AGENCE FRANÇAISE
DE SÉCURITÉ SANITAIRE
DES ALIMENTS
Site of
Maisons-Alfort

LABORATORY FOR STUDIES
AND RESEARCH ON
FOODS QUALITY AND
FOOD-PROCESSING
(LERQAP)

EU CRL for
Coagulase Positive
Staphylococci

Study of an alternative to the confirmation step of the Standard EN ISO 6888-1 for enumeration of coagulase-positive staphylococci (BP agar)

April 2007–June 2008

Report version 1 – 23 April 2009

Keyword : Coagulase- positive staphylococci, confirmation step

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ABBREVIATIONS

BHI: Brain Heart Infusion
BP: Baird Parker
RPF: Rabbit Plasma Fibrinogen
TS: Tryptone Salt
TSAYE: Tryptone Soya Agar Yeast
CPS: Coagulase Positive Staphylococci

I. INTRODUCTION AND PURPOSE

Staphylococci are facultative anaerobic, Gram-positive, catalase positive cocci in the family *Staphylococcaceae*. Staphylococci form part of the normal bacterial flora of the skin, mucous membranes and alimentary and urogenital tracts of a wide variety of mammals and brieds. As well as making up part of the normal flora, some species of staphylococci may also cause a wide variety of usually pyogenic processes in various parts of the body in animals and humans.

Among the microbiological criteria fixed by the EC Regulation 2073/2005 for foodstuffs, criteria are fixed for coagulase-positive staphylococci –CPS (mainly *Staphylococcus aureus*) in:

- Cheeses made from raw milk or from heat-treated milk, ripened cheeses and unripened soft cheeses,
- Milk/whey powder
- Cooked crustaceans and molluscan shellfish

The confirmation procedure of the Standard method EN ISO 6888-Part 1, cited as reference in Regulation 2073/2005, is based on coagulase test in tube, performed with rabbit plasma. An alternative procedure has been proposed and already introduced in a French Standard (NF V08-057-1, 2004): a stabbing test on RPF agar, based upon the combination of a production of a black coloration (reduction of tellurite to tellurium) and of positive coagulase reaction, detected on selective isolation media BP + RPF. This procedure appears to be easier, quicker and less expensive to perform than the coagulase tube test.

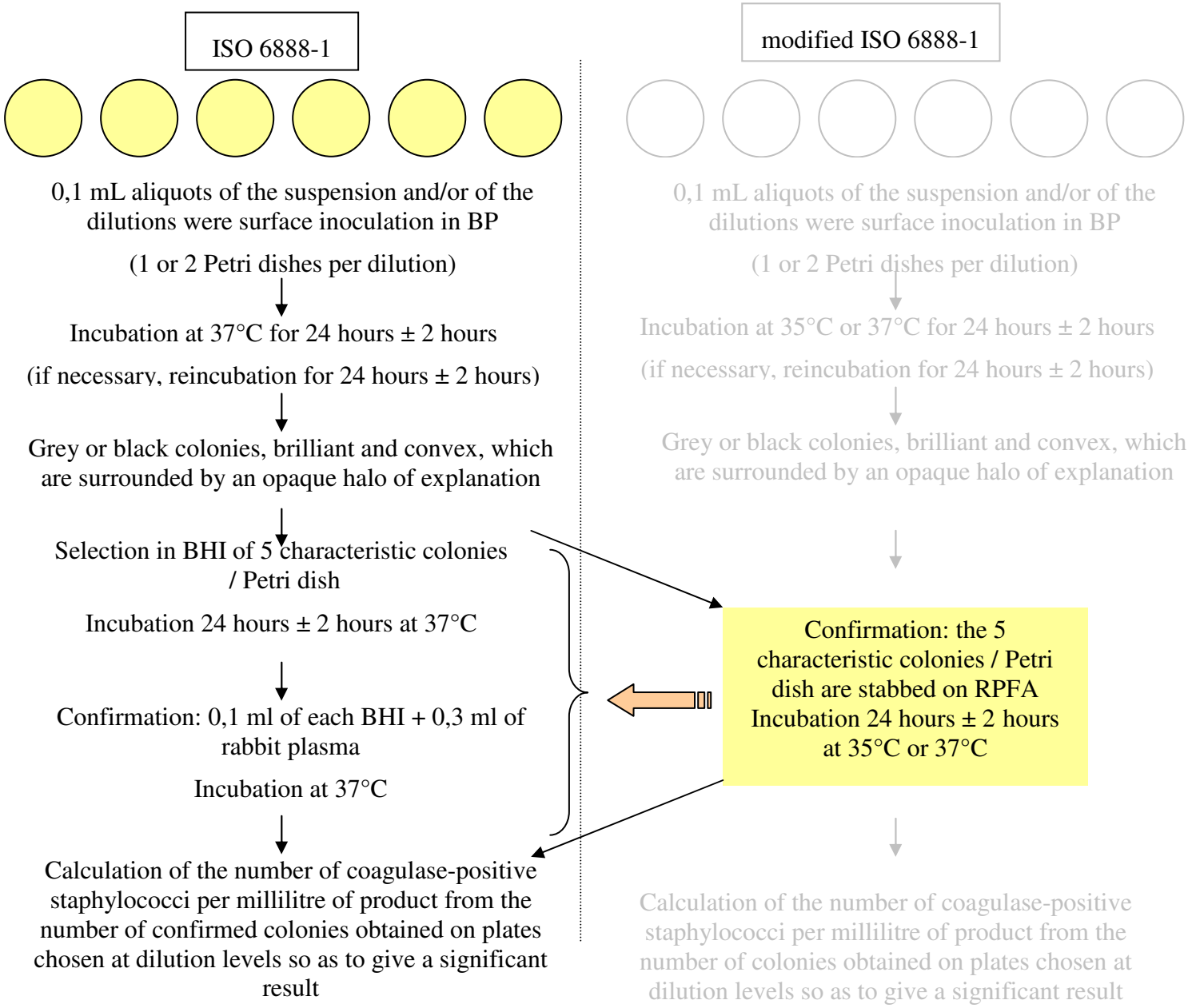
The purpose of this study was to evaluate the performance of the RPFA stabbing test confirmation procedure, which may be introduced as an alternative to the coagulase tube test in the reference method EN ISO 6888-Part 1 for the CPS enumeration.

In a first phase of the study, the following performance criteria were assessed : (i) the inclusivity and exclusivity (the ability that the method detects a wide range of CPS strains and that the method does not cross-react with other closely related bacterial strains); and (ii) the method performance for the analysis of naturally contaminated samples when the two methods are used in parallel.

The results are synthetized within tables and interpreted according to the Standard EN ISO 16140 defining a validation protocol for alternative methods in food microbiology. The CRL for CPS (Unit HMPA) compared the alternative confirmation procedure with the reference EN ISO 6888-1 method.

II. METHODS

See the following diagram.



III. INCLUSIVITY AND EXCLUSIVITY

1- Aim

The purpose of this study was to check that all CPS types were well identified by the altenative confirmation procedure and that there was no crossed reaction with other bacterial strains.

2- Procedure

Bacterial strains and preparation of inocula

Experiments were carried out with several strains of *Staphylococcus* isolated characterised in our laboratory.
A stock culture of *Staphylococcus* was maintained frozen at –80°C using Cryobank tubes. The culture was resuscitated by plating into TSAYE, and then propagated in BHI before use. Appropriate dilutions of cultures grew in BHI for 24h at 37°C. The final BHI culture usually contained around $1x10^9$ $CFU.mL^{-1}$. All decimal dilutions were prepared in TS. To determine the inoculum level, appropriate dilutions were enumerated on TSAYE.

- Reference method (coagulase test)

Inclusivity and exclusivity were studied by the analysis of:
- ✓ 31 pure CPS strains
- ✓ 23 pure non CPS strains

- BP+RPF

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- ✓ 31 pure CPS strains
- ✓ 23 pure non CPS strains

Each strain was further tested in our laboratory with a biochemical test kit, using ID 32 STAPH (bioMérieux). Moreover *Staphylococcus aureus* was tested with a biomolecular test. All gave an excellent identification percentage. CPS were mainly isolated from food having caused food-borne illnesses. Other *Staphylococcus* strains were selected to represent the major *Staphylococcus* species found in milk products, environment of milk processing.

Enumeration procedure

CPS enumeration procedure was performed according to EN ISO 6888- part 1 (see diagram in II). Briefly, inoculation of 0,1 mL aliquot of suspension and/or successive dilution was performed in BP plates (AES Laboratories) and incubation at 37°C for 24h, followed by confirmation step. The confirmation procedure was performed with the EN ISO 6888- part 1 method and the alternative method.

Reference confirmation procedure: inoculation of 5 characteristic colonies in Brain Heart Infusion (BHI) and incubation at 37°C for 24h, followed by coagulase test in tube with rabbit plasma, incubated at 37°C for 24h.

Alternative confirmation procedure: Stab inoculation of 5 characteristic colonies on RP + BPF plates, incubated at 37°C for 24h.

Inclusivity (sensitivity) and exclusivity (specificity) performance criteria were calculated for the two methods:

- inclusivity is defined as the number of true positives divided by the sum of true positives plus false negatives, expressed as a percentage;
- exclusivity is defined as the number of true negatives divided by the sum of true negatives plus false positives, expressed as a percentage.

Positive predictive values (PPV) and negative predictive values (NPV) were also calculated:
PPV is defined as the number of presumptive positive results confirmed as CPS, divided by the number of presumptive positive results;
- NPV is defined as the number of presumptive negative results confirmed as *Staphylococcus* other than CPS, divided by the number of presumptive negative results, expressed as a percentage.

3- Results

The results are detailed in Annex 1 and synthesized in Table 1.

Table 1 : Comparison of performance criteria of the two confirmation procedures

Strains (31 CPS strains and 21 other *Staphylococcus* strains)			
Presumptive values of methods		Reference method (EN ISO 6888-1)	Alternative method (BP + RPF)
	Inclusivity	87%	87%
	Exclusivity	100%	100%
	PPV	100%	100%
	NPV	84%	84%

Inclusivity (%) = true positives / (true positives + false negatives) x 100
Exclusivity (%) = true negatives / (true negatives + false positives) x 100
Positive Predictive Values (PPV %) = true positives / (true positives + false positives) x 100
Negative Predictive values (NPV %) = true negatives / (true negatives + false negatives) x 100

- Reference method (coagulase test)

On 31 tested CPS strains, four strains didn't show a coagulase reaction with rabbit plasma. Presumptive inclusivity of the method was 87%.

All 23 negative strains didn't give typical colonies on Baird Parker plates and didn't show a coagulase reaction with rabbit plasma. Presumptive exclusivity of the method was 100%.

The PPV of the method was 100% and the NPV was 84%.

- BP+RPF

The results are detailed in Annex 1.

On 31 tested pure CPS strains, four strains didn't give typical colonies on BP+RPF plates. Alternative method presumptive inclusivity was 87%.

All 23 negative strains didn't give typical colonies on Baird Parker plates and didn't give typical colonies on BP+RPF plates. Presumptive exclusivity of the method was 100%.

The PPV of the method was 100% and the NPV was 84%.

Compared to the EN ISO 6888- part1 reference method, the alternative method had a satisfactory inclusivity (87 % for reference method and BP-RPF method) and exclusivity (100% reference method and BP-RPF method). No difference was observed between the reference method and the alternative method.

However two strains of *Staphylococcus aureus* were not detected with both the alternative method and the reference method.
The strain 07HMPA51 having caused food-poisoning was characterized with the sequence ARN 23s as a toxinogenic *Staphylococcus aureus*, but did not produce coagulase. The strain 85 CHPL1 was characterized as *Staphylococcus aureus* and produced coagulase.

Moreover two strains of *Staphylococcus intermedius* were not detected with both the alternative method and the reference method (strains n° 409F and 408).

IV. ANALYSIS OF NATURALLY CONTAMINATED SAMPLES

The purpose of this study was to verify that the results obtained with the alternative method had no bias compared with the results obtained with the reference method.

31 foods samples, implicated in a food-borne outbreak, were submitted to the entire enumeration protocol; 10g test portions were analysed for each sample. The two confirmation methods were used in parallel for each sample.

Details on the sample preparation are provided in Annex 2.

The results of the alternative method are not scattered around the regression line (figure 1).
The alternative method's response increases with an increase in the reference method's response according to a linear relation (equation of the linear relationship: alternative method ($\log(cfug.^{-1})$) = 0.9962 reference method ($\log(cfu.g^{-1})$) +0.0369, R^2= 0.9912).
One result observed deviates from the linear relationship. Nevertheless in Annex1, Table 1 shows that the tested method gave similar result. Intercept is very near to zero (0.0339) and confidence interval is [-0.1735;0.2474] at 5 %.. The regression line is very near to one (0.9962) and confidence interval is [0.9592;1.0332] at 5 %.

Typical and atypical colonies were observed on the BP plates. However, atypical colonies were tested with the two methods. All atypical colonies (grey-black not surrounded by a clear zone and without opaque rings) showed a negative coagulase test.

V. CONCLUSION

Performance of the alternative method and the reference method were similar. Consequently these two methods could be used interchangeably as confirmatory step in the EN ISO 6888-1 method.

A number of the CPS strains tested (13%) did not produce a positive coagulase reaction with both the alternative method and the reference method, which decreased the performance of both procedures in terms of sensitivity.

The absence of coagulase positive reaction has already been reported for some *Staphylococcus aureus* strains, and some strains produce a lower quantity of coagulase than necessary for detection with the confirmation method. Moreover one *Staphylococcus aureus* strain initially characterized as coagulase positive, did not produce a positive coagulase reaction, suggesting that its biochemical profile could have been altered during its storage.

In this context, additional tests besides the coagulase reaction, such as sequencing of 23s RNA, PFGE, ribotyping, or PCR techniques, could be useful in addition to the biochemical identification in order to provide further evidence of the identity of the isolates.

As a result of this study, we can recommend to ISO/TC 34/SC 9, in charge of the Standard EN ISO 6888-1, the following modification of the confirmation step in the standard: the possibility to use a stabbing on BP +RPF medium, as an alternative to the coagulase test tube method.

The alternative confirmation step procedure could be used as an optional confirmation procedure, in alternative to the coagulase tube test of the current EN ISO 6888-1, that would be easier, quicker and less expensive to perform than the coagulase test.

VI. BIBLIOGRAPHY

[1] Standard EN ISO 16140: 2003 " Microbiology of food and animal feeding stuffs -- Protocol for the validation of alternative methods"

[2] Standard ISO 6888-1: 1999 "Horizontal method for the enumeration of coagulase positive staphylococci (*Staphylococcus aureus* and other species) -- Part 1: Technique using Baird-Parker agar medium"

ANNEX 1

RESULTS OF THE STUDY OF THE INCLUSIVITY AND EXCLUSIVITY

	Strain	Reference designation	Origin	Reference Method (NF EN ISO 6888-1)	BP-RPF method
				log (cfu.g^{-1})	log (cfu^{-1})
Posistive strain	*Staphylococcus aureus*	80CHPL1	French pastry	8,2	8,2
	Staphylococcus aureus	01CHPL2	goat milk	7,8	7,8
	Staphylococcus aureus	07HMPA50A	"Camembert" cheese	9,1	9,1
	Staphylococcus aureus	HMPL58	unknown	9,2	9,2
	Staphylococcus aureus	07HMPA49	unknown	9,2	9,2
	Staphylococcus aureus	05CHPL68	cheese	9,1	9,1
	Staphylococcus aureus	01CHPL4	chocolate cake	9,1	9,1
	Staphylococcus aureus	92CHPL1	contaminated carrier	9,1	9,1
	Staphylococcus aureus	07HMPA48	unknow	8,8	8,8
	Staphylococcus aureus	81CHPL01	fresh cheese	9,1	9,1
	Staphylococcus aureus	01CHPL3	"Cantal" cheese	8,9	8,9
	Staphylococcus aureus	05CHPL69	mussel	9,0	9,0
	Staphylococcus aureus	02CHPL128	raw milk	8,7	8,7
	Staphylococcus aureus	84CHPL1	pasta	7,7	7,7
	Staphylococcus aureus	86CHPL1	ewe cheese	9,1	9,1
	Staphylococcus aureus	07CEB159	crushed "Tome" cheese	9,9	9,9
	Staphylococcus aureus	08CEB320	cheese	10,9	10,9
	Staphylococcus aureus	08CEB341	spiny lobster	10,4	10,4
	Staphylococcus intermedius canin	303E	unknown	9,4	9,4
	Staphylococcus aureus	07CEB93	hamburger	10,1	10,1
	Staphylococcus intermedius	410 F	unknown	9,7	9,7
	Staphylococcus aureus	07CEB100	cheese	10,0	10,0
	Staphylococcus aureus	07CEB91	cake	10,0	10,0
	Staphylococcus intermedius	288D	dog skin	9,7	9,7
	Staphylococcus aureus	38A	unknown	9,4	9,4
	Staphylococcus aureus	07HMPA51	coconut cake	<1	<1
	Staphylococcus aureus	85CHPL1	cheese	<1	<1
	Staphylococcus intermedius	409 F	unknown	<1	<1
	Staphylococcus intermedius	408	unknown	<1	<1
	Staphylococcus aureus	07HMPA244	"Reblochon“ cheese	8,2	8,2
	Staphylococcus aureus	07HMPA132	cheese	8,0	8,0

	Strain	Reference designation	Origin	Reference Method (NF EN ISO 6888-1)	BP-RPF method
				log (cfu.g^{-1})	log (cfu.$^{-1}$)
Negative strain	*Staphylococcus epidermidis*	06CHPL03	horse skin	<2	<2
	Staphylococcus caprae	CHPL22	goat milk	<2	<2
	Staphylococcus lentus	CHPL52	goat's udder	<2	<2
	Staphylococcus equorum	CHPL20	horse skin	<2	<2
	Staphylococcus sciuri	06CHPL15	bull skin	<2	<2
	Staphylococcus haemolyticus	02CHPL125	raw milk	<2	<2
	Staphylococcus simulous	CHPL53	human skin	<2	<2
	Staphylococcus copitis	CHPL 49	unknown	<2	<2
	Staphylococcus hominis	CHPL51	human skin	<2	<2
	Staphylococcus warnari	06CHPL14	human skin	<2	<2
	Staphylococcus haemolyticus	02CHPL123	raw milk	<2	<2
	Staphylococcus chromogénos	06CHPL24	pig skin	<2	<2
	Staphylococcus lentus	02CHPL122	raw milk	<2	<2
	Staphylococcus cohnii	06CHPL23	human skin	<2	<2
	Staphylococcus haemolyticus	02CHPL126	raw milk	<2	<2
	Staphylococcus haemolyticus	CHPL50	human skin	<2	<2
	Staphylococcus haemolyticus	CHPL124A	raw milk	<2	<2
	Staphylococcus haemolyticus	02CHPL127	raw milk	<2	<2
	Staphylococcus	07CEB243	cheese	<2	<2
	Staphylococcus	07CEB147	rice salad with vegetable	<2	<2
	Staphylococcus pasteurii	53B	goat milk	<2	<2
	Staphylococcus hyicus	40A	unknown	<2	<2
	Staphylococcus epidermidis	172C	goat milk	<2	<2

ANNEX 2

ANALYSIS OF NATURALLY CONTAMINATED SAMPLES

1- Procedure

Most of the naturally contaminated samples were received from LCSV (Laboratoire Central des Services Vétérinaires), which performed bacterial analysis for official controls, prescribed by the French competent authority. Samples were received and stored frozen at –18°C. They were thawed a night before use at 3°C +/- 2°C.
The same 10 fold dilution of naturally contaminated foods, was analysed in parallel both with the reference method and alternative method.

2- Results

The raw results per category are given in table see below.
The regression line are given in Figure 1, for data $\log_{10}$-transformed.

CPS was detected in 18 samples tested. Most of sample was performed twice. 13 analysis was negative, which could be explained by the low level of contamination of this product and the decrease of the inoculums size during the freezing.

All typical colonies obtained on BP plates gave an excellent identification percentage (100%).

Results of naturally contaminated samples

N°sample	Date of analysis	Product	Reference Method (ISO 6888-1)				BP-RPF method			
			duplicat a (cfu.g^{-1})	duplicat a log (cfu.g^{-1})	duplicat b (cfu.g^{-1})	duplicat b log(cfu.g^{-1})	duplicat a (cfu.g^{-1})	duplicat a log (cfu.g^{-1})	duplicat b (cfu.g^{-1})	duplicat b log(cfu.g^{-1})
07HMPA250	19/04/2007	Milk powder	<100	<2,0	<100	<2,0	<100	<2,0	<100	<2,0
07HMPA251	19/04/2007	cottage cheese	<100	<2,0	<100	<2,0	<100	<2,0	<100	<2,0
07HMPA252	19/04/2007	cheese strainer	<100	<2,0	<100	<2,0	<100	<2,0	<100	<2,0
07HMPA253	19/04/2007	cottage cheese	<100	<2,0	<100	<2,0	<100	<2,0	<100	<2,0
07HMPA254	19/04/2007	fresh cheese	<100	<2,0	<100	<2,0	<100	<2,0	<100	<2,0
07HMPA292	14/05/2007	ravioli	2,60E+05	5,4	-	-	2,60E+05	5,4	-	-
07HMPA557	11/07/2007	semolina with vegetable	4,50E+03	3,7	<10000	<4,0	3,20E+04	4,5	<10000	<4,0
07HMPA558	11/07/2007	semolina with vegetable	<10000	<4,0	<10000	<4,0	<10000	<4,0	<10000	<4,0
07HMPA559	11/07/2007	cheese	5,00E+04	4,7	1,80E+04	4,3	5,00E+04	4,7	1,80E+04	4,3
07HMPA560	11/07/2007	cheese	<100	<2,0	<100	<2,0	<100	<2,0	<100	<2,0
07HMPA561	11/07/2007	cottage cheese	<100	<2,0	<100	<2,0	<100	<2,0	<100	<2,0
07HMPA567	30/07/2007	pasta	<100	<2,0	<100	<2,0	<100	<2,0	<100	<2,0
07HMPA569	30/07/2007	bechamel sauce	<100	<2,0	<100	<2,0	<100	<2,0	<100	<2,0
07HMPA758	22/10/2007	coconut cake	9,90E+07	8,0	-	-	8,60E+07	7,9	-	-
08HMPA64	28/01/2008	coconut cake	5,20E+06	6,7	3,70E+06	6,6	5,20E+06	6,7	3,70E+06	6,6
08HMPA65	28/01/2008	coconut cake	8,50E+07	7,9	1,30E+08	8,1	8,50E+07	7,9	1,50E+08	8,2
08HMPA66	28/01/2008	coconut cake	1,90E+08	8,3	1,30E+08	8,1	1,90E+08	8,3	1,30E+08	8,1
08HMPA67	28/01/2008	coconut cake	1,10E+08	8,0	9,20E+07	8,0	1,10E+08	8,0	9,20E+07	8,0
08HMPA68	28/01/2008	coconut cake	1,70E+04	4,2	2,10E+04	4,3	1,70E+04	4,2	2,10E+04	4,3
08HMPA69	28/01/2008	coconut cake	4,50E+01	1,7	9,00E+01	2,0	<100	<2,0	4,50E+01	1,7
08HMPA70	28/01/2008	coconut cake	3,10E+02	2,5	1,30E+03	3,1	3,10E+02	2,5	1,30E+03	3,1
08HMPA352	26/05/2008	cheese	3,20E+05	5,5	3,30E+05	5,5	3,20E+05	5,5	3,30E+05	5,5
08HMPA353	26/05/2008	cheese	>150	>2,2	>150	>2,2	>150	>2,2	>150	>2,2
08HMPA354	26/05/2008	cheese	2,90E+04	4,5	3,30E+04	4,5	2,90E+04	4,5	3,30E+04	4,5
08HMPA355	26/05/2008	cheese	9,30E+04	5,0	1,10E+05	5,0	9,30E+04	5,0	1,10E+05	5,0
08HMPA356	26/05/2008	fish hash	1,10E+05	5,0	1,20E+05	5,1	1,10E+05	5,0	1,20E+05	5,1
08HMPA357	26/05/2008	cottage cheese	-	-	-	-	-	-	-	-
08HMPA358	26/05/2008	cheese	<100	<2,0	<100	<2,0	<100	<2,0	<100	<2,0
08HMPA359	26/05/2008	cheese	<100	<2,0	<100	<2,0	<100	<2,0	<100	<2,0
08HMPA360	26/05/2008	cheese	3,80E+04	4,6	1,90E+04	4,3	3,80E+04	4,6	1,60E+04	4,2
08HMPA361	26/05/2008	sausage	3,60E+03	3,6	<100	<2,0	3,60E+03	3,6	<100	<2,0

Figure 1 : regression line

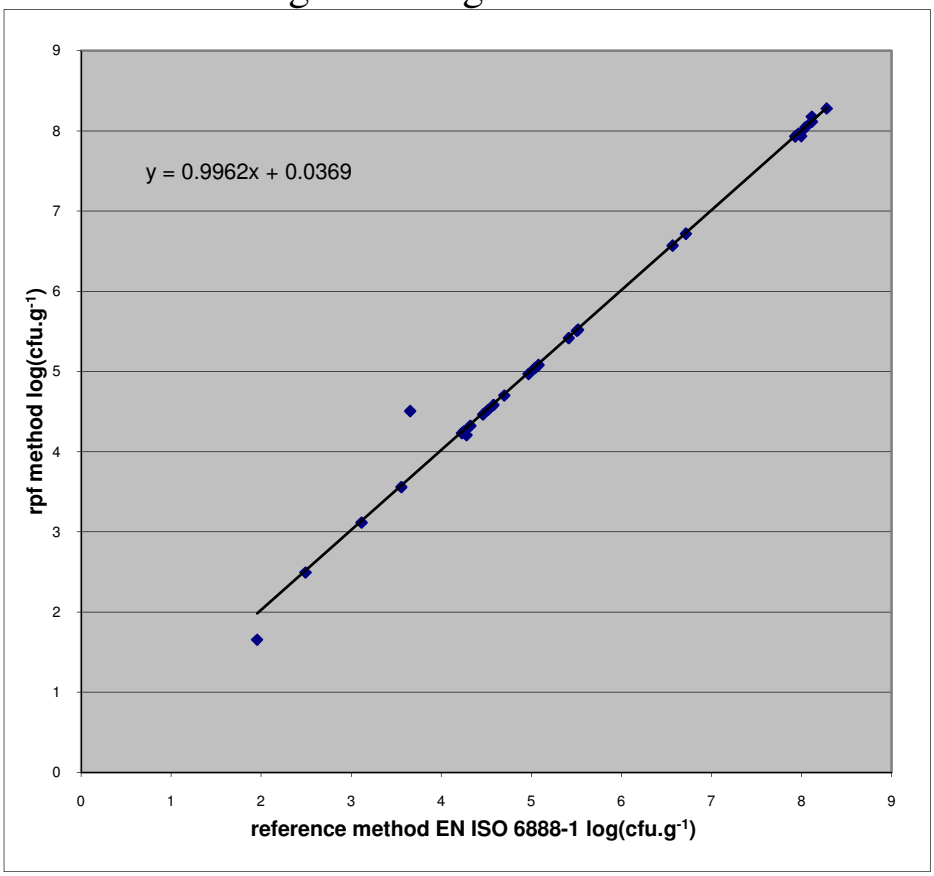

Tableau 2: Results

Regression Statistics	
Multiple R	0,9956
R-Square	0,9912
Ajusted R-Square	0,9909
Standard error	0,1743
Observations	29

ANOVA

	degree of freedom	Square sums	Square means	F	Significance R
Regression	1	92,5910	92,5910	3047,2853	2,6478E-29
Residual	27	0,8204	0,0304		
Total	28	93,4114			

	Coefficients	Standard Error	T Stat	P-values	Lower 95%	Upper 95%
Intercept	0,0369	0,1026	0,3600	0,7217	-0,1735	0,2474
X variable	0,9962	0,0180	55,2022	2,65E-29	0,9592	1,0332