NF VALIDATION 16140 ™

AFNOR CERTIFICATION VALIDATION OF THE METHOD

Petrifilm Staph Express (STX)

For the enumeration of coagulase positive *Staphylococcus*

Protocol for all food products and pet food

Comparison with the method described in the standard EN ISO 6888-2/A1

SUMMARY REPORT – MARCH 2015 – V1

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Appendices

1. Introduction

1.1. Date(s) and validation history

The 3M Petrifilm Staph Express Count system (STX system) has been validated in April 2003. The reference method was the EN ISO 6888-1:1999 standard and its 2004 amendment, including some accuracy data.
Complementary assays were performed in July 2003 by the 3M Company to allow the freezing at -20°C of the Petrifilm STX Plate between 18 hours and 7 days before confirmation with the Petrifilm STX Disk.

In 2007, the reference method was the EN ISO 6888-2:1999 standard (technique using rabbit plasma fibrinogen agar), and its 2003 amendment. Some assays in comparison with the EN ISO 6888-2:1999 standard, with its amendment of 2003, were presented in 2008.
Results of the studies from 2003 were collected in the part « inclusivity/exclusivity ».

In 2007, the reference protocol was the EN ISO 16140 standard (2003). The alternative method has not been modified since 2007.

The 2011 renewal study took into account the new amendment of the ISO 16140 standard, published in January 2010 (EN ISO 16140/A1: Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods – Amendment 1: Interlaboratory study on quantitative methods, including new calculations for Interlaboratory study).

The interlaboratory study was therefore reviewed to fulfil the new amendment (new statistical assessment).

The results of the comparative study and of the interlaboratory study reported in this report were produced during the validation tests conducted by IPL Santé Développement Durable Nord under the mark NF validation accreditation, in accordance with the requirements in force.

1.2. Principle and protocol of the alternative method

1.2.1. Principle

The Petrifilm Staph Express Count System (STX system) consists in a Petrifilm Staph Express Count Plate (STX plate) and a Petrifilm Staph Express Disk (STX disk).

The Petrifilm Staph Express Count Plate is a sample-ready culture medium system which contains a cold-water-soluble gelling agent. The chromogenic modified Baird-Parker medium in the plate is selective and differential for *Staphylococcus aureus*, *S. hyicus* and *S. intermedius*.
The Petrifilm Staph Express disk contains toluidine blue-O that facilitates the visualization of deoxyribonuclease (DNase) reactions. DNase-positive organisms detected on the Petrifilm Staph Express plate are *S. aureus*, *S. hyicus* and *S. intermedius*. These three microorganisms represent the majority of the group of organisms commonly known as coagulase-positive staphylococci.

1.2.2. Protocol

From an initial suspension realized according to the prescriptions of the ISO 6887 standard, or directly from a liquid sample, decimal dilutions are realized and 3M Petrifilm Staph Express Count system (STX) is inoculated as described below:

- Place the Petrifilm Staph Express Plate on a flat, level surface,
- Lift the top film and, with the pipette perpendicular, dispense 1 mL of sample suspension or dilution onto the center bottom film,

- Roll the top film down onto the sample to prevent trapping air bubbles,
- Place the plastic spreader with the flat side down on the center of the plate. Press gently on the center of the spreader to distribute the sample evenly. Spread the inoculum over the entire Petrifilm Plate growth area before the gel is formed.
- Remove the spreader and leave the plate undisturbed for at least one minute allowing the gel to form.
- Incubate plates in a horizontal position with the clear side up in stacks of no more than 20 plates. Petrifilm Plates are incubated for 24 ± 2 h at 37 ± 1°C.

After incubation, Petrifilm Staph Express Plates are counted with a standard colony counter or other illuminated magnifier according to coloured colonies observations:

- no colonies : the test is complete,
- observation of only red-violet colonies after 24 ± 2 h : count these colonies as *S. aureus*, *S. hyicus* and *S. intermedius*, the test is complete.
- observation of any coloured colony except red-violet colonies (for example : black or blue-green): use a Petrifilm Staph Express Disk. Black colonies may be stressed microorganisms.

The Petrifilm Staph Express Count system (Petrifilm + Disk) is incubated for 3 hours at 37 ± 1°C.
Count all pink zones. Pink zones are usually associated with *S. aureus* but may indicate *S. hyicus* or *S. intermedius*. Colonies not associated with a pink zone are not DNase producing staphylococci, and should not be counted. If the entire disk area is pink with no distinct zones, large numbers of DNase producing colonies are present. Record the result as too numerous to count (TNC) and dilute the sample further to obtain a more accurate count.

Calculate the number of microorganisms present in the test sample according to ISO 7218 for one plate per dilution.
Counting range is:

- less than or equal to 150 red-violet colonies and/or less than or equal to 300 total colonies
- less than or equal to 150 pink zones. (Note : read the plates after 3 hours of incubation time is complete).

Analytical diagrams are presented in appendix 1.

1.3. Reference method

The initial validation in 2003 was performed according to the EN ISO 6888-1 : 1999 standard : Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) – Part 1: Technique using Baird-Parker agar medium.

The first renewal and extension in 2007 was performed according to the EN ISO 6888-2/A1 : 2004 : Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) – Technique using rabbit plasma fibrinogen agar medium. Amendment 1: Inclusion of precision data.

This method is a method without confirmation of the characteristic colonies using coagulase activity test.
Analytical diagram is presented in appendix 2.

1.4. Application scope

The application scope is the following: all human food products and pet food.

2. Comparative study

The following criteria were determined :

- relative accuracy,
- linearity,
- inclusivity and exclusivity,
- interlaboratory study,
- and practicability.

2.1. Relative accuracy

The relative accuracy is the closeness of agreement between a test result and the accepted reference value.

2.1.1. Number and nature of the samples

According to the EN ISO 16140 reference document, food products (naturally contaminated or spiked samples) were analyzed in duplicate according to the two methods:

- reference method EN ISO 6888-2/A1, using rabbit plasma fibrinogen agar medium,
- alternative method: Petrifilm STX system.

In total, 197 products were analyzed so as to obtain at least 10 usable results in each food category.

The categories and the types of samples studied are presented in table 1.

Category	Type	Analyzed samples	Exploited results
Meat products	Raw meat	26	11
	Prepared & seasoned (raw) meat	12	7
	Delicatessen	17	6
	TOTAL	**55**	**24**
Dairy products	Raw milk cheese	30	8
	Raw milk and raw cream	14	7
	Ice cream	5	5
	TOTAL	**49**	**20**
Seafood products	Raw fish	10	5
	Shellfish	9	5
	Prepared fish	12	6
	TOTAL	**31**	**16**
Vegetables	Raw vegetables	4	2
	Seasoned vegetables	16	9
	Cooked vegetables	3	1
	TOTAL	**26**	**12**
Pastries Egg products	With butter cream	8	6
	With custard	6	3
	Egg products	3	1
	TOTAL	**17**	**10**
Petfood	Dry food	14	7
	Raw meat	4	4
	Cat/doog food	4	2
	TOTAL	**22**	**13**
TOTAL		**197**	**95**

Table 1 : number and nature of samples

The 102 samples whose results were not used, exhibited :

- Colony counts below 10 CFU/g or 100 CFU/g with both methods in 58 cases,
- Colony counts below 10 CFU/g or 100 CFU/g with one method in 21 cases,
- Uninterpretable results in 23 cases.

2.1.2. Artificial contaminations

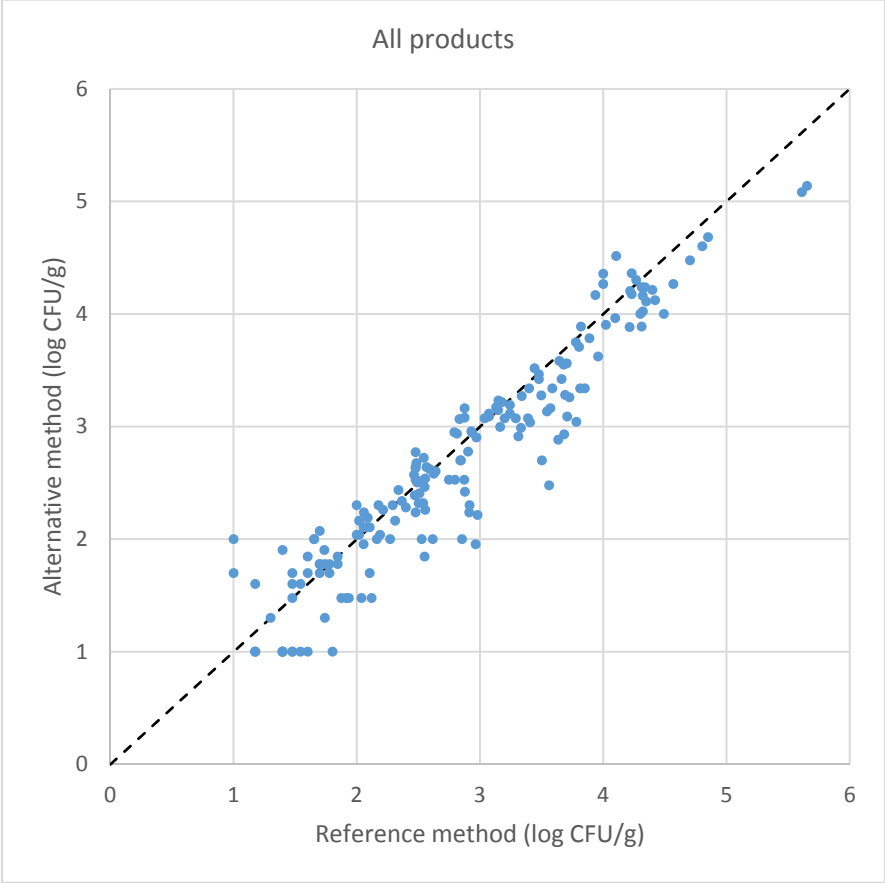
Artificial contaminations were achieved on 14 samples using stressed contaminating suspensions whose stress treatment and efficiency were determined.
The percentage of artificial contaminations was globally 15% for the samples with interpretable results.

2.1.3. Results

Each sample was analyzed in duplicate by the alternative method and the reference method.

Following the EN ISO 16140 standard, the values for each sample were plotted on a two-dimensional graph. The vertical axis (y) is used for the alternative method and the horizontal axis (x) for the reference method. The data were then tested by a linear regression program in order to determine the intercept value (a) and the slope value (b).
The relative accuracy relationship is evaluated according to the model: $y = bx + a$.
For each of the two methods, robust repeatability standard deviations were calculated (Rob.sr(x) and Rob.sr(y)).
As a function of the ratio of these standard deviations, Rob.R = Rob.sr(y)/Rob.sr(x), the linear regression to be used for the interpretation is defined in the EN ISO 16140 standard.

The following graphs represent the raw values obtained for the samples analyzed.
The straight line represented is the first bisector (y = x).



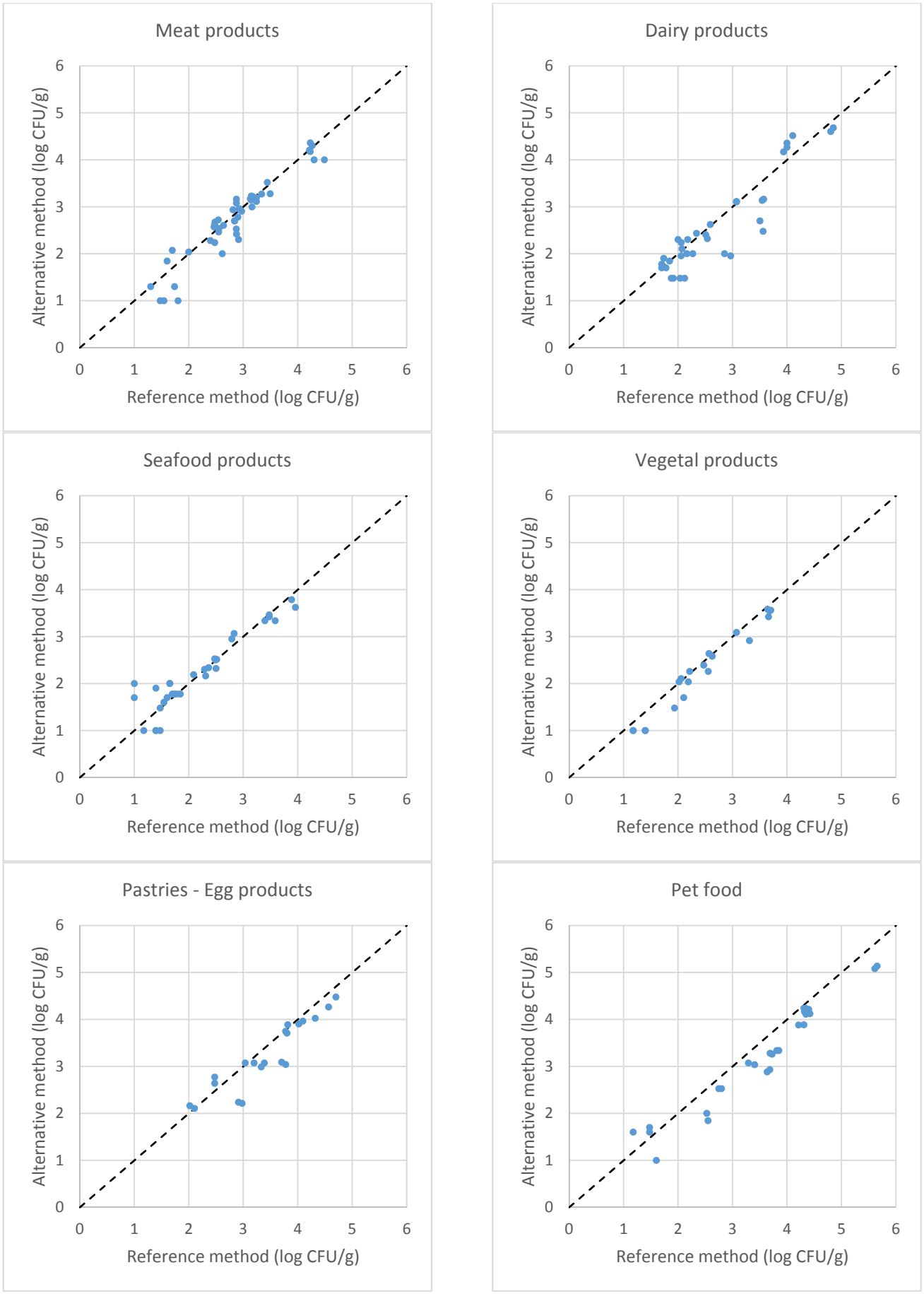


Figure 1 : relative accuracy two-dimensional graphs per category and for all products

2.1.4. Statistical interpretation

In order to check whether the relative accuracy is satisfactory, the two following hypotheses must be verified for a risk α = 5%:

- **Intercept {a = 0}**

The alternative method exhibits a systematic bias compared with the reference method:

- if the value t = a / Sa with (q-2) degrees of freedom is higher than the T-critical value, obtained in Student's table, or
- if the probability p{a = 0} < α (=0.05), p{a = 0} being defined by Student's law.

- **Slope {b = 1}**

If the alternative method does not yield the same values as the reference method:

- the value t = (b-1) / Sb with (q-2) degrees of freedom is higher than the T-critical value, obtained in Student's table, or
- if the probability p{b = 1} < α (=0,05), p{b = 1} being defined by Student's law.

Different values needed in the EN ISO 16140 standard are clarified in table 2. It allowed to compare 3M Petrifilm Staph Express enumeration system with reference method.

Matrix	Rob.R	Regression used	a	t(a)	p(t ;a=0)	b	t(b)	p(t ;b=1)	Conclusion
Meat products	0,848	GMFR	-0,352	2,133	0,044	1,088	1,564	0,132	{a=0} accepted {b=1} rejected
Dairy products	1,171	GMFR	-0,432	1,652	0,116	1,090	0,962	0,349	{{a=0} accepted {b=1} accepted
Seafood products	1,368	GMFR	+0,176	0,977	0,345	0,935	0,851	0,409	{a=0} accepted {b=1} accepted
Vegetables	1,054	GMFR	-0,313	1,733	0,114	1,048	0,782	0,452	{a=0} accepted {b=1} accepted
Pastries - Egg products	0,784	GMFR	-0,047	0,098	0,924	0,954	0,341	0,742	{a=0} accepted {b=1} accepted
Pet food	0,696	GMFR	-0,089	0,382	0,710	0,934	1,055	0,314	{a=0} accepted {b=1} accepted
All products	**1,044**	**GMFR**	**-0,075**	**0,895**	**0,373**	**0,974**	**0,929**	**0,355**	**{a=0} accepted {b=1} accepted**

Table 2 : statistical data

The equation for the regression lines between the alternative method and the reference method, for each category of products are in table 3.

Matrix	Equation
Meat products	log (Alt) = 1,088 log (Ref) – 0,352
Milk products	log (Alt) = 1,090 log (Ref) – 0,432
Seafood products	log (Alt) = 0,935 log (Ref) + 0,176
Vegetables	log (Alt) = 1,048 log (Ref) – 0,313
Pastries - Egg products	log (Alt) = 0,954 log (Ref) – 0,047
Petfood	log (Alt) = 0,934 log (Ref) – 0,089
All products	**log (Alt) = 0,974 log (Ref) – 0,075**

Table 3 : equation of the regression lines by category

Other parameters are presented in table 4.

- the limits of robust repeatability (log values) obtained for the alternative method and the reference method,
- the bias between the two methods (alternative method –reference method).

Matrix	Robust repeatability		Bias (D) (log CFU/g) (alternative – reference)		Contamination range (log)
	Ref.	Alt.	Average	Median	
Meat products	0,25	0,21	-0,102	-0,086	1,00 - 4,36
Dairy products	0,28	0,32	-0,193	-0,127	1,48 - 4,85
Seafood products	0,26	0,35	+0,033	-0,046	1,00 - 3,96
Vegetables	0,17	0,18	-0,177	-0,187	1,00 - 5,56
Pastries – Egg products	0,22	0,17	-0,204	-0,121	2,10 - 4,70
Pet foods	0,15	0,10	-0,321	-0,293	1,00 - 5,63
All products	**0,22**	**0,23**	**-0,149**	**-0,129**	**1,00 - 5,63**

Table 4 : bias, repeatability and contamination range by category

2.1.5. Conclusion

For all product categories except for meat products, the two hypotheses {a=0} and {b=1} are accepted. There is no systematic bias between the two methods. For raw meat products, the correlation coefficient of the regression line is correct.

The repeatability log values obtained with the alternative method and the reference method are 0,23 for the alternative method and 0,22 for the reference method.

The median bias calculated between the alternative method and the reference method is D= – 0,13 log.

2.2. 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.

2.2.1. Protocols

Six food products were contaminated, at five contamination levels. For each product and each contamination level, the alternative and the reference methods were performed with two repetitions.
The analyzed products were the following

- raw ground meat,
- raw fish,
- shredded carrots,
- raw milk,
- custard,
- pet food.

The contamination levels were:

- 100 to 500 CFU/g
- 500 to 1000 CFU/g
- 1000 to 5000 CFU/g
- 5000 to10 000 CFU/g
- 10 000 to 100 000 CFU/g

Different strains of *Staphylococcus aureus* were used, as presented in table 5.

Product	**Strain and origin**
Raw ground meat	*Staphylococcus aureus* from ground raw meat
Raw milk	*Staphylococcus aureus* from yoghurt
Raw fish	*Staphylococcus aureus* from smoked salmon
Shredded carrots	*Staphylococcus aureus* from vegetables salad
Custard	*Staphylococcus aureus*
Pet food	*Staphylococcus aureus* from meat product

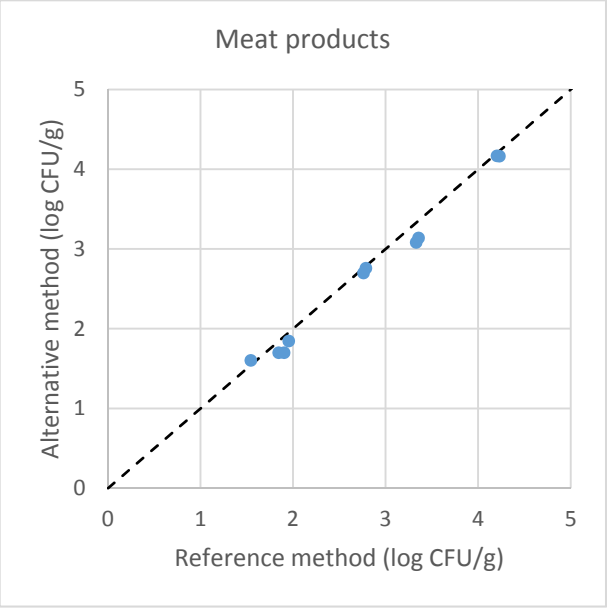
Table 5 : matrix strain couples for linearity study

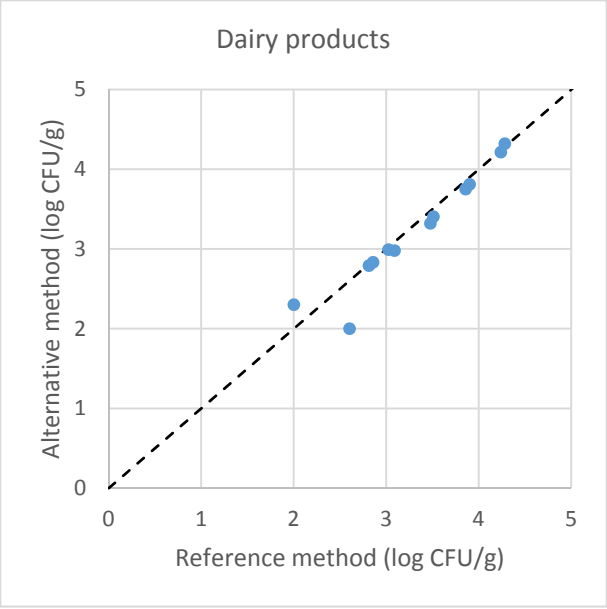
2.2.2. Raw data

Following the EN ISO 16140 standard, the values for each sample were plotted on a two-dimensional graph. The vertical axis (y) is used for the alternative method and the horizontal axis (x) for the reference method.

The data were then tested by a linear regression program in order to determine the intercept value (a) and the slope value (b), like in the relative accuracy part.

The following graphs represent the raw values obtained for each product.





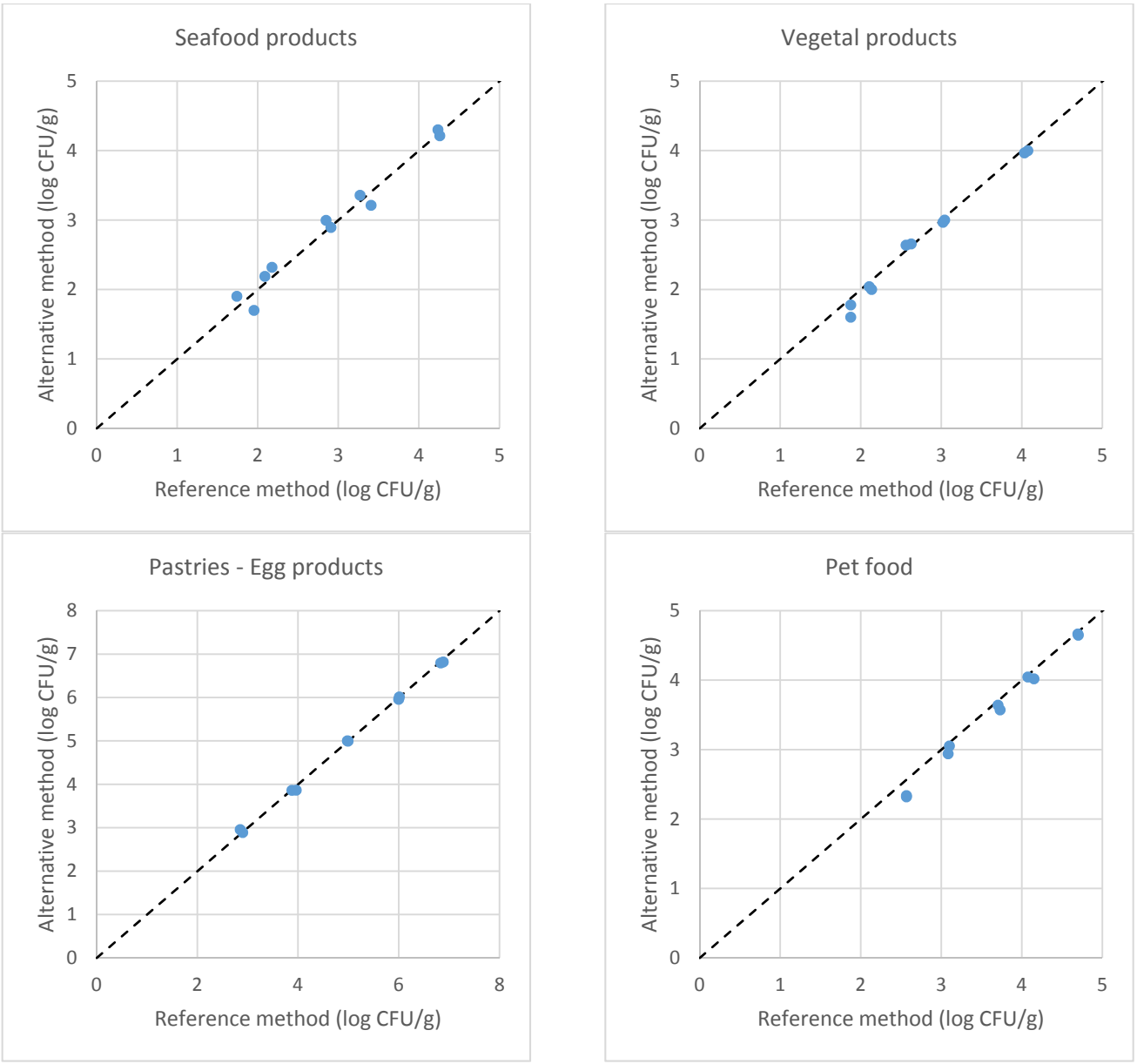


Figure 2 : linearity two-dimensional graphs per category

2.2.3. Statistical interpretation

The linearity is evaluated with the probability of lack-of-fit.

The value Rob.F is calculated as follows: $Rob.F = \frac{(N-2)\,(s^2 y{:}x \,/\, Rob.sr(y)^2) - q\,(n-1)}{q-2}$

with q, number of levels (q = 5)
n, number of repetitions (n = 2)
N, number of samples (N = nq)

The relationship is not linear if:

- [Rob.F > Fcrit (vnum, vden)], or
- p(F, vnum, vden)< α(=0.05)

The type of regression and the Rob.F values are detailed in table 6.

Product	Rob.R	Régression used	F critical value	Rob.F	p (Rob.F) %
Raw ground meat	2,195	OLS	5,41	10,122	1 %
Raw milk	1,643	GMFR	5,19	1,097	44 %
Raw fish	1,506	GMFR	5,41	0,363	78 %
Shredded carrots	1,094	GMFR	5,41	38,225	0 %
Custard	0,490	OLS	5,41	16,167	0 %
Pet food	1,607	GMFR	5,41	11,321	1 %

Table 6 : matrix-strain couples for linearity study

The equations for the regression lines between the alternative method and the reference method are in table 7.

Matrix	Equation	Coefficient of correlation R^2
Raw ground meat	log Alt = 0,996 log Ref - 0,095	R^2 = 0,996
Raw milk	log Alt = 1,031 log Ref - 0,178	R^2 = 0,997
Raw fish	log Alt = 0,989 log Ref + 0,053	R^2 = 0,997
Shredded carrots	log Alt = 1,042 log Ref - 0,183	R^2 = 0,996
Custard	log Alt = 0,984 log Ref + 0,064	R^2 = 0,999
Pet food	log Alt = 1,079 log Ref- 0,399	R^2 = 0,999

Table 7 : equation of the regression lines for the linearity matrix-strain couples

2.2.4. Conclusion

The statistical tests conclude that the relationship between the alternative method and the reference method is linear for “raw milk” and “raw fish”.

For “raw ground meat”, “shredded carrots”, “pet food”, “custard”, the non-linearity test is highly significant.

But, the correlation coefficients for these three products are very high, about 99%, so the significance of the non- linearity test could be failed.

And considering the different graphs and regression equations, the linearity is satisfactory.

2.3. Relative sensitivity and determination of unknown samples

An estimate of sensitivity is used in EN ISO 16140 in order to ascertain that values given by the alternative method do not differ markedly from the reference method (less than 30% in difference).

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. It is the minimal quantity variation (increase of the analyte concentration x) which gives a significant variation of the measured signal (response y).

For each matrix, the precision profile s(<x(y)>) or CV(<x(y)>) versus x(y) was determined over the whole measurement range.

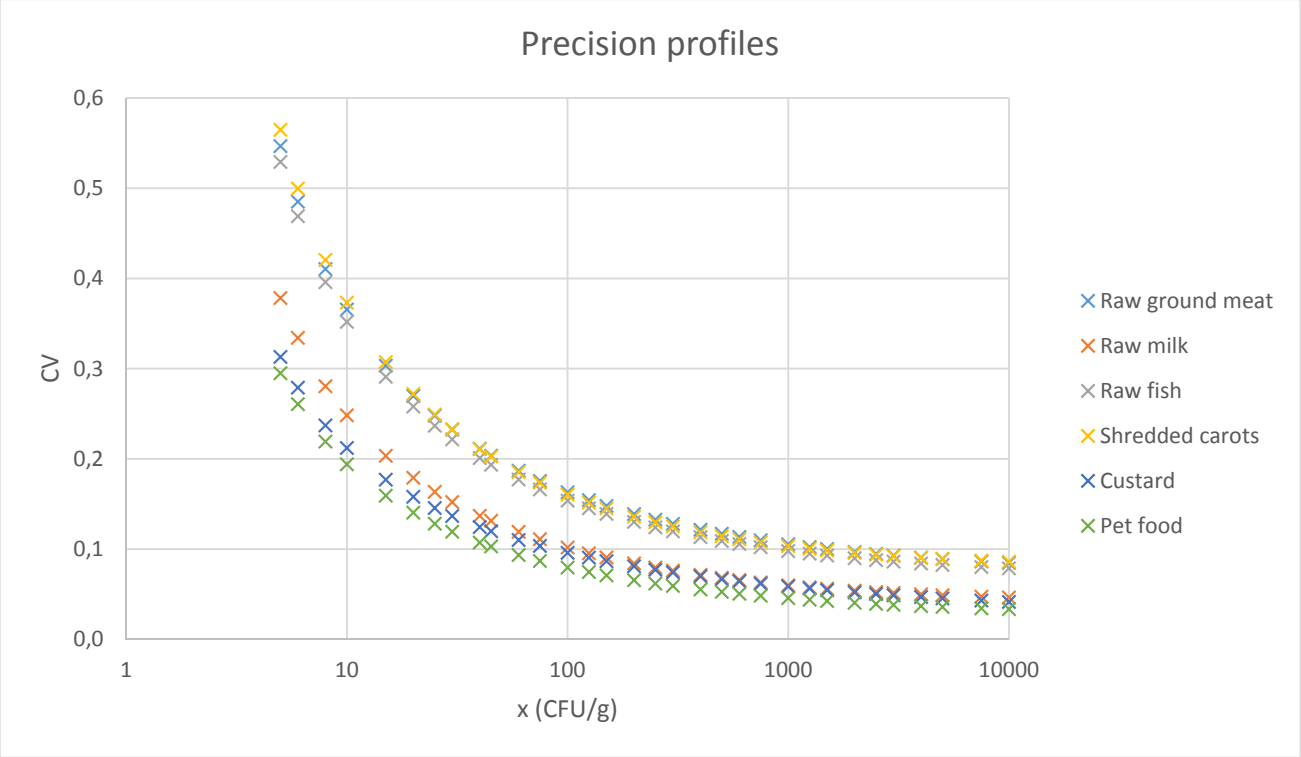


Figure 3 : precision profiles for the matrix-strain couples

2.4. Detection and quantification limits

Detection (LOD) and quantification (LOQ) limits were determined by analysis of a pure culture of *Staphylococcus aureus* with the alternative method.
Four levels of inoculation were tested, with six replicates by level.

Results are synthesized in tables below.

Level / ml	**Positive samples number**	**Standard deviation s_0**	**Bias x_0 (x_{0i} median)**
0	0/6		
0,93	2/6	0,00	0,0
1,24	3/6	0,00	0,5
1,86	4/6	1,19	1,5
12,4	6/6	13,12	11,5

Table 8 : positive samples, standard deviation and bias by level

From s_0 and x_0 values obtained for the first level, the critical limit (LC), the detection limit (LOD) and the quantification limit (LOQ) were determined:

Parameter	Formula	Value obtained (CFU/mL)
LC	1,65 **s0** + **x0**	3,5
LOD	3,3 **s0** + **x0**	5,4
LOQ	10 **s0** + **x0**	13,4

Table 9 : positive samples, standard deviation and bias by level

2.5. Selectivity

The aim of this study is to check that all coagulase positive *Staphylococcus* strains are detected, and that no cross reaction exists with other species of *Staphylococcus* (except *S. hyicus* and *S. intermedius*) or with other genus strains.

2.5.1. Protocol

Strains were cultivated in brain heart infusion during 18 to 24 hours at 37°C.
Different dilutions are realized and inoculated on 3M Petrifilm Staph Express test and on Baird Parker agar medium.
To study the system specificity, whatever the utilization conditions, the disk was inserted in all cases. The growth, the coloring and the DNase reaction of colonies were observed.

2.5.2. Results and conclusion

Results are listed in appendix 3.

- All 28 *Staphylococcus aureus* tested strains gave red-violet colonies.

After disk insertion, all colonies were surrounded with a pink zone.

- The other coagulase-positive strains of staphylococci, *S. hyicus* and *S. intermedius*, presented typical aspect as *Staphylococcus aureus* : the colonies were red-violet or dark, and after revelation with STX disk, the colonies were surrounded with a pink zone.

- Other 15 staphylococci tested strains, which are not coagulase-positive strains and the 11 strains of other genus didn't give any culture or typical colonies (no red-violet color, no pink zone after disk insertion).

Inclusivity and exclusivity are satisfactory. 3M Petrifilm Staph Express enumeration system permitted to detect all coagulase-positive *Staphylococcus* inoculated strains. All colonies had a typical aspect after incubation and after revelation with STX disk.

2.6. Practicability

Practicability is assessed according to criteria which are defined by the AFNOR Technical Committee.
The 3M Petrifilm Staph Express system is compared to the reference method NF ISO 6888-2/A1 in terms of 13 criteria.

Criterion	**Communication on the criterion**
1. Packaging 2. Reagents volumes	In **sealed pouches**. Petrifilm tests: packages of 2 x 25 units or 20 x 25 units. Disks: packages of 1 x 20 units or 5 x 20 units.
3. Storage conditions – Expiration date of unopened tests	Store unopened Petrifilm plates and disks pouches refrigerated or frozen at **temperature less than or equal to 8°C**. Expiration date is noted on each package of Petrifilm plates and disks. (Period of validity of 18 month after the plant leaving).
4. Utilization procedure after first utilization	**Petrifilm**: Return unused plates to pouch. Seal by folding the end of the pouch over and taping shut. To prevent exposure to moisture, do not refrigerate opened pouches. Store resealed pouches **in a cool dry place for no longer than one month.** Petrifilm Staph Express **disks** are individually packaged within a foil pouch. They are sensitive to both moisture and light. Remove only those individually packaged disks that will be used immediately; store

4. Utilization procedure after first utilization	remaining disks in the foil pouch by folding the end of the pouch and taping it shut. Place the resealed disk pouch in a sealable container and store **in a freezer for no longer than six months.** These informations are indicated in the 3M Petrifilm Staph Express Count System instructions.
5. Specific necessary equipment and premises	Usual configuration and equipment of a microbiological laboratory. Nothing specific except the **Petrifilm Flat Spreader** available at 3M. Easier reading with magnifying glass utilization.
6. Ready for use reagents or to restore	The Petrifilm™ system is **ready for use**.
7. Duration of training for a non initiated operator	For a laboratory technician fully formed to standard microbiological techniques, technique training requires **less than one day**.
8. Real time handling and technique flexibility in comparison with number of samples to analyze	**Saving of time** of 8 minutes maximum per positive sample in comparison with standard method which needs tests of confirmation (coagulase activity of 20 colonies at the most). The time of handling is the same for a great or a small series of samples.
9. Response lead time	Result is obtained in **24 hours (D1**) whatever the test response: presence or not of coagulase-positive *Staphylococci* in the sample. The analysis of 1 sample according to standard EN ISO 6888-2 gives a result after 24 to 48 hrs (D2) for a sample.
10. Operator qualification type	The user must be trained to good laboratory practices (indicated in the STX instructions).
11. Joint stages with standard method	Initial suspension preparation, grinding and dilutions.
12. Analysis results traceability	**Batch number** is noted on each package of STX Petrifilm system. The batch number is also noted on individual plates and on the individual disk packages.
13. Laboratory maintenance	No particular service.

3. Interlaboratory study

The aim of the interlaboratory study was to determine the variability of the results obtained in different laboratories using identical samples and to compare these results with those obtained during the methods comparison study.
The interlaboratory study was conducted in 2007 according to NF EN ISO 16140 (2003).
A new statistical assessment was done in 2011 (renewal study) based on all data collected in 2007 according to the NF EN ISO 16140 (amendment 1, 2010) standard.

3.1. Study organization

Thirteen laboratories took part in the interlaboratory study.
Pasteurized milk has been inoculated by a coagulase-positive *Staphylococcus aureus* strain, isolated from a dairy product.
Eight samples were prepared per laboratory, two flasks per inoculation level.
Interlaboratory study laboratories and the expert laboratory carried out the analyses with the alternative and reference methods.
The analyses have been performed two days after sending the samples.

3.2. Control of experimental parameters

3.2.1. Contamination levels obtained after artificial contamination

The four contamination levels are presented in table 10.

Level	Sample	Targeted level (CFU/mL)	Real level (CFU/mL)
Level 0	1 and 8	0	0
Level 1	2 and 7	100	81
Level 2	3 and 6	1 000	810
Level 3	4 and 5	10 000	8100

Table 10 : contamination levels

3.2.2. Strain stability

In order to evaluate the *Staphylococcus aureus* strain variability during transport, bacterial count of inoculated flasks at level 2, has been checked at different time, during storage at 7°C.

Results (CFU/mL) are reported in table 11.

	J0	J1	J2
Sample 1	1400	1500	2000
Sample 2	1500	1400	1900
Sample 3	1900	2000	1700

Table 11 : determination of the strain stability (CFU/mL)

No evolution of the strain has been observed after 48 h of storage at 7°C.

3.2.3. Problems of temperature recorded during transport, temperature on reception an reception times

The temperatures during transport have been registered and checked in order to verify their stability.
All temperature probes showed a temperature between 0°C and 8°C.
Measured temperatures on receipt are listed in table 12.

Laboratory	Receipt Temperatures (°C)		Comments
	Measured by the laboratory	Temperature probe record	
A	6,5	1,9	/
B	5,0	2,4	/
C	1,2	2,4	/
D	3,0	0,4	/
E	1,7	0,0	/
F	4,0	4,5	/
G	9,4	7,9	Receipt at D+2
H	5,0	4,9	/
I	*12,3*	*4,4*	/
J	/	3,0	/
K	3,7	3,5	/
L	1,5	1,9	/
M	0,7	1,4	/

Table 12 : temperature recordings

3.2.4. Conclusion

The laboratory G received the samples at D+2, the day when the labs had to carry out the analyses. The temperature checking showed that, for his package, the temperature stayed below 8°C.
The laboratory I announced a temperature higher than 8°C, but the temperature probe showed a temperature of 4,4°C on receipt.
The conditions of temperature for these two labs were within the correct range, so their results were exploited.

The temperature curve during the storage after receipt and before performing the analyses was above the correct range: temperature upper than 8°C. So the results of laboratory M had not been exploited.

Finally, according to temperature conditions, the results of 12 laboratories have been included to the statistical interpretations.

3.3. Results

3.3.1. Expert laboratory

Results obtained by the expert laboratory with EN ISO 6888-2 method (EN ISO 6888-2:1999 and EN ISO 6888-2/A1:2003) and Petrifilm Staph Express system are presented in the table 13.

	EN ISO 6888-2		Petrifilm STX system	
	Duplicate 1	**Duplicate 2**	**Duplicate 1**	**Duplicate 2**
Level 0	<10	<10	<10	<10
Level 1	95	30	40	40
Level 2	650	620	370	600
Level 3	6700	6700	6100	6200

Table 13 : expert laboratory results (CFU/mL)

Results according to standard EN ISO 6888-2 and alternative method were in agreement.

3.3.2. Collaborative laboratories

Results of the 12 laboratories which realized the analysis are presented in the table 14 below.

	Level 0				Level 1				Level 2				Level 3			
Lab	**RM**		**AM**		**RM**		**AM**		**RM**		**AM**		**RM**		**AM**	
	D1	**D2**	**D1**	**D2**	**D1**	**D2**	**D1**	**D2**	**D1**	**D2**	**D1**	**D2**	**D1**	**D2**	**D1**	**D2**
A	<10	<10	<10	<10	70	55	80	100	780	870	590	720	8800	10000	7900	4600
B	<10	<10	<10	<10	110	65	40	90	580	680	500	620	8100	7200	5500	4800
C	<10	<10	<10	<10	85	90	40	90	1000	1000	580	710	16000	14000	9700	7600
D	<10	<10	<10	<10	75	75	70	90	1000	1000	780	830	11000	11000	8500	10000
E	<10	<10	<10	<10	80	70	40	40	840	810	560	680	7900	6900	5500	5800
F	<10	<10	<10	<10	55	60	70	60	990	860	670	710	8500	9600	4100	6300
G	<10	<10	<10	<10	85	65	30	40	930	720	630	670	7900	8600	6500	7500
H	<10	<10	<10	<10	100	ni	80	60	ni	880	920	860	ni	30000	8500	8900
I	<10	<10	<10	<10	110	95	80	170	900	970	600	860	9200	11000	11000	10000
J	<10	<10	<10	<10	100	91	110	70	720	900	520	540	9000	11000	5200	5100
K	<10	<10	<10	<10	110	100	50	50	890	1100	680	620	8500	8000	6300	7100
L	<10	<10	<10	<10	90	118	90	60	950	1000	680	740	10000	10000	8400	7500
M	<10	<10	<10	<10	ni	ni	<10	60	ni	ni	740	600	ni	ni	8300	8600

Table 14 : collaborative laboratory results in CFU/mL (RM: reference method, AM: alternative method, D: duplicate, ni: not interpretable)

3.3.3. Conclusion

For the laboratory M, the results are presented but have not been taken into account because of temperature problems during storage of the samples. In addition, this lab encountered problems using the standard method. Nevertheless the results obtained with the alternative method were correct.

The laboratory H encountered problem for the interpretation of the dishes due to the interfering flora with the standard method. No result has been taken into account for this lab.

Therefore, results of 11 laboratories have been statistically exploited.

3.4. Calculations

Statistical interpretations have been calculated according to NF EN ISO 16140: 2003 and amendment 1, 2010 (NF EN ISO 16140/A1: 2010 document), per level of contamination.
Results were converted in log for the calculations.

3.4.1. Determination of the accuracy and fidelity characteristics

The application of the NF EN ISO 16140:2003 standard and of its amendment entails to determine the “robust values” to avoid excluding laboratories presenting extreme values.
The synthesis of obtained values is presented in the table below and the detailed calculations in appendix 4.

Level	Parameter	Formula	Reference method	Alternative method
Level 1	Unbiased Rousseeuw's scale estimator Q_{intra}	$Q_{intra} = C_{2p}.Q_{2p}$	0,036	0,094
	Unbiased Rousseeuw's scale estimator Q_{inter}	$Q_{inter} = C_{p}.Q_{p}$	0,090	0,168
	Mediane **m** :	**m**	1,920	1,820
	Standard deviation of repeatability S_r :	$S_r = \sqrt{2}\, Q_{intra}$	0,050	0,134
	Relative standard deviation of repeatability RSD_r :	$RSD_r = S_r / m$	0,026	0,074
	Repeatability limit r:	$r = 2.8\, S_r$	0,141	0,375
	Interlaboratory standard deviation S_L:	$S_L = \sqrt{(Q^2_{inter} - Q^2_{intra})}$ *or* $S_L = 0$ if $Q^2_{inter} - Q^2_{intra} < 0$	0,083	0,138
	Standard deviation of reproducibility S_R :	$S_R = \sqrt{(S_L^2 + S_r^2)}$	0,097	0,192
	Relative standard deviation of reproducibility RSD_R :	$RSD_R = S_r / m$	0,051	0,106
	Reproducibility limit R:	$R = 2.8\, S_R$	0,272	0,539
Level 2	Unbiased Rousseeuw's scale estimator Q_{intra}	$Q_{intra} = C_{2p}.Q_{2p}$	0,026	0,038
	Unbiased Rousseeuw's scale estimator Q_{inter}	$Q_{inter} = C_{p}.Q_{p}$	0,041	0,069
	Mediane **m** :	**m**	2,970	2,820
	Standard deviation of repeatability S_r :	$S_r = \sqrt{2}\, Q_{intra}$	0,036	0,054
	Relative standard deviation of repeatability RSD_r :	$RSD_r = S_r / m$	0,012	0,019
	Repeatability limit r:	$r = 2.8\, S_r$	0,102	0,150
	Interlaboratory standard deviation S_L:	$S_L = \sqrt{(Q^2_{inter} - Q^2_{intra})}$ *or* $S_L = 0$ if $Q^2_{inter} - Q^2_{intra} < 0$	0,032	0,058
	Standard deviation of reproducibility S_R :	$S_R = \sqrt{(S_L^2 + S_r^2)}$	0,049	0,079
	Relative standard deviation of reproducibility RSD_R :	$RSD_R = S_r / m$	0,016	0,028
	Reproducibility limit R:	$R = 2.8\, S_R$	0,136	0,220
Level 3	Unbiased Rousseeuw's scale estimator Q_{intra}	$Q_{intra} = C_{2p}.Q_{2p}$	0,017	0,038
	Unbiased Rousseeuw's scale estimator Q_{inter}	$Q_{inter} = C_{p}.Q_{p}$	0,080	0,138
	Mediane **m** :	**m**	3,980	3,850
	Standard deviation of repeatability S_r :	$S_r = \sqrt{2}\, Q_{intra}$	0,024	0,054
	Relative standard deviation of repeatability RSD_r :	$RSD_r = S_r / m$	0,006	0,014
	Repeatability limit r:	$r = 2.8\, S_r$	0,066	0,150
	Interlaboratory standard deviation S_L:	$S_L = \sqrt{(Q^2_{inter} - Q^2_{intra})}$ *or* $S_L = 0$ if $Q^2_{inter} - Q^2_{intra} < 0$	0,079	0,133
	Standard deviation of reproducibility S_R :	$S_R = \sqrt{(S_L^2 + S_r^2)}$	0,082	0,143
	Relative standard deviation of reproducibility RSD_R :	$RSD_R = S_r / m$	0,021	0,037
	Reproducibility limit R:	$R = 2.8\, S_R$	0,230	0,400

Table 15 : accuracy and fidelity characteristics

3.4.2. Control of the coherence of the results of measurement

Two graphs of coherence are applied to identify the results of measurement or the inconsistent laboratories with regard to the other results of measurement or the laboratories: the statistics interlaboratory h and intra-laboratory k coherence of Mandel.

In case of interlaboratory coherence, only 5 % or 1 % respectively h values are over the horizontal lines representing the indicators of this statistics.

In case of intra-laboratory coherence, only 5 % or 1 % respectively k values are over the horizontal lines representing the indicators of this statistics.

Figures showing the h and k plots for the reference method and the alternative method are presented in appendix 5. Horizontal lines at the 5% and 1% significance indicators are added.

For the reference method, the robust analysis indicates some inconsistencies between measurement results or laboratories for interlaboratory coherence (h values):

- at level 1, no laboratory over thresholds,
- at level 2, 1 laboratory is over 5% and 1% threshold,
- at level 3, 1 laboratory is over 5% threshold.

For the alternative method, the robust analysis does not indicate inconsistencies between measurement results or laboratories for interlaboratory coherence (h values).

For the reference method and the alternative method, the robust analysis indicates some inconsistencies between measurement results or laboratories for intralaboratory coherence (k values).

- Reference method:
  - at level 1, 1 laboratory is over 5% t and 1% thresholds,
  - at level 2, 2 laboratories are over 5% threshold,
  - at level 3, 3 laboratories are over 5% threshold.

- Alternative method:
  - at level 1, 2 laboratories are over 5% threshold,
  - at level 2, 1 laboratory is over 5% threshold,
  - at level 3, 2 laboratories are over 5% threshold, including 1 laboratory over 1% threshold,.

The alternative method showed satisfactory interlaboratory coherence, slightly better than the reference method. Intralaboratory results are comparable and less satisfactory for both methods, although no laboratory showed systematically bad intralaboratory performance for all levels.

3.4.3. Bias calculation

For each level, difference between duplicate means obtained by the alternative method and the reference method has been calculated, that allows the determination of bias Dij ($D_{ij} = y^*_{ij}$, Alt – y^*_{ij}, Ref.), with y* = mean of two values of measurement.

In order to verify if the relative accuracy is correct, the {D=0} hypothesis was tested for each level with the calculation of the parameter t(d).
The bias is significant if the t(d) > 2, i.e. the alternative method lacks accuracy, relative to the reference method for the considered level.

The bias D (alternative-reference) values and the t(d) values obtained by level are reported in the following table:

Level	Bias (Dij) (log)	t(d)	Conclusion Bias (alternative – reference)
Level 1	-0,15	2,99	{D=0} not accepted
Level 2	-0,10	2,21	{D=0} not accepted
Level 3	-0,12	2,41	{D=0} not accepted

Table 16 : bias values

For the three higher levels, the {D=0} hypothesis is statistically not accepted. But, the bias values are similar to those obtained in the comparative study. So, the alternative method accuracy, relative to the reference method is satisfactory.

The bias value (alternative – reference) obtained during the comparative study was (– 0,13 log CFU/g).

3.4.4. Repeatability calculation

For each method and each level, the repeatability limits r have been computed: r= 2.8 Sr, with Sr: repeatability standard deviation, Sralt for alternative method and Srref for reference method.

So, for each level, repeatability standard deviations (Sr_{alt} and Sr_{ref}) were calculated.
If at level j, the standard deviation report (Sr_j, Alt/Sr_j, Ref) is upper to 2, fidelity of alternative method in repeatability conditions is considered as lower than that of the reference method.
If this report is lower than 0.5, fidelity of alternative method in repeatability conditions is considered as upper than that of the reference method.

Values obtained for the repeatability limit are reported in the following table:

Level	Repeatability limits r (log CFU/ml)		Sr_{alt}/ Sr_{ref}	Conclusion
	Reference method	Alternative method		
Level 1	0,141	0,375	2,65	Different repeatability
Level 2	0,102	0,150	1,47	Equivalent repeatability
Level 3	0,067	0,150	2,27	Different repeatability

Table 17 : repeatability values

Standard deviation reports of repeatability are included between 1,47 and 2,64; at all three levels, they are larger than 0,5; but smaller than 2 only for level 2.
The alternative method and the reference method repeatability limits are statistically comparable for medium level (L2) and different (lower) for low (L1) and high (L3) levels.
The repeatability limits r values obtained during the comparative study were 0,22 log (CFU/g) for reference method and 0,23 (CFU/g) for alternative method.

3.4.5. Reproducibility calculation

For each method and each level, the reproducibility limits r have been computed: R= 2.8 SR, with SR: reproducibility standard deviation, SRalt for alternative method and SRref for reference method.

So, for each level, reproducibility standard deviations (Sralt and Srref) were calculated.
If at level j, the standard deviation ratio (SR, Alt/SRj, Ref) is upper to 2, fidelity of alternative method in reproducibility conditions is considered as lower than that of the reference method.
If this ratio is lower than 0.5, fidelity of alternative method in reproducibility conditions is considered as upper than that of the reference method.

Values obtained for the r reproducibility limit are reported in the following table:

Level	Reproducibility limits R (log CFU/ml)		SR_{alt}/ SR_{ref}	Conclusion
	Reference method	Alternative method		
Level 1	0,272	0,539	1,98	Equivalence
Level 2	0,136	0,220	1,62	Equivalence
Level 3	0,230	0,400	1,74	Equivalence

Table 18 : reproducibility values

At all three levels, standard deviation reports of reproducibility are included between 1,62 and 1,98, they are larger than 0,5 and smaller than 2.
The alternative method and the reference method reproducibility limits are statistically comparable for all levels.

3.4.6. Conclusion

The precision under conditions of repeatability and reproducibility of both methods is considered to be equal and is not dependent on the levels of microorganisms.

4. General conclusion

The validation study of 3M Petrifilm Staph Express (STX) method was conducted according to the reference document EN ISO 16 140/A1 (2011).

The 3M Petrifilm Staph Express system for coagulase-positive *Staphylococcus* enumeration is a miniaturized test with a chromogenic medium selective and differential for coagulase-positive *Staphylococcus*, which doesn't need complementary confirmation tests. So, the Petrifilm Staph Express system is an easy to use method. And it allows a saving of space in the incubators.

Some reading difficulties can happen when products are contaminated with important levels of associated flora as for standard method.
The Petrifilm Staph Express system for coagulase-positive *Staphylococcus* enumeration allows a saving of time with regard to standard method, in particular for positive samples (result at Day 1 with regard to Day 1- Day 2 for standard method).

The comparison of Petrifilm Staph Express system with EN ISO 6888-2 standard allows concluding that the alternative method gives accurate results with regard to standard method.

The linearity of the alternative method is satisfactory.

The method is coagulase-positive *Staphylococcus* specific as for standard method.

Statistical interpretations of collaborative study have been calculated according to NF EN ISO 16140: 2003 and amendment 1, 2010 (NF EN ISO 16140/A1).

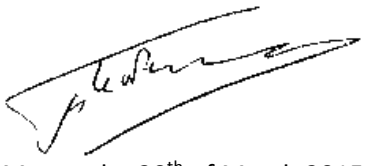
Interlaboratory study (accuracy) gave satisfactory values of repeatability and of reproducibility.

The alternative method and the reference method repeatability limits were statistically comparable for medium level (L2) and lower for low (L1) and high (L3) levels.

The precision under conditions of repeatability and reproducibility of both methods is considered to be equal and is not dependent on the levels of microorganisms.

For the alternative method, the robust analysis showed a satisfactory interlaboratory coherence, slightly better than the reference method.
Intralaboratory results were comparable and less satisfactory for both methods.

For all the contamination levels, the alternative method accuracy, relative to the reference method is satisfactory.
The bias values (alternative – reference) were similar to those obtained in the comparative study was (– 0,10/-0,15 log(CFU/ml or g)).



Massy, the 30th of March 2015
François Le Nestour
Innovation Biology Unit Manager

APPENDIX 1 – ALTERNATIVE METHOD PROTOCOL

3M Petrifilm Staph Express count plate

Initial suspension preparation (1/10)
Realization of decimal dilutions

⬇

Inoculation of 1 3M Petrifilm Staph Express Plate
with 1 mL of initial suspension or decimal dilutions
Adjust pH from 6,0 to 8,0 if needed

⬇

Incubate for 24±2 h at
37±1°C

⬇

Interpretation:
Observation of the colonies colour

⬇	⬇	⬇
No colony	Only red-violet colonies	Other colonies than red-violet ones
⬇	⬇	⬇
The test is complete	Colonies are enumerated and recorded as *S. aureus*, *S. hiycus* and *S. intermedius*	The STX disk is inserted to complete the 3M Petrifilm Staph Express System
	⬇	
	The test is complete	

3M Petrifilm Staph Express Disk Insertion

Lift the top film of the STX plate and insert the STX disk

⬇

Incubate for 3 h at 37±1°C

⬇

Enumerate the pink zones without or around colonies as *S. aureus*, *S. hiycus* and *S. intermedius*

APPENDIX 2 – REFERENCE METHOD PROTOCOL

NF EN ISO 6888-2

Weigh an aliquot sample – Realize the initial suspension and decimal dilutions

⬇

Transfer 1 mL in duplicate in two Petri dishes

⬇

Add 12 to 15 mL of RPFA medium

⬇

Let solidify about 10 minutes at room temperature

⬇

Invert the plates

⬇

Incubate for 24±2 h at 37±1°C , and if necessary 24 h more (absence of characteristic colonies)

⬇

Enumerate characteristic colonies

APPENDIX 3 - SELECTIVITY

Strains	Origin	Results after 1st incubation	Results after disk insertion
Staphylococcus aureus	ATCC 6538	red-violet	pink zone
Characteristic colonies on Baird-Parker agar medium	ATCC 9144	red-violet	pink zone
	Dairy product	red-violet	pink zone
	Meat product	red-violet	pink zone
	Raw milk	red-violet	pink zone
	Raw milk cheese	red-violet	pink zone
	Dairy product	red-violet	pink zone
	Dairy product	red-violet	pink zone
	Raw milk cheese	red-violet	pink zone
	Raw milk cheese	red-violet	pink zone
	Chipolatas	red-violet	pink zone
	Meat product	red-violet	pink zone
	Meat product	red-violet	pink zone
	Meat product	red-violet	pink zone
	Meat product	red-violet	pink zone
	Meat product	red-violet	pink zone
	CIP 7625	red-violet	pink zone
	Cake	red-violet	pink zone
	Cake	red-violet	pink zone
	Smoked salmon	red-violet	pink zone
	Milk	red-violet	pink zone
	CIP 53154	red-violet	pink zone
	Fish filet	red-violet	pink zone
	Salad	red-violet	pink zone
	Toast	red-violet	pink zone
Staphylococcus aureus	Meat product	red-violet	pink zone
No characteristic colonies on Baird-Parker agar medium	Poultry liver	red-violet	pink zone
	Goat milk	red-violet	pink zone
St.hyicus	Collection	red-violet	pink zone
St.hyicus	Meat product	black	pink zone
St.hyicus	Meat product	black	pink zone
St.hyicus	Meat product	black	pink zone
St.hyicus	Collection	black	pink zone
St intermedius	Collection	red-violet	pink zone
St intermedius	Collection	violet	pink zone
St.xylosus	Munster (cheese)	black	no pink zone
St.epidermidis	Dairy product	no colonie	/
St.epidermidis	ATCC 12228	no colonie	/
St.scuiri	Collection	no colonie	/
St.saprophyticus	Collection	black	no pink zone
St.cohnii	Smoked salmon	no colonie	/
St.epidermidis	Clinical	no colonie	/
St.epidermidis	Smoked salmon	no colonie	/
St.epidermidis	Collection	no colonie	/
St.simulans	Salad	black	no pink zone
St.warneri	Ham	no colonie	/
St.warneri	Bacon	no colonie	/
St.warneri	Bayonne ham	no colonie	/
St.xylosus	Salad	black	no pink zone
St.xylosus	Offal	black	no pink zone
Other genus			
Listeria innocua	Smoked fish	blue	no pink zone
Enterococcus faecalis	Meat product	no colonie	/
Micrococcus spp	Vegetables	no colonie	/
E.coli	Dairy product	no colonie	/
Micrococcus spp	Environment	no colonie	/
Micrococcus luteus	Environment	no colonie	/
Micrococcus roseus	Environment	no colonie	/
Enterococcus faecalis	Eggs	blue-green	no pink zone
Enterococcus faecium	ATCC 3286	blue-green	no pink zone
Enterococcus faecium	CIP 5433	no colonie	/
Enterococcus durans	Meat product	no colonie	/

APPENDIX 4 – MANDEL STATISTICAL CALCULATIONS

Calculations
Level 1

Table 10 Reference method NF EN ISO 6888-2

1	2	3	4	5	6
Laboratory	**Measurement**		**Mean**	**Deviation**	
A	1.85	1.74	1.79	0.05	-0.05
B	2.02	1.81	1.92	0.10	-0.10
C	1.93	1.95	1.94	-0.01	0.01
D	1.88	1.88	1.88	0.00	0.00
E	1.90	1.85	1.87	0.03	-0.03
F	1.74	1.78	1.76	-0.02	0.02
G	1.93	1.81	1.87	0.06	-0.06
H	2.02	1.98	2.00	0.02	-0.02
I	2.00	1.96	1.98	0.02	-0.02
K	2.02	2.00	2.01	0.01	-0.01
L	1.95	2.07	2.01	-0.06	0.06

p	11	**m**	**1.92**
	Repeatability	**Sr**	**0.05062591**
		RSDr	0.02640822
		r	0.14175255
	Inter-labo	SL	0.0830553
	Reproducibility	**SR**	**0.09726852**
		RSDR	0.05073861
		R	0.27235187

Table 10 Alternative method

1	2	3	4	5	6
Laboratory	**Measurement**		**Mean**	**Deviation**	
A	1.90	2.00	1.95	-0.05	0.05
B	1.60	1.95	1.78	-0.18	0.18
C	1.60	1.95	1.78	-0.18	0.18
D	1.85	1.95	1.90	-0.05	0.05
E	1.60	1.60	1.60	0.00	0.00
F	1.85	1.78	1.82	0.04	-0.03
G	1.48	1.60	1.54	-0.06	0.06
H	1.90	1.78	1.84	0.06	-0.06
I	1.90	2.24	2.07	-0.17	0.17
K	1.70	1.70	1.70	0.00	0.00
L	1.95	1.78	1.87	0.09	-0.09

p	11	**m**	**1.82**
	Repeatability	**Sr**	**0.13397152**
		RSDr	0.07381351
		r	0.37512026
	Inter-labo	SL	0.13818444
	Reproducibility	**SR**	**0.19246638**
		RSDR	0.10604208
		R	0.53890587

Calculations
Level 2

Table 10 reference method NF EN ISO 6888-2

1	2	3	4	5	6
Laboratory	**Measurement**		**Mean**	**Deviation**	
A	2.89	2.94	2.91	-0.02	0.02
B	2.76	2.83	2.80	-0.04	0.04
C	3.01	3.01	3.01	0.00	0.00
D	3.00	3.01	3.01	-0.01	0.01
E	2.92	2.91	2.92	0.01	-0.01
F	3.00	2.94	2.97	0.03	-0.03
G	2.97	2.86	2.91	0.06	-0.06
H	2.95	2.99	2.97	-0.02	0.02
I	2.86	2.95	2.91	-0.05	0.05
K	2.95	3.03	2.99	-0.04	0.04
L	2.98	3.00	2.99	-0.01	0.01

p	11	**m**	**2.97**
	Repeatability	**Sr**	**0.03644876**
		RSDr	0.01228811
		r	0.10205654
	Inter-labo	SL	0.03207628
	Reproducibility	**SR**	**0.04855306**
		RSDR	0.01636887
		R	0.13594858

Table 10 Alternative method

1	2	3	4	5	6
Laboratory	**Measurement**		**Mean**	**Deviation**	
A	2.77	2.86	2.82	-0.04	0.04
B	2.70	2.79	2.75	-0.04	0.04
C	2.76	2.85	2.81	-0.04	0.05
D	2.89	2.92	2.91	-0.02	0.01
E	2.74	2.83	2.79	-0.04	0.04
F	2.93	2.85	2.89	0.04	-0.04
G	2.80	2.83	2.82	-0.02	0.02
H	2.96	2.94	2.95	0.01	-0.01
I	2.78	2.94	2.86	-0.08	0.08
K	2.83	2.79	2.81	0.02	-0.02
L	2.83	2.87	2.85	-0.02	0.02

p	11	**m**	**2.82**
	Repeatability	**Sr**	**0.05358861**
		RSDr	0.01903681
		r	0.1500481
	Inter-labo	SL	0.05764768
	Reproducibility	**SR**	**0.07870829**
		RSDR	0.02796032
		R	0.2203832

Calculations
Level 3

Table 10 reference method NF EN ISO 6888-2

1	2	3	4	5	6
Laboratory	**Measurement**		**Mean**	**Deviation**	
A	3.95	4.02	3.98	-0.03	0.03
B	3.91	3.86	3.88	0.02	-0.02
C	4.20	4.15	4.18	0.03	-0.03
D	4.06	4.06	4.06	0.00	0.00
E	3.90	3.84	3.87	0.03	-0.03
F	3.93	3.98	3.96	-0.03	0.03
G	3.90	3.94	3.92	-0.02	0.02
H	3.97	4.03	4.00	-0.03	0.03
I	3.96	4.02	3.99	-0.03	0.03
K	3.93	3.90	3.92	0.01	-0.01
L	4.02	4.01	4.01	0.01	-0.01

p	11	**m**	**3.98**
	Repeatability	**Sr**	**0.02359511**
		RSDr	0.00592444
		r	0.06606629
	Inter-labo	SL	0.07870959
	Reproducibility	**SR**	**0.08217012**
		RSDR	0.0206319
		R	0.23007632

Table 10 Alternative method

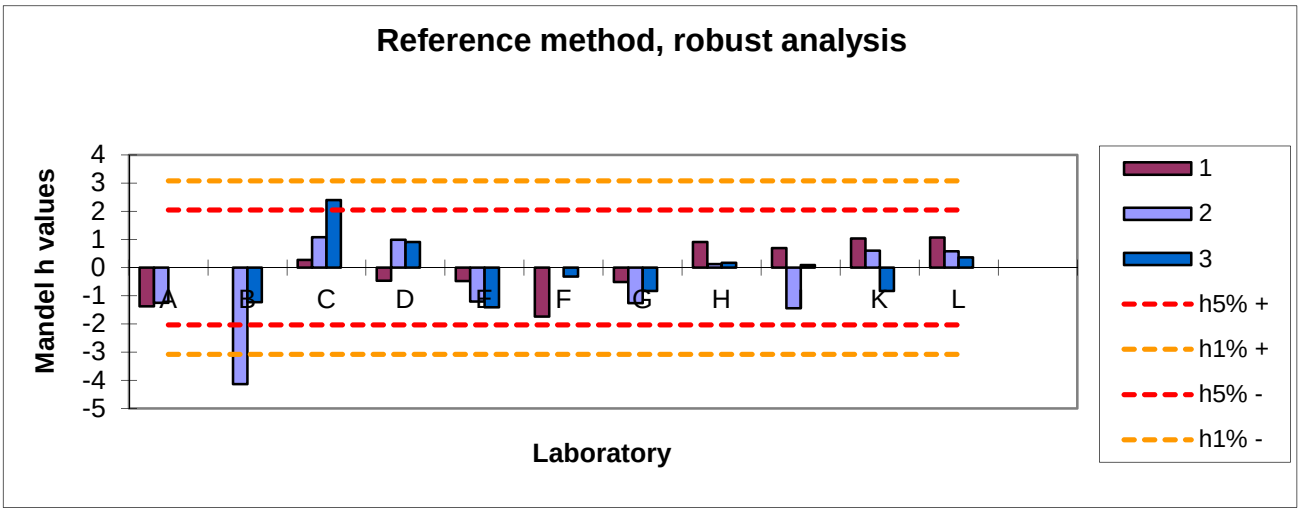
1	2	3	4	5	6
Laboratory	**Measurement**		**Mean**	**Deviation**	
A	3.89	3.67	3.78	0.11	-0.11
B	3.74	3.68	3.71	0.03	-0.03
C	3.99	3.88	3.94	0.06	-0.06
D	3.93	4.01	3.97	-0.04	0.04
E	3.74	3.76	3.75	-0.01	0.01
F	3.61	3.80	3.71	-0.10	0.09
G	3.81	3.88	3.85	-0.03	0.04
H	3.93	3.95	3.94	-0.01	0.01
I	4.03	4.00	4.02	0.01	-0.02
K	3.80	3.85	3.83	-0.03	0.02
L	3.92	3.87	3.90	0.02	-0.02

p	11	**m**	**3.85**
	Repeatability	**Sr**	**0.05358861**
		RSDr	0.01393722
		r	0.1500481
	Inter-labo	SL	0.13266737
	Reproducibility	**SR**	**0.14308169**
		RSDR	0.0372124
		R	0.40062872

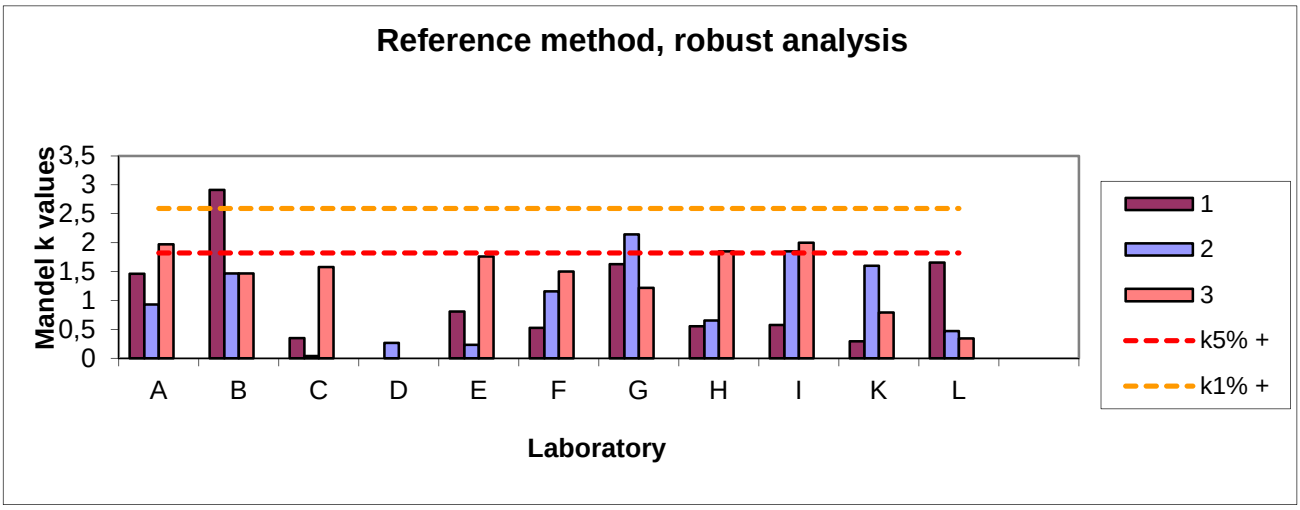
APPENDIX 5 – MANDEL GRAPHS

Control of the coherence of the reference method

Mandel h_{ij} inter-laboratory statistical coherence

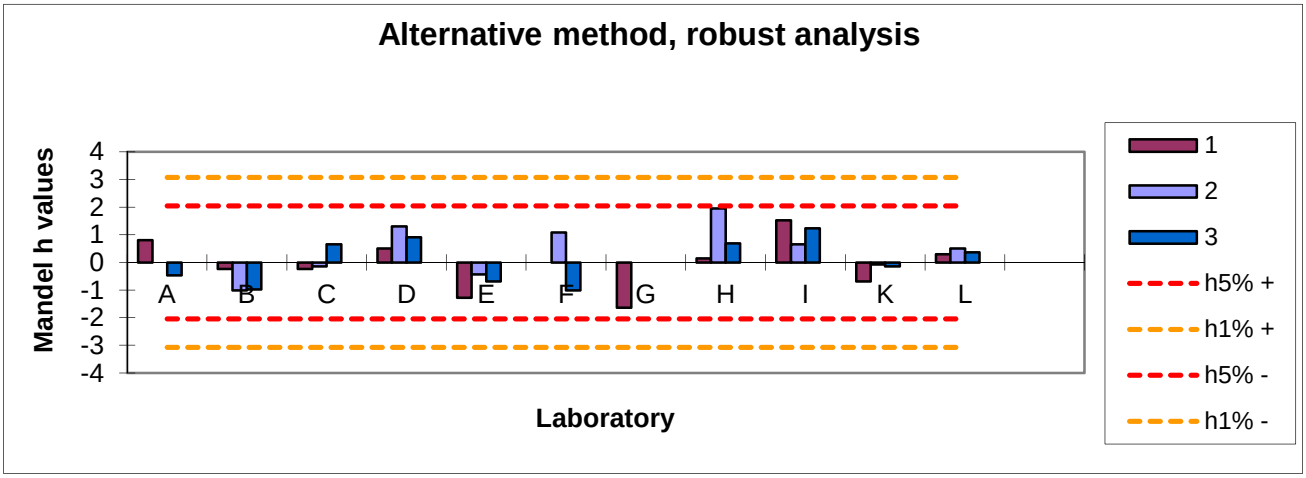


Mandel k_{ij} intra-laboratory statistical coherence



Control of the coherence of the alternative method

Mandel h_{ij} inter-laboratory statistical coherence



Mandel k_{ij} intra-laboratory statistical coherence