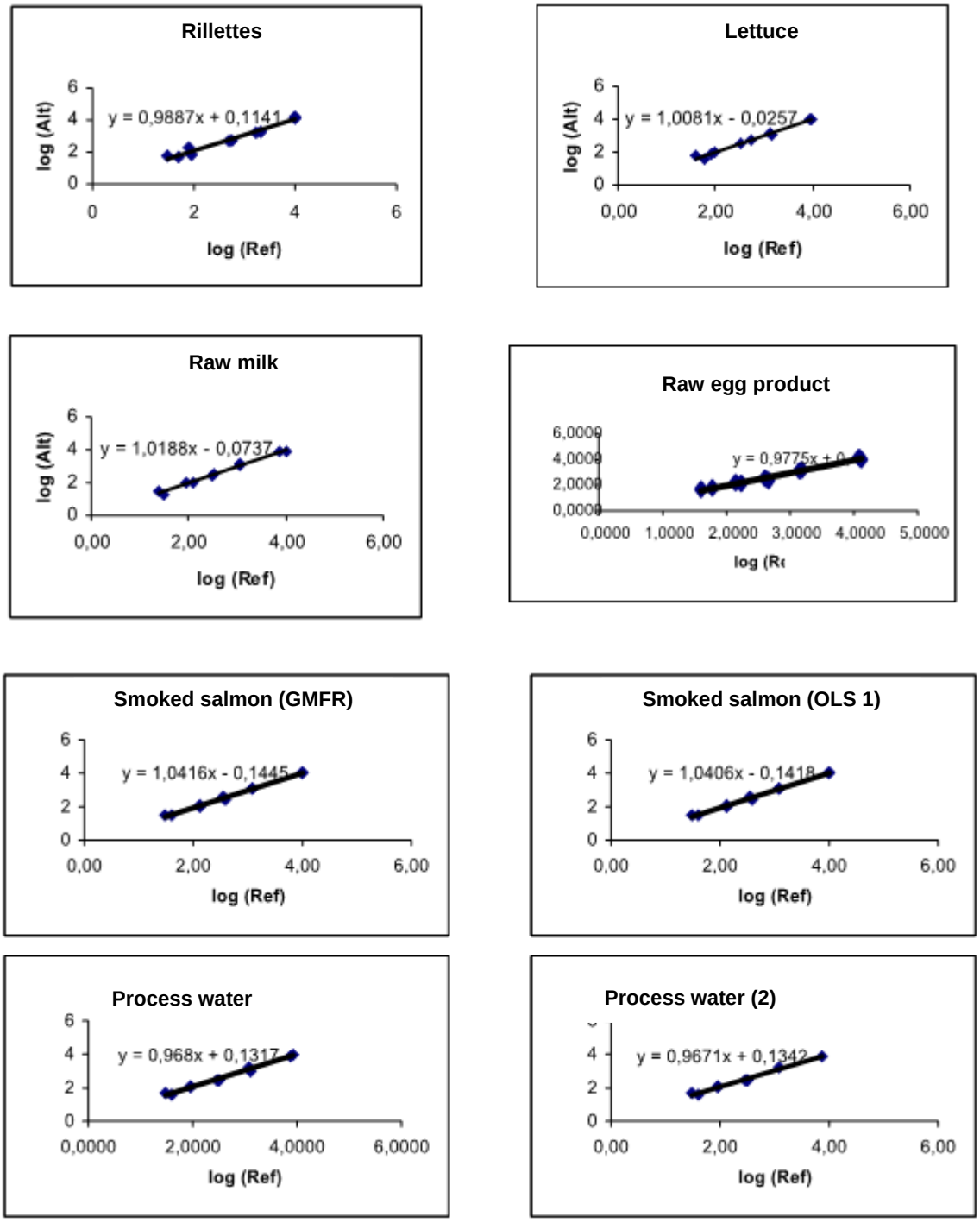
P > 5 %: not significant

1 % < P < 5 %: significant

P < 1 %: highly significant

The regressions straight lines are shown figure 2.

Figure 2 – Regression straight lines



Discussion

The linearity is accepted in all cases, with P values ≥ 5 % and correlation coefficients superior to 0.99.

For the matrix “Rillettes“, the linearity test is significant (P % = 4) with a high correlation coefficient value (0.996).

The Listeria Precis™ method shows satisfying linearity.

2.1.1.2 Relative accuracy

The accuracy is the closeness of agreement between a test result and the accepted reference value.
The bias is the difference between the expectation of the test results and an accepted reference value.

Number and nature of the samples

The repartition per category and types is provided in table 3.

Table 3 – Number and nature of the samples

Categories	Types	Number of samples	
		Analysed	Exploited (1)
Meat products	Raw meat products	6	4
	Ready to cook products	4	2
	Ready to eat delicatessen	10	4
	Total	*20*	*10*
Dairy products	Raw milk cheeses	7	4
	Raw milks	4	3
	Other dairy products	5	5
	Total	*16*	*12*
Fishery products	Raw fishes	5	4
	Processed fishes	7	2
	Ready-to-eat products	6	4
	Total	*18*	*10*
Vegetables	Raw vegetables	5	4
	Frozen vegetables	3	3
	Lettuce, raw vegetables	5	5
	Total	*13*	*12*
Egg products	Raw egg	5	5
	Pastries	3	3
	Pasteurised egg products	4	3
	Total	*12*	*11*
Environmental samples	Wipes, swabs	8	4
	Process water	3	3
	Dusts	3	3
	Total	*14*	*10*
TOTAL		**93**	**65**

(1) Some data cannot be exploited because enumeration was under the detection level for at least one of the method.

Artificial contamination of the samples

Artificial contaminations were realised by spiking after performing injury protocols on pure cultures, or by cross-contaminations.

56 samples were artificially contaminated; 53 gave exploitable results among a total of 65 samples. 81.50 % of the samples were artificially contaminated. The injury protocol and the strains inoculated are provided in Table 4.

Table 4

Stress N°	Injury protocol	Strain	Origin
A	45 min at 50 °C	48 LM -H123 LM-H170	Smoked salmon Lettuce Lettuce
B	30 min at 50 °C and 2 h at –20°C	18 43 LM-O1	Raw milk (environment) Environment Egg product
C	30 min at 55°C	18 48 LM-H123	Raw milk Smoked salmon Lettuce
D	30 min at 50°C et 2 h at –80°C	34 LM-H171 LM-O1	Rillettes Lettuce Egg product
E	30 min at 60 °C	LM-O1	Egg product

Confirmatory tests

For the reference and the alternative methods, confirmations were realised by the tests described in the ISO 12290-2/A1 standard. For the alternative method, only one colony was confirmed while 5 colonies were tested for the reference method.

Results

Samples were analysed in duplicate by the reference and the alternative methods.

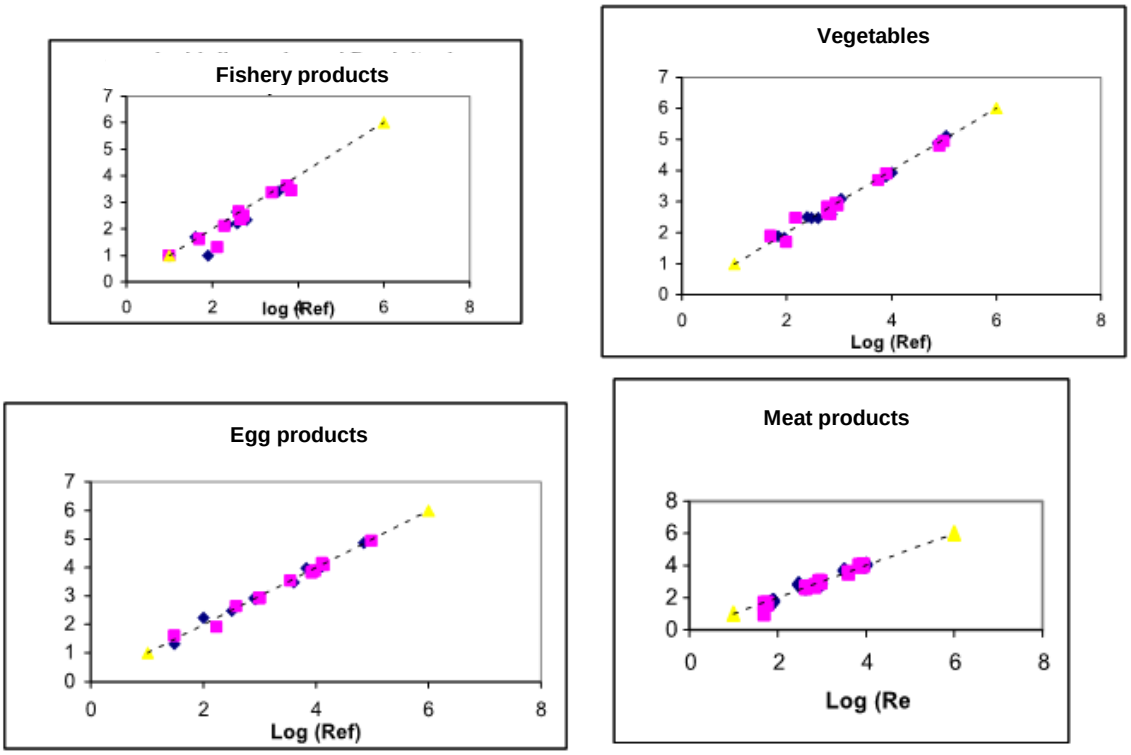
The contamination range is provided in table 5 per tested category.

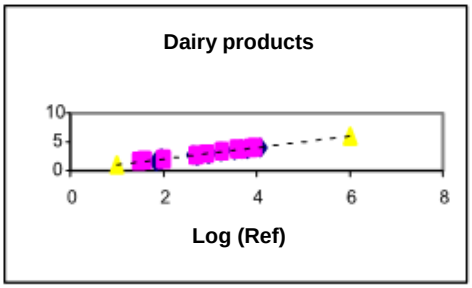
Table 5 – Contamination range

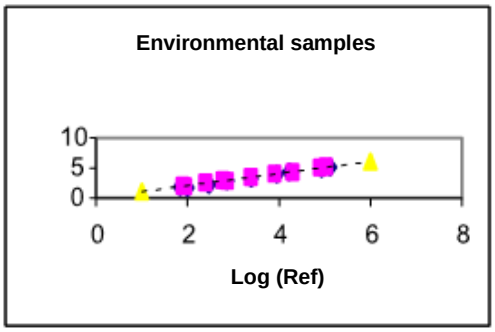
Food category	Contamination level (log CFU/g)
Meat products	1.00 – 4.04
Dairy products	1.48 – 4.08
Fishery products	1.00 – 3.84
Vegetables	1.70 – 5.11
Egg products	1.30 – 4.99
Environmental samples	1.70 – 5.11
All categories	1.00 – 5.11

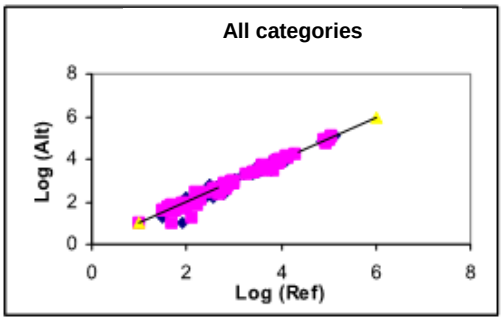
Bi-dimensional graphs are given figure 3.

Figure 3 – Bi-dimensional graphs









Statistical interpretation according to EN ISO16140 method

The results of the statistical interpretation are given in Table 6.

Table 6 – Statistical interpretation results

Category	n	R	Regression used	a	t(a)	b	t(b)	Critical T	P%	
									Ordinate at 0	Slope at 1
Meat products	10	1.58	GMFR	-0.455	3.321	1.15	3.123	2.306	0.01	0.01
Dairy products	12	0.65	GMFR	-0.027	0.385	1.002	0.108	2.228	0.92	0.71
Fishery products	10	1.30	GMFR	-0.305	1.024	1.034	0.312	2.306	0.76	0.34
Vegetables	12	0.86	GMFR	-0.015	0.128	0.993	0.191	2.228	0.85	0.90
Egg products	11	1.58	GMFR	-0.038	0.866	1.001	0.090	2.262	0.93	0.41
Environmental samples	10	1.76	GMFR	-0.139	2.65	1.034	2.31	2.306	0.05	0.03
All products	65	1.18	GMFR	-0.182	3.042	1.040	2.145	2.000	0.04	0.00
All products (estimated number)	65	1.18	GMFR	-0.182	3.042	1.040	2.145	2.000	0.04	0.00
All products (non estimated results)	20	1.15	GMFR	-0.367	1.51	1.138	1.141	2.101	0.27	0.15

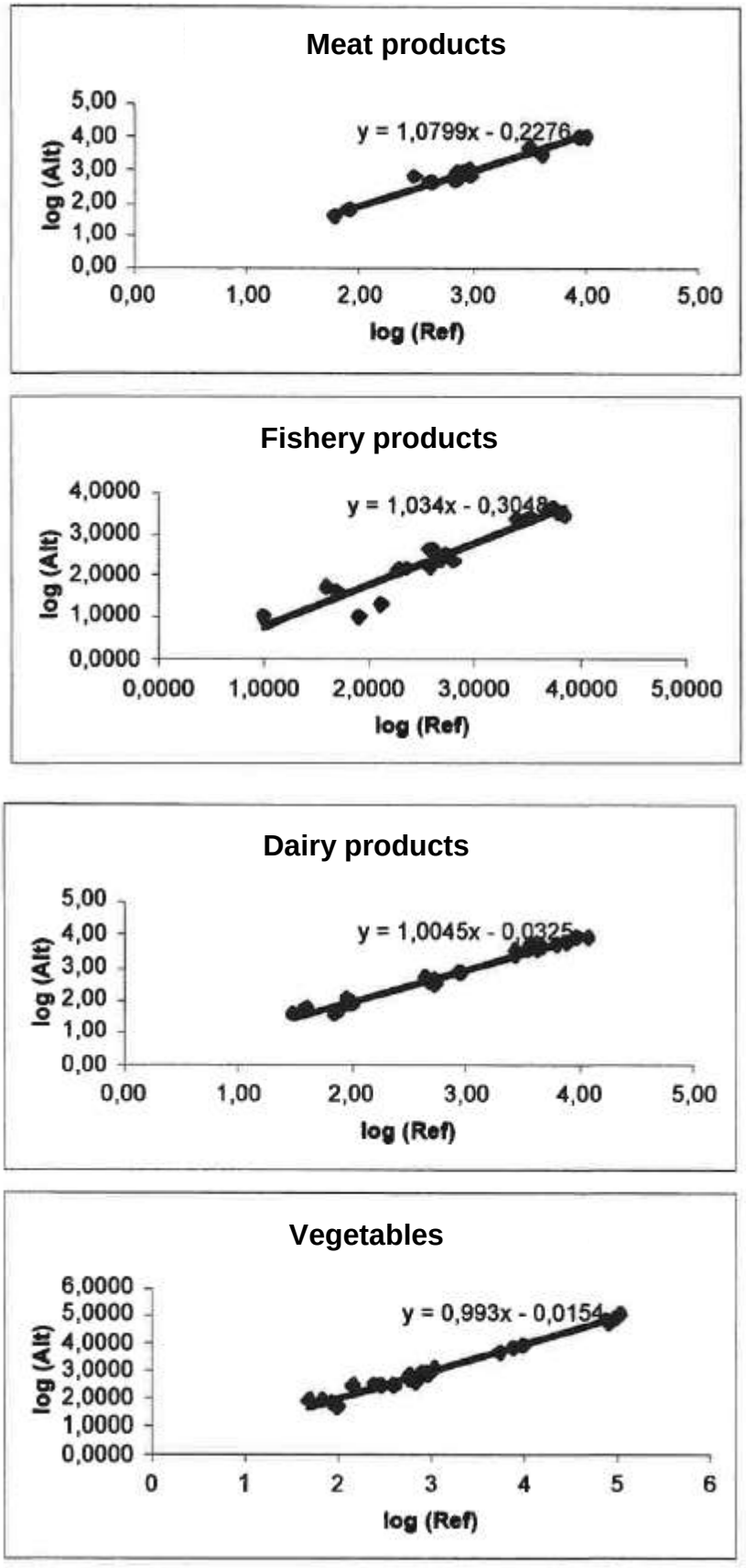
Repeatability limits obtained for the alternative and reference methods as well as bias obtained are presented in Table 7.

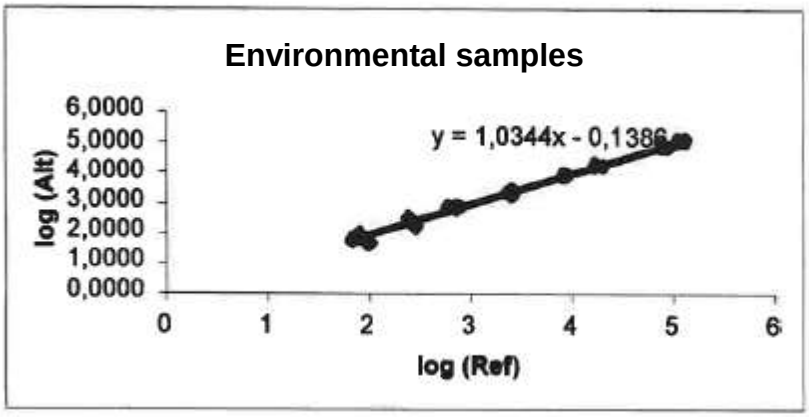
Table 7

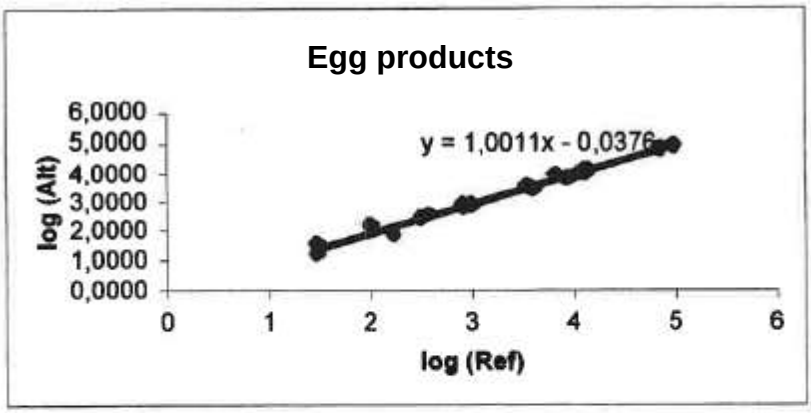
Categories	Bias	Repeatability limits	
		Rob. r	
		Ref.	Alt.
Meat products	0.008	0.314	0.343
Dairy products	-0.031	0.136	0.130
Fishery products	-0.133	0.220	0.214
Vegetables	-0.041	0.180	0.319
Egg products	-0.026	0.219	0.206
Environmental samples	-0.007	0.198	0.119
All products	**-0.032**	**0.196**	**0.215**

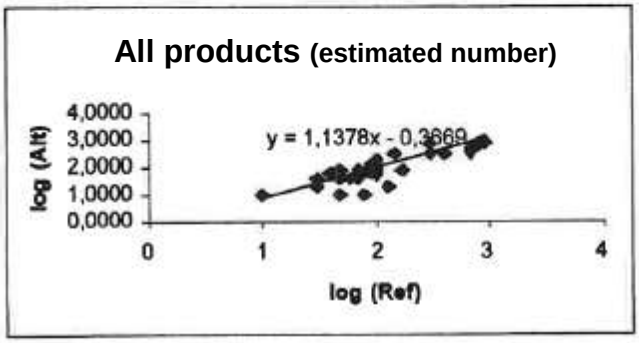
Regression straight lines (graph and equation representations) for each food category and for all products are presented figure 4.

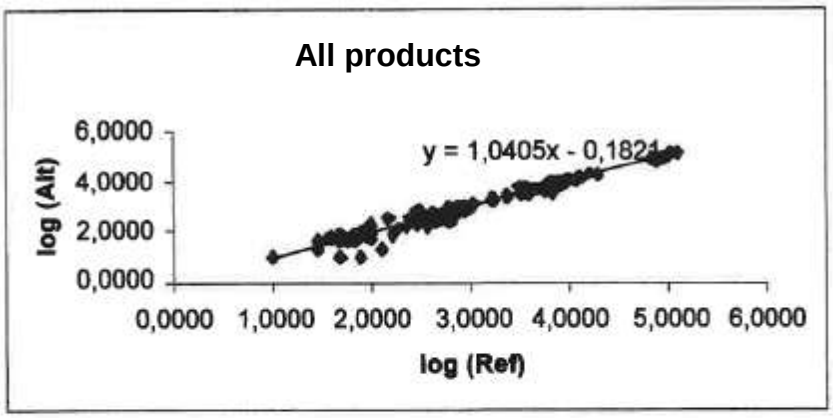
Figure 4 – Regression straight lines

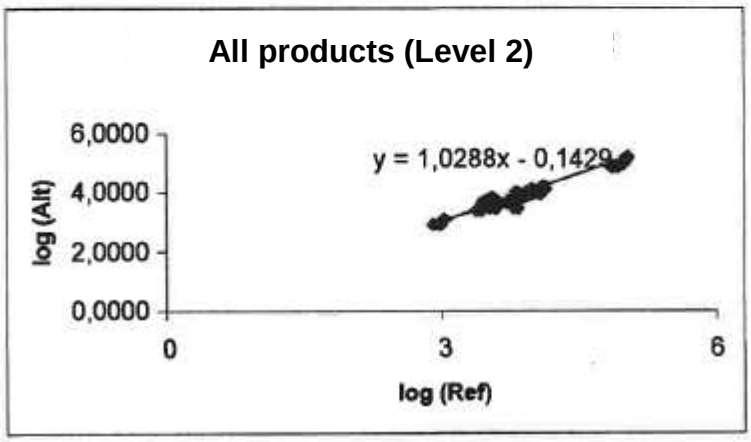



Environmental samples
y = 1,0344x - 0,1386
log (Alt)
log (Ref)
6,0000
5,0000
4,0000
3,0000
2,0000
1,0000
0,0000
0
1
2
3
4
5
6


Egg products
y = 1,0011x - 0,0376
log (Alt)
log (Ref)
6,0000
5,0000
4,0000
3,0000
2,0000
1,0000
0,0000
0
1
2
3
4
5
6


All products (estimated number)
y = 1,1378x - 0,3669
log (Alt)
log (Ref)
4,0000
3,0000
2,0000
1,0000
0,0000
0
1
2
3
4





The hypotheses that the ordinates are close to 0 and the slopes close to 1 are approved in all cases, except for:

- Meat products (slope P % = 1; ordinate P % = 1);
- Environmental samples (ordinate P % = 3);
- All products (ordinate P % = 0).

The bias between the two methods vary from – 0.133 to 0.008 log CFU. For all categories, the bias is – 0.032 log CFU. The repeatability limits of the alternative and the reference method are similar. For all products, the values are respectively: 0.215 and 0.196 log CFU. For all products, these interpretations are provided taking into account data with low contamination level or not (estimated colonies number).

For all food products, the regression straight line is the following:

log (Alt) = 1.040 log Ref. – 0.182

The Listeria Precis™ method shows satisfying relative accuracy.

2.1.1.3 Detection limit (LOD) and quantification limit (LOQ)

The critical level is the defined as the smallest amount which can be detected (not null), but not quantified as an exact value. Below this value, it cannot be sure that the true value is not null.

The detection limit is defined as being higher than the critical level because it involves a power, the probability 1-β, which has to be well over 50 %, for example 95 %.

The quantification limit is defined as the smallest amount of analyte (that is the lowest actual number of organisms) which can be measured and quantified with defined precision and accuracy under the experimental conditions by the method under validation.

- ***Protocol***

The detection limit of the alternative method was realised with pure cultures. Three different inoculation levels were tested, with six replicates per level, i.e. 18 analyses were done by the alternative method.

Listeria monocytogenes 18 was used for the detection limit of the alternative method.

Quantification limit was calculated for six independent blank samples determinations.

- ***Results***

These data are intrinsic to the method used and are presented in the following tables:

Table 8 – Inoculation 1 ml on 3 plates

Level (CFU/ml)	Positive samples number	Standard deviation	Bias
0	0/6	/	/
0.5	2/6	1.211	0
1	3/6	0.816	0.5
5	6/6	2.483	8.5

Table 9 – Inoculation 0.1 ml on 1 plate

Level (CFU/ml)	Positive samples number	Standard deviation	Bias
0	0/6	/	/
5	3/6	0.548	0.5
10	4/6	1.414	2
50	6/6	2.258	5.5

Table 10

	Formulas	Obtained values
LC	$1.65\ S_0 + X_0$	1.8
LOD	$3.3\ S_0 + X_0$	3.2
LOQ	$10\ S_0 + X_0$	8.7

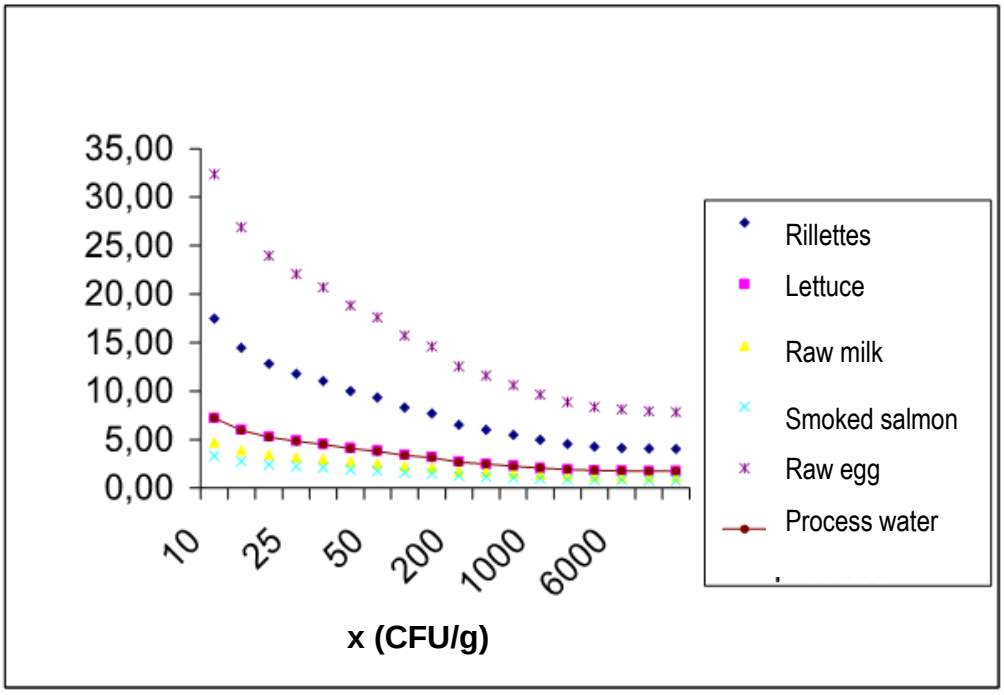
2.1.1.4 Relative Sensitivity

The relative sensitivity is defined as the ability of the alternative method to detect two different amounts of analyte measured by the reference method within a given matrix, at a specified average value, or over the whole measurement range; that is, it is the minimal quantity variation (increase of the analyte concentration x) which gives a significant variation of the measured signal (response y).

These data are intrinsic to the used method and are obtained from the results of the linearity study.

Sensitivity patterns obtained for tested (matrix/strain) pairs are presented in figure 5.

Figure 5 – Sensitivity patterns for the tested (matrix/strain) pairs



2.1.1.5 Specificity/Selectivity

The specificity is defined as the degree to which a method is affected (or not) by the other components present in a multi-component sample. That is the ability of a method to measure exactly a given analyte, or its amount, within the sample without interference from non-target components such as a matrix effect, or background noise.

The selectivity is defined as a measure of the degree of non-interference in the presence of non-target analytes. A method is selective if it can be used to detect the analyte under examination, and that a guarantee can be provided that the detected signal can only be a product by that specific analyte.

The specificity and selectivity study was performed for the Listeria Precis™ detection method; no additional test was realised for the enumeration method. The results were the following:

- Among the *Listeria monocytogenes* strains tested, 2 strains (*Listeria monocytogenes* CIP 78.34 and *Listeria monocytogenes* CIP 105459) gave negative results. These 2 strains were tested twice by running 48 h incubation time of the compared agars. Positive results were then observed by the alternative method. Pale characteristic colonies were observed on the ISO agar by streaking the Half Fraser broth only.

- No cross reaction was observed with the non-target strains, except with *Listeria ivanovii* which gave characteristic colonies on *Brilliance™ Listeria* Agar, but the confirmatory tests provide a clear discrimination between *Listeria monocytogenes* and *Listeria ivanovi* species.

2.1.2 Practicability

1. Packaging	Ready to use *Brilliance Listeria* agar plates are available (x 10 plates)
2. Reagent volume	/
3. Storage conditions– Shelf life of products not opened	The plates are stored at 6 - 12°C in darkness conditions. The expiration date is mentioned on the package and on the plates. It is around 3 months.
4. Modalities of use after first use	/
5. Required equipments	No specific equipment is required.
6. Reagents	Ready to use plates
7. Training	0.5 day
8. Real time handling	Reference method: 9 min for one sample 55 min for 10 samples Alternative method: 7 min for one sample 35 minutes for 10 samples
9. Time to result	Negative results: Reference method: Day 2 Alternative method: Day 2 Negative results after confirmation or positive results: Reference method: Day 4 – Day 7 Alternative method: Day 4 – Day 7 Positive results: Reference method: Day 4 – Day 7 Alternative method: Day 2 – Day 3 (with OBIS Mono Test) or Day 4 – Day 7 (by the classical tests described in the standardised method)
10. Technician skill	Identical to that required for the reference method.
11. Steps common to the reference method	Mother suspension, revivification step and decimal dilutions.
12. Traceability	/
13. Maintenance	/

2.1.3 Inter-laboratory study

2.1.3.1 Organisation

12 laboratories participated to this study. Pasteurised milk was inoculated by *Listeria monocytogenes* 1/2a, isolated from raw milk. The targeted inoculation levels were:

- 0 CFU/ml,
- 50 CFU /ml,
- 500 CFU /ml,
- 5 000 CFU /ml.

Each laboratory received eight flasks of 10 ml sample, i.e. two flasks per inoculation level. Furthermore, one non-inoculated sample has been added to the package for total viable count microflora (NF ISO 4833 method).

Detailed instructions were transmitted to the collaborators by the expert laboratory.

Collaborative study laboratories and the expert laboratory carried out the analyses with the alternative and reference methods.

2.1.3.2 Verification of experimental parameters

- ***Strain stability during transport***

In order to evaluate the *Listeria monocytogenes* strain stability during transport, bacterial count of all inoculated flasks were checked at different time, i.e. inoculation time, after 24 h of conservation at 2° - 8°C. Results are reported in table 11.

Table 11 – *Listeria monocytogenes* count by ISO 11290-2/A1 method (log CFU/ml)

Day of the analyses	Level contamination	Reference method
Day 0 (29/05/2006)	1	45
	2	440
	3	4 500
Day 1 (30/05/2006)	1	60
	2	430
	3	4 900

No evolution of the strain was observed after storage for 24 h at 2°C – 8°C.

- ***Contamination level***

The real contamination levels are provided in table 12.

Table 12 – Real contamination level (CFU/ml)

Target inoculation (CFU/ ml)	Samples N°	Real contamination levels (CFU/ml)
0	1 - 8	0
50	5 - 6	45
500	3 - 7	440
5000	2 - 4	4 500

- ***Sample temperature at receipt***

Measured temperatures at receipt are listed in table 13.

Table 13 - Sample temperature on receipt

Laboratories	Receipt day	Hour receipt	Measured temperature at receipt (°C)	Measured temperature by the temperature probe (°C)
A		10h45	8.2	0.3
B		10h15	5.5	0.3
C		11h15	4.5	-1.3
D		10h00	6.3	0.3
E		09h30	6.0	0.4
F		8h30	6.2	-1.4
G	30/05/2006	11h50	8.1	0.5
H		10h00	3.8	0.4
I		08h30	4.8	0.1
J		14h45	-	/
K		08h30	1.4	0.5
L		09h45	6.7	-1.3
M		08h45	7.6	0.4

- ***Conclusion***

The temperatures during the transport were correct for all the labs, except for Lab J which didn't provide the information.

All the labs received their packages with several broken flasks.

Three labs (B, D and L) received some damaged samples but proceeded to analyses.

All the labs started the analyses at Day 1, except Lab J.

2.1.3.3 Results

- ***Aerobic mesophilic flora***

The count of aerobic mesophilic flora varied from 9.4 10^2 CFU/ml to 3.3 10^3 CFU/ml.

Expert lab results

The results obtained by the expert lab are the following:

Table 14

Target inoculation level (CFU/ml)	Reference method ISO 11290-2		Alternative method: Listeria Precis Enumeration	
	Rep 1	Rep 2	Rep 1	Rep 2
< 10	< 10	< 10	< 10	<10
50	50 (Ne)	60 (Ne)	50 (Ne)	30 (Ne)
500	470	390	490	500
5 000	4 700	5 100	5 900	3 400

Ne: estimated number

The obtained results are in agreement with those expected.

Collaborator Labs results

A synthesis of the results obtained by the collaborator labs is provided Table 15 (CFU/ml) and Table 16 (log CFU/ml).

Table 15 – Synthesis of results obtained by the alternative and the reference methods (CFU/ml)

Labs	Level 1				Level 2				Level 2 bis				Level 3			
	Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method	
A	40	40	70	40	480	410	210	340	1000	300	400	300	4900	4600	4900	5100
B	40	15	40	20	350	300	360	450	350	650	800	600	4100	4700	5100	3700
C	50	20	20	70	440	430	480	450	300	650	300	500	4600	5400	4600	5000
D	40	60	40	40	490	460	600	540	250	450	600	1000	4900	4900	4400	4600
E	50	40	50	20	490	520	530	560	750	550	400	200	5400	4500	4100	5400
F	60	20	60	50	370	250	300	130	300	350	600	300	4400	4700	4900	5900
G	70	35	120	50	440	390	570	370	450	550	700	700	5000	4500	4000	4400
H	110	40	100	90	520	440	450	540	150	650	300	300	4200	4700	5600	4800
L	35	60	20	40	390	390	170	380	650	350	800	200	5300	4600	5600	5900
M	45	45	30	40	450	490	550	410	400	550	800	180	4900	4900	6400	4800

Table 16 – Synthesis of results obtained by the alternative and the reference methods (log CFU/ml)

Labs	Level 1				Level 2				Level 2 bis				Level 3			
	Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method	
A	1.602	1.602	1.845	1.602	2.681	2.613	2.322	2.531	3.000	2.477	2.602	2.477	3.690	3.663	3.690	3.708
B	1.602	1.176	1.602	1.301	2.544	2.477	2.556	2.653	2.544	2.813	2.903	2.778	3.613	3.672	3.708	3.568
C	1.699	1.301	1.301	1.845	2.643	2.633	2.681	2.653	2.477	2.813	2.477	2.699	3.663	3.732	3.663	3.699
D	1.602	1.778	1.602	1.602	2.690	2.663	2.778	2.732	2.398	2.653	2.778	3.000	3.690	3.690	3.643	3.663
E	1.699	1.602	1.699	1.301	2.690	2.716	2.724	2.748	2.875	2.740	2.602	2.301	3.732	3.653	3.613	3.732
F	1.778	1.301	1.778	1.699	2.568	2.398	2.477	2.114	2.477	2.544	2.778	2.477	3.643	3.672	3.690	3.771
G	1.845	1.544	2.079	1.699	2.643	2.591	2.756	2.568	2.653	2.740	2.845	2.845	3.699	3.653	3.602	3.643
H	2.041	1.602	2.000	1.954	2.716	2.643	2.653	2.732	2.176	2.813	2.477	2.477	3.623	3.672	3.748	3.681
L	1.544	1.778	1.301	1.602	2.591	2.591	2.230	2.580	2.813	2.544	2.903	2.301	3.724	3.663	3.748	3.771
M	1.653	1.653	1.477	1.602	2.653	2.690	2.740	2.613	2.602	2.740	2.903	2.255	3.690	3.690	3.806	3.681

Level 2: results obtained taking account the 3 plates inoculated with the mother-suspension

Level 2 bis: results obtained taking account the plates inoculated with 0.1 ml of the mother-suspension

Scrutiny of the measurement results for consistency

In order to identify the measurement results or laboratories that could be inconsistent, two graphical consistency techniques were realised: Mandel's h-ank-statistics. Mandel indicators h and k at 5% significance highlighted some possible inconsistent data (See tables and graphics in **Appendix 3** and **Appendix 4**):

Mandel's values	Number of values observed over the threshold			
	Reference method		Alternative method	
h > 1 %	Lab F	Level 2	Lab F	Level 2
h > 5 %	Lab F	Level 2	Lab A	Level 2
			Lab D	Level 3
	Lab B	Levels 1 and 2	Lab F	Level 2
			Lab L	Level 2
k> 1 %	/		Lab F	Level 3
k > 5 %	Lab F	Level 2	Lab C	Level 2
			Lab F	Level 3
			Lab L	Levels 1 and 3
			Lab M	Level 1

The emphasized results appear (i) coherent with the targeted contamination levels, (ii) concordant between both methods.

All the results were thus kept for the statistical analysis.

- ***Comparison of the trueness and precision characteristics of the reference method and alternative methods***

The statistical values are summarised hereafter:

Level	p	Reference method			Alternative method			Repeatability ratio	Reproducibility ratio
		Median	Repeatability standard deviation	Reproducibility standard deviation	Median	Repeatability standard deviation	Reproducibility standard deviation		
1	10	1.6519	0.2415	0.2425	1.6021	0.2045	0.2433	0.847	1.007
2	10	2.6551	0.0543	0.0659	2.6719	0.0914	0.1325	1.682	2.011
2 bis	10	2.6581	0.2607	0.2607	2.5951	0.1900	0.1953	0.729	0.749
3	10	1.8671	0.0378	0.0378	1.8671	0.0478	0.0611	1.264	1.617

❒ *Bias of the alternative method*

In order to estimate the bias of the alternative method with regard to the reference method for every level, Dij and t are calculated according to the following equations:

$$Dij = \bar{Y}\,ij, \mathrm{Alt} - \bar{Y}\,ij, \mathrm{Ref}$$

$$t = \frac{\left|\text{median } i\,(Dij)\right|}{\sqrt{\pi / (2\ p)\ \varphi\ Diff}}$$

If the t value is superior to 2, the alternative method is significantly biased with regard to the reference method.

The values are given in table 17.

Table 17 – t (d) values obtained by level

Level	Bias	t (d)	Conclusion
1	0.068	1.14	Non significant bias
2	0.021	0.82	Non significant bias
2 bis	- 0.037	0.35	Non significant bias
3	- 0.037	0.43	Non significant bias

The statistical test concludes to a non significant bias for all the inoculation levels. The bias varies from – 0.037 to 0.068 log CFU/ml.

❒ *Comparison of the repeatability standard deviations*

If the ratio Srj, Alt / Srj, Ref. of the repeatability standard deviations of the alternative method and the reference method is larger than 2, the precision under repeatability conditions of the alternative method is considered to be lower than that of the reference method. If this ratio is smaller than 0.5, the precision under repeatability conditions of the alternative method is considered to be greater than that of the reference method.

The ratio values are given in table 18.

Table 18

Level	Repeatability standard deviation		
	Reference method	Alternative method	Alternative method / reference method ratio
1	0.242	0.205	0.847
2	0.054	0.091	1.682
2 bis	0.261	0.190	0.729
3	0.038	0.048	1.264

Level	Repeatability limit	
	Reference method	Alternative method
1	0.676	0.573
2	0.152	0.256
2 bis	0.730	0.532
3	0.106	0.134

The ratios of the repeatability standard deviations are below 2 in all cases. The precision under repeatability conditions of the alternative method is equivalent to that of the reference method.

❒ *Comparison of the reproducibility standard deviations*

If the ratio Srj, Alt / Srj, Ref. of the reproducibility standard deviations of the alternative method and the reference method is larger than 2, the precision under reproducibility conditions of the alternative method is considered to be lower than that of the reference method. If this ratio is smaller than 0.5, the precision under reproducibility conditions of the alternative method is considered to be greater than that of the reference method.

The ratio values are given in table 19.

Table 19

Level	Reproducibility standard deviation		
	Reference method	Alternative method	Alternative method / reference method ratio
1	0.242	0.243	1.007
2	0.066	0.133	2.011
2 bis	0.261	0.195	0.749
3	0.038	0.061	1.617

Level	Reproducibility limit	
	Reference method	Alternative method
1	0.676	0.681
2	0.185	0.371
2 bis	0.730	0.547
3	0.106	0.171

The ratios of the reproducibility standard deviations are below or close to 2. The precision under reproducibility conditions of the alternative method is equivalent to that of the reference method.

2.1.4 Conclusion

- ***Method comparison study***

The Listeria Precis™ enumeration method shows satisfying linearity and relative accuracy.

The alternative method shows satisfying inclusivity and exclusivity results. Two *Listeria monocytogenes* strains gave negative results with the alternative method. These strains grew weakly on the reference method media (O&A, Palcam and Oxford).

The limits of repeatability of the alternative method are similar to those of the reference method.

The bias between both methods are low, between - 0.133 Log CFU/g and - 0.008 Log CFU/g.

The Listeria Precis™ enumeration method offers the saving for confirmatory tests.

Positive results are obtained with the alternative method in 2-3 days (with OBIS Mono test) or in 4-7 days (by the classical tests described in the standardized method), against 4-7 days for the reference method.

- ***Inter-laboratory study***

The bias between the reference and the alternative methods shows satisfying and correct values:

- level 1 (100 - 1 000 CFU/g), bias = + 0.07 log CFU/g
- level 2 (1 000 - 10 000 CFU/g), bias = 0.02 log CFU/g
- level 2 bis (1 000 - 10 000 CFU/g), bias = - 0.04 log CFU/g
- level 3 (10 000 - 100 000 CFU/g), bias = - 0.04 log CFU/g

The repeatability and the reproducibility of the alternative method look similar to those of the reference method.

2.2 Extension study (realised by ASEPT, 2007)

An extension study was performed in 2007 for a new confirmatory test: OBIS MONO.

150 *Listeria monocytogenes* strains and 100 non-target strains were isolated onto TSAYE and *Brilliance*^{TM} Listeria Agar plates. All the *Listeria monocytogenes* and the non-target strains gave the expected results.

Appendix 1 – Flow diagram of the Listeria Precis™ method

x g sample + 9 x ml buffered peptone water

↓

Revivification step for 1 h ± 5 min at 20°C ± 1°C

Streaking 1 ml onto 3 *Brilliance* Listeria Agar plates (mother suspension)
or 0.1 ml onto 1 *Brilliance* Listeria Agar plates (mother suspension and decimal dilutions)

↓

Incubation for 48 h ± 3 h at 37°C ± 1°C

↓

Confirmatory tests on at least one typical colony:
- by the tests described in the ISO method
- by applying the OBIS mono test on an isolated colony

Appendix 2 – ISO 11290-2 (1998) & ISO 11290-2/A1 (2004): Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 2: enumeration of *Listeria monocytogenes* in foods

10 g cheese + 90 ml buffered peptone water or Half Fraser Broth (without supplements) (1/10 dilution)

↓

Stomach 1 min 30 seconds

↓

Keep at 20°C ± 2°C for 1 h ± 5 min

↓

Decimal dilutions in 9 ml peptone salt tubes

↓

Dilution 1/10	1 ml on 3 Chromogenic Listeria medium (ISO) plates (x 2) (-1) 0.1 ml on 1 Chromogenic Listeria medium (ISO) plate (x 2) (-2)
Dilution 1/100	0.1 ml on 1 Chromogenic Listeria medium (ISO) plate (x 2) (-3)
Dilution 1/1000	0.1 ml on 1 Chromogenic Listeria medium (ISO) plate (x 2) (-4)

↓

Incubation at 37°C ± 1°C

24 h - 48 h

↓

Numerate typical colonies at 24 h

(blue/green surrounded by an opaque halo)

↓

Numerate at 48 h

Streak typical colonies on TSYEA

(5 colonies/plate, 2 successive dilutions)

↓

Confirmation tests:

Catalase

Gram stain

For confirmation of *L. monocytogenes*:

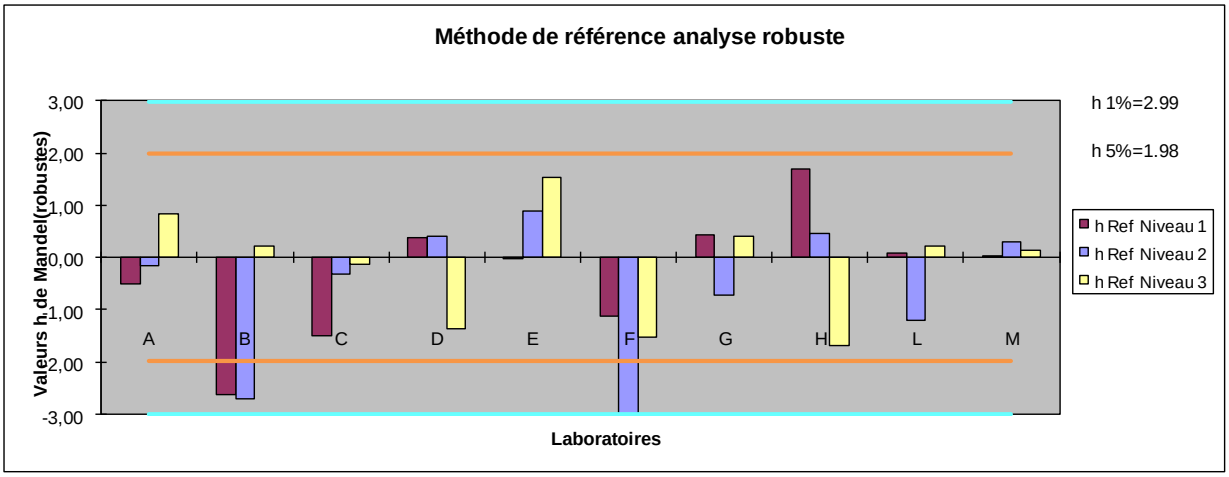
Haemolysis

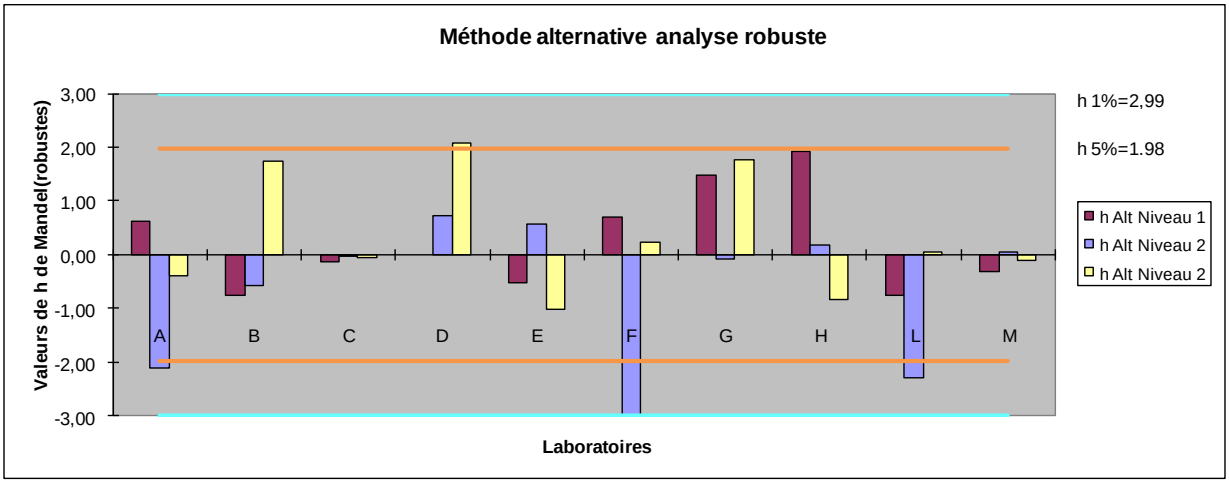
Carbohydrate (rhamnose and xylose) utilisation

CAMP Test

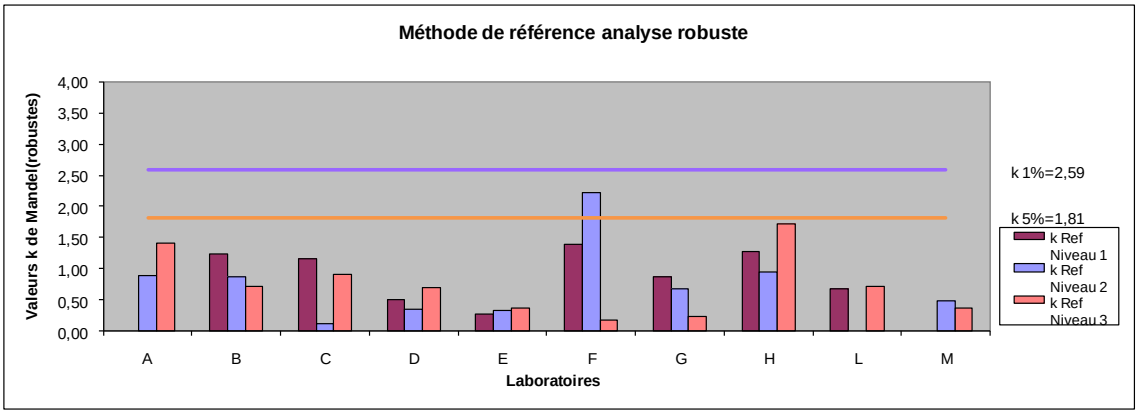
Appendix 3 – Mandel's graphics

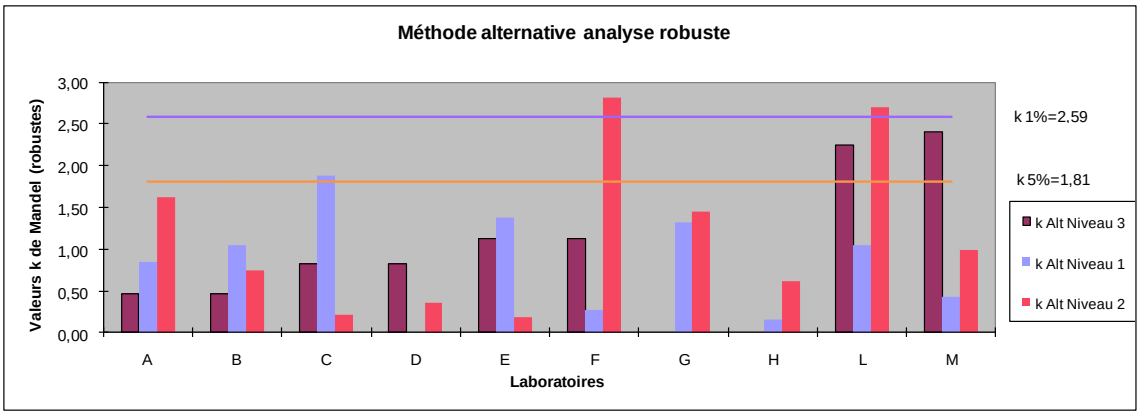
Laboratoire	h Ref Niveau 1	h Ref Niveau 2	h Ref Niveau 3	h Alt Niveau 1	h Alt Niveau 2	h Alt Niveau 3	h5%	h1%	h5%	h1%
A	-0,50	-0,15	0,83	0,62	-2,12	-0,39	1,98	2,99	-1,98	-2,99
B	-2,61	-2,70	0,21	-0,77	-0,58	1,73	1,98	2,99	-1,98	-2,99
C	-1,51	-0,31	-0,14	-0,15	-0,04	-0,05	1,98	2,99	-1,98	-2,99
D	0,38	0,40	-1,37	0,00	0,72	2,07	1,98	2,99	-1,98	-2,99
E	-0,01	0,90	1,55	-0,52	0,56	-1,01	1,98	2,99	-1,98	-2,99
F	-1,12	-3,21	-1,53	0,70	-3,25	0,23	1,98	2,99	-1,98	-2,99
G	0,42	-0,71	0,40	1,47	-0,09	1,76	1,98	2,99	-1,98	-2,99
H	1,69	0,46	-1,69	1,92	0,18	-0,83	1,98	2,99	-1,98	-2,99
L	0,09	-1,20	0,21	-0,77	-2,31	0,05	1,98	2,99	-1,98	-2,99
M	0,01	0,31	0,14	-0,32	0,04	-0,11	1,98	2,99	-1,98	-2,99





Laboratoire	k Ref Niveau 1	k Ref Niveau 2	k Ref Niveau 3	k Alt Niveau 1	k Alt Niveau 2	k Alt Niveau 3	k1%	k5%
A	0,00	0,89	1,42	0,84	1,62	0,46	2,59	1,81
B	1,25	0,87	0,73	1,04	0,75	0,46	2,59	1,81
C	1,17	0,13	0,91	1,88	0,22	0,83	2,59	1,81
D	0,52	0,36	0,69	0,00	0,35	0,83	2,59	1,81
E	0,28	0,34	0,37	1,38	0,19	1,12	2,59	1,81
F	1,40	2,22	0,18	0,27	2,81	1,12	2,59	1,81
G	0,88	0,68	0,24	1,31	1,45	0,00	2,59	1,81
H	1,29	0,94	1,73	0,16	0,61	0,00	2,59	1,81
L	0,69	0,00	0,73	1,04	2,70	2,24	2,59	1,81
M	0,00	0,48	0,38	0,43	0,99	2,41	2,59	1,81





Appendix 4 – Statistical results

Level 1

Nombre de laboratoires (p) 10

Laboratoires	Niveau 1 Ref		Moyenne	si	Deviation		h	k
A	1,602	1,602	1,602	0,00	0,000	0,000	-0,495	0,000
B	1,602	1,176	1,389	0,30	0,213	-0,213	-2,613	1,247
C	1,699	1,301	1,500	0,28	0,199	-0,199	-1,510	1,165
D	1,602	1,778	1,690	0,12	-0,088	0,088	0,380	0,516
E	1,699	1,602	1,651	0,07	0,048	-0,048	-0,013	0,284
F	1,778	1,301	1,540	0,34	0,239	-0,239	-1,116	1,397
G	1,845	1,544	1,695	0,21	0,151	-0,151	0,425	0,881
H	2,041	1,602	1,822	0,31	0,220	-0,220	1,689	1,286
L	1,544	1,778	1,661	0,17	-0,117	0,117	0,092	0,685
M	1,653	1,653	1,653	0,00	0,000	0,000	0,013	0,000

	m	1,65186
	Cn(2p)	1,867
	Cn(p)	1,6101
n=2*p	n	20
	f	11
	l	55
n=p	n	10
	f	6
	l	15
	Qn(2p)	0,091465
	Qn(p)	0,062469
	Qintra	0,17078
	Qinter	0,10058
	Sr(écart-type de répétabilité)	0,2415
	RSDr(coefficient de variation de la r	14,62%
	r(repeatability limit))	0,676
	SL(écart-type interlaboratoire)	-0,01905 SL<0
	SR(écart-type de reproductibilité)	0,2415
	RSDR(coefficient de variation de la	0,146
	R(limite de reproductibilité))	0,6762

Laboratoires	Niveau 1 Alt		Moyenne	si	Deviation		h	k	D
A	1,845	1,602	1,724	0,17	0,122	-0,122	0,621	0,840	0,122
B	1,602	1,301	1,452	0,21	0,151	-0,151	-0,769	1,041	0,062
C	1,301	1,845	1,573	0,38	-0,272	0,272	-0,148	1,881	0,073
D	1,602	1,602	1,602	0,00	0,000	0,000	0,000	0,000	-0,088
E	1,699	1,301	1,500	0,28	0,199	-0,199	-0,522	1,376	-0,151
F	1,778	1,699	1,739	0,06	0,040	-0,040	0,698	0,274	0,199
G	2,079	1,699	1,889	0,27	0,190	-0,190	1,467	1,315	0,194
H	2,000	1,954	1,977	0,03	0,023	-0,023	1,917	0,158	0,155
L	1,301	1,602	1,452	0,21	-0,151	0,151	-0,769	1,041	-0,210
M	1,477	1,602	1,540	0,09	-0,062	0,062	-0,319	0,432	-0,114

	m	1,60206	
	Cn(2p)	1,867	
	Cn(p)	1,6101	
n=2*p	n	20	
	f	11	
	l	55	
n=p	n	10	
	f	6	
	l	15	
	Qn(2p)	0,07745	
	Qn(p)	0,121519	
	Qintra	0,14461	
	Qinter	0,19565	
	Sr	0,2045	
	RSDr	12,77%	
	r	0,573	
	SL	0,01737	
	SR	0,2433	
	RSDR	0,152	
	R(limite de reproductibilit	0,68123	

***mediane* D**		0,0678
Cp		1,6101
Qn(D)		0,092926
Qdiff		0,1496169
t		1,14

Level 2

Nombre de laboratoires (p) 10

Laboratoires	Niveau 2 Ref		Moyenne	si	Deviation		h	k
A	2,681	2,613	2,647	0,05	0,034	-0,034	-0,151	0,891
B	2,544	2,477	2,511	0,05	0,033	-0,033	-2,699	0,872
C	2,643	2,633	2,638	0,01	0,005	-0,005	-0,311	0,130
D	2,690	2,663	2,676	0,02	0,014	-0,014	0,400	0,357
E	2,690	2,716	2,703	0,02	-0,013	0,013	0,897	0,336
F	2,568	2,398	2,483	0,12	0,085	-0,085	-3,214	2,217
G	2,643	2,591	2,617	0,04	0,026	-0,026	-0,707	0,682
H	2,716	2,643	2,680	0,05	0,036	-0,036	0,460	0,945
L	2,591	2,591	2,591	0,00	0,000	0,000	-1,196	0,000
M	2,653	2,690	2,672	0,03	-0,018	0,018	0,311	0,481

	m	2,655082
	Cn(2p)	1,867
	Cn(p)	1,6101
n=2*p	n	20
	f	11
	l	55
n=p	n	10
	f	6
	l	15
	Qn(2p)	0,020570
	Qn(p)	0,0332437
	Qintra	0,03841
	Qinter	0,05352
	Sr(écart-type de répétabilité)	0,0543
	RSDr(coefficient de variation de l	2,05%
	r(repeatability limit))	0,152
	SL(écart-type interlaboratoire)	0,00139
	SR(écart-type de reproductibilité)	0,0659
	RSDR(coefficient de variation de	0,025
	R(limite de reproductibilité))	0,18446

Laboratoires	Niveau 2 Alt		Moyenne	si	Deviation		h	k	D
A	2,322	2,531	2,427	0,15	-0,105	0,105	-2,119	1,620	-0,220
B	2,556	2,653	2,605	0,07	-0,048	0,048	-0,581	0,750	0,094
C	2,681	2,653	2,667	0,02	0,014	-0,014	-0,040	0,217	0,029
D	2,778	2,732	2,755	0,03	0,023	-0,023	0,721	0,354	0,079
E	2,724	2,748	2,736	0,02	-0,012	0,012	0,556	0,185	0,033
F	2,477	2,114	2,296	0,26	0,182	-0,182	-3,255	2,811	-0,188
G	2,756	2,568	2,662	0,13	0,094	-0,094	-0,085	1,453	0,045
H	2,653	2,732	2,693	0,06	-0,040	0,040	0,181	0,613	0,013
L	2,230	2,580	2,405	0,25	-0,175	0,175	-2,307	2,704	-0,186
M	2,740	2,613	2,677	0,09	0,064	-0,064	0,040	0,987	0,005

	m	2,6719001	***mediane* D**	0,0209
	Cn(2p)	1,867	Cp	1,6101
	Cn(p)	1,6101	Qn(D)	0,039911
n=2*p	n	20	Qdiff	0,0642591
	f	11	t	0,82
	l	55		
n=p	n	10		
	f	6		
	l	15		
	Qn(2p)	0,0345985		
	Qn(p)	0,0718158		
	Qintra	0,06460		
	Qinter	0,11563		
	Sr	0,0914		
	RSDr	3,42%		
	r	0,256		
	SL	0,00920		
	SR	0,1325		
	RSDR	0,050		
	R(limite de reproductit	0,3708618		

Level 2 bis

Nombre de laboratoires (p) 10

Laboratoires	Niveau 2 bis Ref		Moyenne	si	Deviation		h	k
A	3,000	2,477	2,739	0,37	0,261	-0,261	0,832	1,418
B	2,544	2,813	2,678	0,19	-0,134	0,134	0,211	0,729
C	2,477	2,813	2,645	0,24	-0,168	0,168	-0,135	0,911
D	2,398	2,653	2,526	0,18	-0,128	0,128	-1,370	0,692
E	2,875	2,740	2,808	0,10	0,067	-0,067	1,547	0,365
F	2,477	2,544	2,511	0,05	-0,033	0,033	-1,525	0,182
G	2,653	2,740	2,697	0,06	-0,044	0,044	0,400	0,236
H	2,176	2,813	2,495	0,45	-0,318	0,318	-1,692	1,727
L	2,813	2,544	2,678	0,19	0,134	-0,134	0,211	0,729
M	2,602	2,740	2,671	0,10	-0,069	0,069	0,135	0,375

	m	2,658114
	Cn(2p)	1,867
	Cn(p)	1,6101
n=2*p	n	20
	f	11
	l	55
n=p	n	10
	f	6
	l	15
	Qn(2p)	0,098745
	Qn(p)	0,06007
	Qintra	0,18437
	Qinter	0,09672
	Sr(écart-type de répétabilité)	0,2607
	RSDr(coefficient de variation de	9,81%
	r(repeatability limit))	0,730
	SL(écart-type interlaboratoire)	-0,02464 SL<0
	SR(écart-type de reproductibilité	0,2607
	RSDR(coefficient de variation de	0,098
	R(limite de reproductibilité))	0,72996

Laboratoires	Niveau 2 bis Alt		Moyenne	si	Deviation		h	k	D
A	2,602	2,477	2,540	0,09	0,062	-0,062	-0,391	0,465	-0,199
B	2,903	2,778	2,841	0,09	0,062	-0,062	1,732	0,465	0,162
C	2,477	2,699	2,588	0,16	-0,111	0,111	-0,049	0,826	-0,057
D	2,778	3,000	2,889	0,16	-0,111	0,111	2,074	0,826	0,363
E	2,602	2,301	2,452	0,21	0,151	-0,151	-1,012	1,120	-0,356
F	2,778	2,477	2,628	0,21	0,151	-0,151	0,230	1,120	0,117
G	2,845	2,845	2,845	0,00	0,000	0,000	1,764	0,000	0,148
H	2,477	2,477	2,477	0,00	0,000	0,000	-0,832	0,000	-0,017
L	2,903	2,301	2,602	0,43	0,301	-0,301	0,049	2,241	-0,076
M	2,903	2,255	2,579	0,46	0,324	-0,324	-0,112	2,411	-0,092

	m	2,59505	***mediane***	**D** -0,0372
	Cn(2p)	1,867	Cp	1,6101
	Cn(p)	1,6101	Qn(D)	0,1656915
n=2*p	n	20	Qdiff	0,2667753
	f	11	t	0,35
	l	55		
n=p	n	10		
	f	6		
	l	15		
	Qn(2p)	0,07195		
	Qn(p)	0,08805		
	Qintra	0,13435		
	Qinter	0,14176		
	Sr	0,1900		
	RSDr	7,32%		
	r	0,532		
	SL	0,00205		
	SR	0,1953		
	RSDR	0,075		
	R(limite de reproduct	0,54686		

Level 3

Nombre de laboratoires (p) 10

Laboratoires	Niveau 3 Ref		Moyenne	si	Deviation		h	k
A	3,690	3,663	3,676	0,02	0,014	-0,014	-0,311	0,513
B	3,613	3,672	3,642	0,04	-0,030	0,030	-1,851	1,109
C	3,663	3,732	3,698	0,05	-0,035	0,035	0,645	1,302
D	3,690	3,690	3,690	0,00	0,000	0,000	0,311	0,000
E	3,732	3,653	3,693	0,06	0,040	-0,040	0,429	1,480
F	3,643	3,672	3,658	0,02	-0,014	0,014	-1,157	0,536
G	3,699	3,653	3,676	0,03	0,023	-0,023	-0,328	0,856
H	3,623	3,672	3,648	0,03	-0,024	0,024	-1,615	0,913
L	3,724	3,663	3,694	0,04	0,031	-0,031	0,461	1,150
M	3,690	3,690	3,690	0,00	0,000	0,000	0,311	0,000

	m	3,68334
	Cn(2p)	1,867
	Cn(p)	1,6101
n=2*p	n	20
	f	11
	l	55
n=p	n	10
	f	6
	l	15
	Qn(2p)	0,014323
	Qn(p)	0,013719
	Qintra	0,02674
	Qinter	0,02209
	Sr(écart-type de répétabilité)	0,0378
	RSDr(coefficient de variation de la répéta	1,03%
	r(repeatability limit))	0,106
	SL(écart-type interlaboratoire)	-0,00023 SL<0
	SR(écart-type de reproductibilité)	0,0378
	RSDR(coefficient de variation de la reproc	0,010
	R(limite de reproductibilité))	0,105894

Laboratoires	Niveau 3 Alt		Moyenne	si	Deviation		h	k	D
A	3,690	3,708	3,699	0,01	-0,009	0,009	0,354	0,257	0,022
B	3,708	3,568	3,638	0,10	0,070	-0,070	-0,844	2,061	-0,005
C	3,663	3,699	3,681	0,03	-0,018	0,018	0,000	0,536	-0,017
D	3,643	3,663	3,653	0,01	-0,010	0,010	-0,545	0,286	-0,037
E	3,613	3,732	3,673	0,08	-0,060	0,060	-0,162	1,769	-0,020
F	3,690	3,771	3,731	0,06	-0,040	0,040	0,975	1,193	0,073
G	3,602	3,643	3,623	0,03	-0,021	0,021	-1,141	0,612	-0,053
H	3,748	3,681	3,715	0,05	0,033	-0,033	0,664	0,990	0,067
L	3,748	3,771	3,760	0,02	-0,011	0,011	1,544	0,335	0,066
M	3,806	3,681	3,744	0,09	0,062	-0,062	1,234	1,848	0,054

	m	3,68086	
	Cn(2p)	1,867	
	Cn(p)	1,6101	
n=2*p	n	20	
	f	11	
	l	55	
n=p	n	10	
	f	6	
	l	15	
	Qn(2p)	0,01811	
	Qn(p)	0,03164	
	Qintra	0,03381	
	Qinter	0,05094	
	Sr	0,0478	
	RSDr	1,30%	
	r	0,134	
	SL	0,00145	
	SR	0,0611	
	RSDR	0,017	
	R(limite de reproduct	0,17119	

***mediane* D**	0,0089
Cp	1,6101
Qn(D)	0,0325359
Qdiff	0,0523852
t	0,43