

**LABORATOIRES 3M SANTE**  
Boulevard de l'Oise  
95029 CERGY PONTOISE CEDEX

**AFNOR Validation of Alternative Methods**  
*FOOD MICROBIOLOGY*

**REPORT**

*(Preliminary and Collaborative studies  
according to the NF EN ISO 16140 standard)*

**ISO 16140 validation of  
3M™ Petrifilm™ Coliforms count plates (CC)  
for gas producing colonies enumeration**

Quantitative method

This report includes 25 pages, with 4 annexes.  
Only copies including the totality of this report are authorised.

**PCC Coliforms gas producers Synthesis  
(Version 2) September 3, 2008**

*Cancel and replace the previous version which must be returned  
to ADRIA Développement or destroyed internally*

**ADRIA DEVELOPPEMENT**

Creac'h Gwen - F. 29196 QUIMPER Cedex - Tél. (33) 02.98.10.18.18 - Fax (33) 02.98.10.18.08  
E-mail : [adria.developpement@adria.tm.fr](mailto:adria.developpement@adria.tm.fr) - Site web : <http://www.adria.tm.fr> - Site réservé adhérents : <http://www.clubiaa.net>  
ASSOCIATION LOI DE 1901 - N° SIRET 306 964 271 00036 - N° EXISTENCE 532900006329 - N° TVA FR4530696427100036

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The modifications brought to the report are indicated by a double line in the margin on the left.

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## *Foreword*

Competences of the laboratory are certified by COFRAC accreditation for the analysis marked with symbol♦.

Any element which allows analysis quality certification can be consulted by 3M SANTE.

The results are presented according to the NF EN ISO 16140 standard.

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- ✓ **Firm:** 3M SANTE  
Boulevard de l'Oise  
F-95029 CERGY PONTOISE CEDEX
  
- ✓ **Expert laboratory:** ADRIA Développement  
ZA Creac'h Gwen  
F\*29196 QUIMPER Cedex
  
- ✓ **Studied method:** ISO 16140 validation of 3M™ Petrifilm™ Coliforms count plates (PCC) for gas producing colonies enumeration
  
- ✓ **Validation standard:** NF EN ISO 16140 (October 2003) : Food microbiology – Protocol for the validation of alternative methods
  
- ✓ **Standard method♦ :** NF ISO 4831 (August 2006): Microbiology of food and animal feeding stuffs. Horizontal method for the detection and enumeration of coliforms. Most probable number technique
  
- ✓ **Validation area:** All food products except raw shellfish

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♦ NF ISO 4831 : analysis performed according to the COFRAC accreditation

# 1 INTRODUCTION

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## 1.1 Validation standard

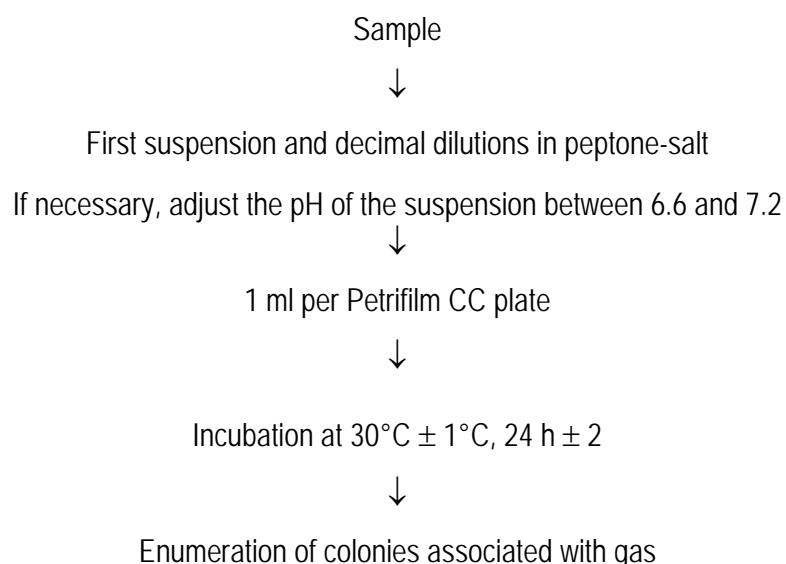
The validation study has been performed according to the NF EN ISO 16140 (October 2003) : Food microbiology – Protocol for the validation of alternative methods.

## 1.2 Protocol and principle of the alternative method

The Petrifilm CC plate is a ready-to-use system for the enumeration of the total coliforms. It is constituted by a cold water soluble dehydrated gel fixed between a support of polyethylene and a sheet leaf in polypropylene. The Petrifilm CC plate is based on the VRBL medium formulation.

After 24 h  $\pm$  2 h of incubation (at 30°C  $\pm$  1°C), gas producing coliform appear as red colonies associated with gas bubbles within one colony diameter , whereas non gas producing coliform appear as red colonies not associated with gas bubbles.

General use instructions are shown in Annex 1 ; the general protocol is presented below :



## 1.3 Application area

All food products except raw shellfish

## 1.4 Standard method

The reference method is the ISO 4831 method “Microbiology of food and animal feeding stuffs. Horizontal method for the detection and enumeration of coliforms. Most probable number technique” (Cf. Annex 2).

## 2 COMPARATIVE STUDY

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### 2.1 Bibliographical study

No papers related to the alternative method have been published since the last study which has been done in May 2002.

### 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 Food matrices and protocols

Five food categories (category/strain) were tested. Five contamination levels were evaluated and two repetitions were realised by sample

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

Category	Souche	Level (CFU/g)	Method to be tested
Minced beef	<i>Enterobacter cloacae</i> 58	100 - 500 - 1 000 - 5 000 - 50 000	ISO 4831 ; PCC 30°C
Milk	<i>Enterobacter sakazakii</i> 95	100 - 500 - 1 000 - 5 000 - 50 000	ISO 4831 ; PCC 30°C
Egg product	<i>Klebsiella pneumoniae</i> 89	100 - 500 - 1 000 - 5 000 - 50 000	ISO 4831 ; PCC 30°C
Raw fish	<i>Escherichia coli</i> Ad 228	100 - 500 - 1 000 - 5 000 - 50 000	ISO 4831 ; PCC 30°C
Green peas	<i>Escherichia coli</i> 19	100 - 500 - 1 000 - 5 000 - 50 000	ISO 4831 ; PCC 30°C

#### 2.2.2 Results

The bi-dimensional graphs are shown in Annex 3.

### 2.2.3 Statistical interpretations

Statistical interpretation results are shown in this table.

Matrice	R	Selected regression	Rob.F	Critical value	P%	Correlation coefficient	Regression equation*
Minced beef	0,06	OLS2	3,1.10 <sup>7</sup>	5,41	0	0,98	log Ref. = 1,088 log Alt. - 0,335
Milk	0,12	OLS2	4,80	5,41	6	0,93	log Ref. = 0,865 log Alt. - 0,677
Egg product	0,29	OLS2	5,22	5,41	5	0,95	log Ref. = 1,245 log Alt. - 0,672
Green peas	3,33	OLS1	0,753	5,41	57	0,98	log Alt. = 1,077 log Ref. - 0,432
Raw fish	0,26	OLS2	0,000	5,41	100	0,98	log Ref. = 1,033 log Alt. + 0,094

\* x-axis and y-axis choice depends on the selected regression.

Statistical interpretation:

P > 5 % : not significant

1 % < P < 5 % : significant

P < 1 % : highly significant

The regressions lines are shown in Annex 3.

### 2.2.4 Conclusion

The linearity is accepted for the following categories: milk, egg, product, green peas and raw fish.

Coefficient correlations are equal to 0,98 for minced beef, green peas and raw fish, these high values decrease the linearity test robustness. The linearity cannot be refuted for the matrix minced beef.

## 2.3 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.*

The results obtained in 1997 were interpreted according to the ISO 16140. The samples were the following:

	PCC 30°C / ISO 4831
Meat products	47
Dairy products	51
Egg products and pastries	26
Seafood	20
Vegetables	38
Cooked products	44
Total analysed	226
Total exploited	76

### 2.3.1 *Number and nature of the samples*

Categories and types of analysed food are presented table 1.

**Table 1 – Number and nature of the samples**

Categories	Types	Number of samples analysed	Number of results used
Meat products	Raw meat, raw and cooked delicatessen, cooked products	73	50
Dairy products	Raw milk, cheeses, ice creams, creams	51	30
Egg products and pastries	Mayonnaise, egg products, pastries	26	18
Vegetables	Frozen vegetables, salads, spices, flours, cooked vegetables	41	19
Seafood	Fishes and raw shellfish, smoked products, cooked products	35	12

Only naturally contaminated samples were analysed.

### 2.3.2 *Results*

Samples were analysed in duplicate by the Petrifilm CC plate (colonies associated with gas) and in simple by the ISO 4831 method.

The analysed samples presented the following levels of contamination:

**Table 2 - Petrifilm CC plate (colonies associated with gas) / ISO 4831**

Food products	Contamination scale (log)
Meat products	0,56 to 5,97
Dairy products	1,00 to 5,04
Egg products and pastries	0,46 to 6,16
Vegetables	0,63 to 6,04
Seafood	0,95 to 5,66

Bidimensional graphs for each category and for all analysed samples are presented Annex 4.

### 2.3.3 Interpretation

**Table 3 - Petrifilm CC plate (colonies associated with gas) / ISO 4831**

Category	n	R	Regression used	a	t(a)	b	t(b)	Critical T	P%	
									Ordinate at 0	Slope at 1
Meat products	50	/	GMFR	0,295	0,832	0,916	0,918	2,011	41	36
Dairy products	30	/	GMFR	- 0,682	1,560	1,123	0,880	2,048	13	39
Egg products and pastries	18	/	GMFR	0,030	0,122	0,940	0,981	2,120	90	34
Vegetables	19	/	GMFR	0,453	1,250	0,871	1,167	2,110	23	26
Seafood	12	/	GMFR	- 0,073	0,284	0,939	0,864	2,228	78	41
All products	129	/	GMFR	- 0,009	0,068	0,964	1,023		95	31

*Statistical interpretation:*

P > 5 % : not significant

1 % < P < 5 % : significant

P < 1 % : highly significant

Category	Bias D	Alternative method repeatability limit	Reference method repeatability limit
Meat products	- 0,089	0,291	/
Dairy products	- 0,396	0,383	/
Egg products and pastries	- 0,180	0,330	/
Vegetables	- 0,021	0,275	/
Seafood	- 0,299	0,351	/
All products	- 0,220	0,365	/

### 2.3.4 Conclusion

According to the statistical tests, the intercept at 0 and the slopes at 1 are validated in every case. The Petrifilm CC plate shows satisfying relative accuracy.

NB : the bi-dimensional graphs show a light underestimate of the coliforms gas producers populations by the alternative method for the category Dairy products: the slope is equal to 1,123 and the intercept to 0 is - 0,682; the Petrifilm CC plate and the ISO 4831 method are comparable according to the P values, i.e. respectively 39 % and 13 %. For the category Vegetables, the intercept is 0,453 and the slope is 0,871: the Petrifilm CC plate and the ISO 4831 method are comparable according to the P Values, i.e. respectively 23 % and 26 %.

Graphs including ISO 4831 confidence intervals are presented figures 1 and 2. The Petrifilm CC plate results are all close to or integrated into the ISO 4831 confidence intervals.

Two exceptions are only observed for dairy products; the ISO 4832 values for the total coliforms enumeration are given:

- 1 sample of cheese (Morbier) :
  - enumeration by Petrifilm CC plate: 3,85 et 4,04 Log (CFU/g)
  - enumeration by ISO 4831 method: 2,87 [2,11-3,30] Log (CFU/g)
  - enumeration by ISO 4832 method: not available
- 1 sample of raw milk:
  - enumeration by Petrifilm CC plate: 4,18 et 4,30 Log (CFU/g)
  - enumeration by ISO 4831 method: 1,96 [1,18-2,54] Log (CFU/g)
  - enumeration by ISO 4832 method: 4,76 et 4,95 Log (CFU/g)

Four exceptions can be noted in the Vegetables category:

- 2 samples of raw frozen vegetables:
  - enumeration by Petrifilm CC plate: 3,02 and 3,03 Log (CFU/g)
  - enumeration by ISO 4831 method: 1,88 [1,23-2,30]
  - enumeration by ISO 4832 method: 2,69 et 2,56 Log (CFU/g)
  - and
  - enumeration by Petrifilm CC plate: 3,26 and 3,36 Log (CFU/g)
  - enumeration by ISO 4831 method : 1,97 [1,26-2,56]
  - enumeration by ISO 4832 method: 3,36 and 3,16 Log (CFU/g)

- 2 samples with vegetables preparation :
  - enumeration by Petrifilm CC plate: 1,60 and 1,48 Log (CFU/g)
  - enumeration by ISO 4831 method: 0,63 [-0,05-1,26]
  - enumeration by ISO 4832 method : not available
 and
  - enumeration by Petrifilm CC plate: 2,64 et 2,65 Log (CFU/g)
  - enumeration by ISO 4831 method: 1,18 [0,60-1,58]
  - enumeration by ISO 4832 method: 2,37 and 2,57 Log (CFU/g)

*The possibility of an artefact is avoided by the repeatability of the results obtained by the Petrifilm CC plate. The values generated by the Petrifilm CC plate and the ISO 4832 method are comparable, confirming that colonies associated with gas bubbles by the Petrifilm CC plate correspond to coliforms colonies.*

*Finally, in the same way, two enumerations differences are mainly observed in the Meat products category on 50 exploited results:*

- 1 sample of merguez sausage:
  - enumeration by Petrifilm CC plate: 2,70 and 2,60 Log (CFU/g)
  - enumeration by ISO 4831 method: 4,66 Log (CFU/g)
  - dénombrements par la méthode ISO 4832 : 3,92 and 3,90 Log (CFU/g)
- 1 sample of duck pâté:
  - enumeration by Petrifilm CC plate: 4,73 and 4,64 Log (CFU/g)
  - enumeration by ISO 4831 method: 2,04 Log (CFU/g)
  - enumeration by ISO 4832 method: 5,20 and 5,13 Log (CFU/g)

*The values obtained by the Petrifilm CC plate and the ISO 4832 method are comparable, suggesting that the enumeration of gas producing colonies by the alternative method belong to the coliforms group. But, in that case, the differences could be also considered as outliers points among 80 results.*

Figure 1

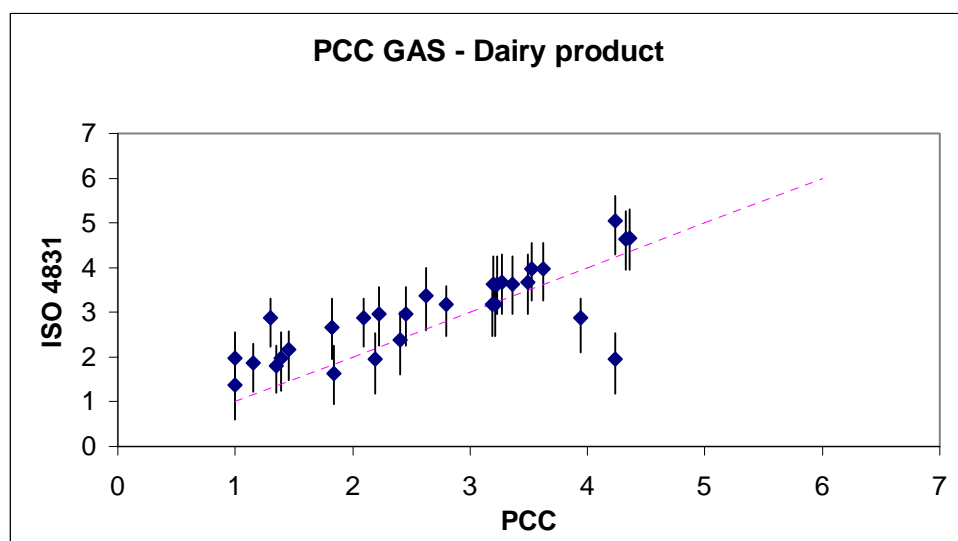
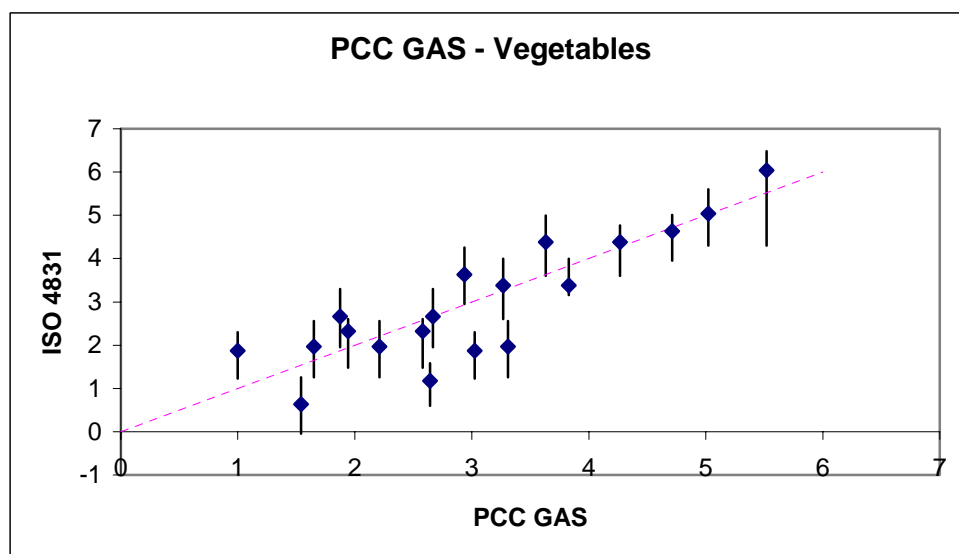


Figure 2



Regression straight lines (graph and equation representations) for each food category and for all matrices are presented in Annex 4.

## 2.4 Detection limit (LOD) and quantification limit (LOQ)

*The critical level is 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  $1-\beta$ , 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.*

### 2.4.1 Protocol

The detection limit of the alternative method has been done with pure cultures. Three different levels of inoculation have been tested, with six replicates per level, i.e. a total of 18 analyses by the alternative method. One *E. coli* strain has been used for the limit of detection of the Petrifilm CC plate. Quantification limit has been calculated for six independent blank samples determinations.

### 2.4.2 Results

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

**Table 4**

Level	Positive samples nb	Standard deviation	Bias
0,5	3 / 6	0,816	0,5
1	3 / 6	1,549	0,5
5	6 / 6	1,751	2

Table 5

	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

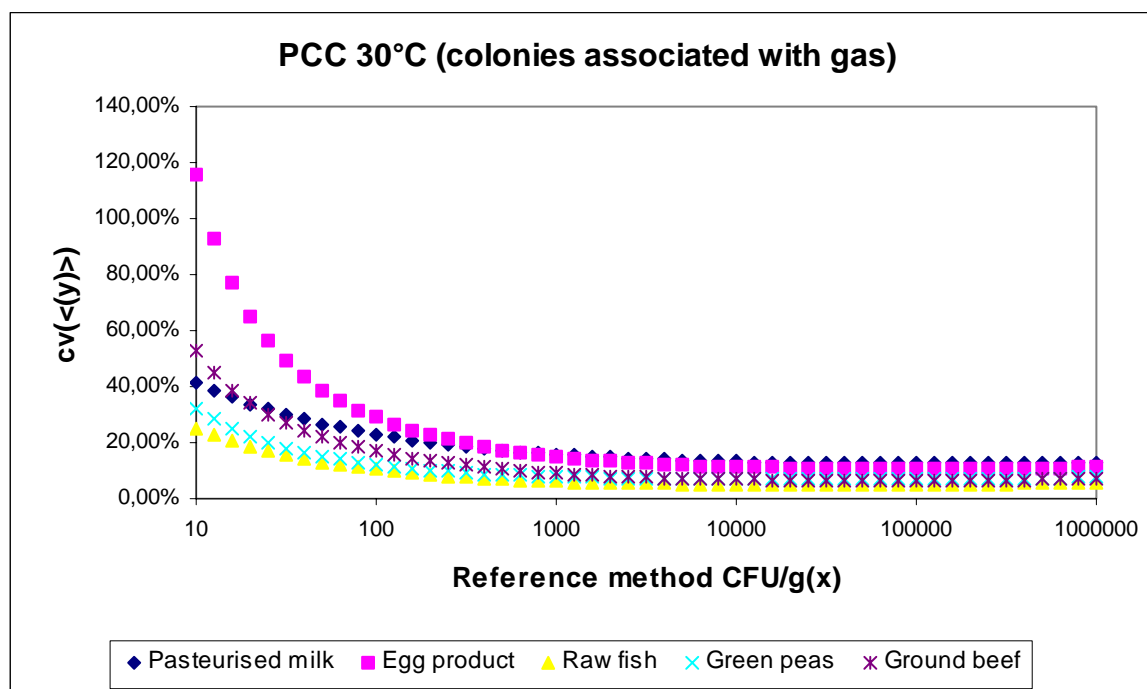
## 2.5 Relative Sensitivity

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  $y$ ).

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

Accuracy patterns obtained for different matrices are presented in figure 3.

Figure 3 – Accuracy patterns for the different matrices used



## 2.6 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 results obtained during the previous validation have been completed in order to fit with the ISO 16140 standard requirements.

### 2.6.1 Inclusivity

30 targeted strains have been tested. The results are similar between both method, except for *Enterobacter sakazakii* 90 which produces gas only on Petrifilm CC plates.

### 2.6.2 Exclusivity

All the 20 tested strains show a negative result by both method.

<b>The Petrifilm CC plate shows a satisfying specificity and selectivity</b>
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## 2.7 Praticability

Petrifilm CC plate allows the saving of time since it is a ready-to-use method.

Time delay to obtain results is much shorter by using the Petrifilm CC plate, contrary to the ISO 4831 standard :

- 24 h  $\pm$  2 h are required by the Petrifilm CC plate for positive and negative results,
- 24 to 48 are required by the ISO standard for negative results,
- 96 h are required by the ISO standard for positive results

No specific maintenance or traceability procedures are required.

### 3 CONCLUSION

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The linearity and the relative accuracy of the Petrifilm CC plate are satisfying.

The Petrifilm CC plate selectivity and specificity are similar to ISO 4831 standard.

The Petrifilm CC plate minimizes :

- the time of manipulation
- the time to obtain results
- the wastes management
- the incubation space
- the manipulation between different steps.

## Annex 1 – Alternative method instruction use

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### 3M™ Petrifilm™

(English)

#### Coliform Count Plate

##### DESCRIPTION

The 3M™ Petrifilm™ Coliform Count (CC) Plate is a sample-ready-culture-medium system which contains Violet Red Bile (VRB) nutrients, a cold-water-soluble gelling agent, and a tetrazolium indicator that facilitates colony enumeration. Petrifilm CC plates are used for the enumeration of coliforms in the food and beverage industries. Petrifilm CC plate components are decontaminated though not sterilized. 3M Microbiology is certified to ISO (International Standards Organization) 9001.

AOAC INTERNATIONAL and the U.S. FDA Bacteriological Analytical Manual (BAM) define coliforms as gram-negative rods, which produce acid and gas from lactose fermentation. Coliform colonies growing on the Petrifilm CC plate produce acid, which causes the pH indicator to deepen the gel color; gas trapped around red colonies indicates coliforms.

ISO defines coliforms by their ability to grow in method-specific, selective media. ISO method 4832, enumerating coliforms by colony-count technique, defines coliforms as acid producers on VRB with lactose (VRBL) agar. On Petrifilm CC plates these acid-producing coliforms are indicated by red colonies with or without gas production. ISO method 4831, enumerating coliforms by the most probable number (MPN) method, defines coliforms by their ability to grow and produce gas from lactose in a selective broth. On Petrifilm CC plates these coliforms are indicated by red colonies associated with gas. AFNOR has validated Petrifilm CC plate in comparison to ISO method 4831 and ISO method 4832 for enumeration of total coliforms. AFNOR has also validated Petrifilm CC plate in comparison to NF V08-017, for enumeration of thermotolerant coliforms.

##### CAUTIONS

3M has not documented Petrifilm CC plates for use in industries other than food and beverage. For example, 3M has not documented Petrifilm CC plates for testing water, pharmaceuticals or cosmetics.

Petrifilm CC plates have not been tested with all possible food products, food processes, testing protocols or with all possible strains of coliform or other bacteria.

The Petrifilm CC plates have been evaluated following AOAC / AFNOR / NordVal guidelines and met validation criteria in representative samples of the following food categories: vegetables, meat, poultry, seafood, dairy, and processed foods.

1

Do not use Petrifilm CC plates in the diagnosis of conditions in humans or animals.

For information on documentation of product performance contact your official 3M Microbiology representative.

##### USER RESPONSIBILITY

No one culture medium will always recover the exact same strains or enumerate a particular strain exactly as does another medium. In addition, external factors such as sampling methods, testing protocols, preparation time and handling may influence recovery and enumeration. The food sample itself may influence results. For example, foods with high sugar content may increase the potential for gas production from non-coliform Enterobacteriaceae.

It is the user's responsibility in selecting any test method to evaluate a sufficient number of samples with particular foods and microbial challenges to satisfy the user that the chosen test method meets the user's criteria.

It is also the user's responsibility to determine that any test methods and results meet its customers' or suppliers' requirements.

As with any culture medium, Petrifilm CC plate results do not constitute a guarantee of quality of food or beverage products or processes that are tested with the plates.

The user must train its personnel in proper testing techniques: for example, Good Laboratory Practices (U.S. Food and Drug Administration, Title 21, Part 58 of the Code of Federal Regulations) or ISO 17025.

##### DISCLAIMER OF WARRANTIES / LIMITED REMEDY

**UNLESS OTHERWISE PROHIBITED BY LAW, 3M DISCLAIMS ALL EXPRESS AND IMPLIED WARRANTIES, INCLUDING, BUT NOT LIMITED TO, ANY WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR USE.** If any 3M Petrifilm plate is proven to be defective, 3M or its authorized distributor will replace or, at its option, refund the purchase price of any plate. These are your exclusive remedies. You must promptly notify 3M within sixty days of discovery of any suspected defect in a product and return the product to 3M. Please call Customer Service (1-800-328-1671 in the U.S.) or your official 3M Microbiology representative for a Returned Goods Authorization.

##### LIMITATION OF 3M LIABILITY

**UNLESS OTHERWISE PROHIBITED BY LAW, 3M WILL NOT BE LIABLE TO USER OR OTHERS FOR ANY LOSS OR DAMAGE, WHETHER DIRECT, INDIRECT, SPECIAL,**

2



**INCIDENTAL OR CONSEQUENTIAL DAMAGES, INCLUDING, BUT NOT LIMITED TO, LOST PROFITS.** Except where prohibited by law, in no event shall 3M's liability under any legal theory exceed the purchase price of the plates alleged to be defective. Customer may have additional rights and should seek advice in country of purchase.

### STORAGE AND DISPOSAL

Store unopened Petrifilm plate pouches refrigerated or frozen at temperatures  $\leq 8^{\circ}\text{C}$  ( $46^{\circ}\text{F}$ ). Just prior to use, allow unopened pouches to come to room temperature before opening. 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. It is recommended that resealed pouches of Petrifilm plates be stored in a freezer (see below) if the laboratory temperature exceeds  $25^{\circ}\text{C}$  ( $77^{\circ}\text{F}$ ) and/or the laboratory is located in a region where the relative humidity exceeds 50% (with the exception of air-conditioned premises).

To store opened pouches in a freezer, place Petrifilm plates in a sealable container. To remove frozen Petrifilm plates for use, open the container, remove the plates that are needed and immediately return remaining plates to the freezer in the sealed container. Plates should not be used past their expiration date. The freezer that is used for open pouch storage must not have an automatic defrost cycle as this would repeatedly expose the plates to moisture which can damage the plates.

Do not use plates that show discoloration. Expiration date and lot number are noted on each package of Petrifilm plates. The lot number is also noted on individual plates.

After use, Petrifilm CC plates may contain microorganisms that may be a potential biohazard. Follow current industry standards for disposal.

### INSTRUCTIONS FOR USE

#### Sample Preparation

1. Use appropriate sterile diluents:

Butterfield's phosphate buffer<sup>1,2</sup> 0.1% peptone water, peptone salt diluent,<sup>3</sup> buffered peptone water,<sup>4</sup> saline solution (0.85-0.90%), bisulfite-free letheen broth or distilled water.

**Do not use diluents containing citrate, bisulfite or thiosulfate with Petrifilm plates; they can inhibit growth.** If citrate buffer is indicated in the standard procedure, substitute with one of the buffers listed above, warmed to  $40\text{--}45^{\circ}\text{C}$ .

AFNOR validated methods:

3

Only ISO diluents listed above and referenced are in the scope of the validation.

2. Blend or homogenize sample.
3. For optimal growth and recovery of microorganisms, adjust the pH of the sample suspension to 6.6 - 7.2. For acidic products, adjust the pH with 1N NaOH. For alkaline products, adjust the pH with 1N HCl.

#### Plating

1. Place the Petrifilm CC plate on a flat, level surface (see figure a).
2. Lift the top film and with the pipette perpendicular dispense 1 mL of sample suspension onto the center of bottom film (see figure b).
3. Roll the top film down onto the sample to prevent trapping air bubbles (see figure c).
4. Place the plastic spreader with the flat side down on the center of the plate (see figure d). 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. Do not slide the spreader across the film.
5. Remove the spreader and leave the plate undisturbed for at least one minute to permit the gel to form.

#### Incubation

Incubate plates in a horizontal position with the clear side up in stacks of no more than 20 plates. Several incubation times and temperatures can be used depending on current local reference methods.

#### For example:

AOAC® Official Methods™ (986.33 Bacteria and Coliform Counts in Milk, Dry Rehydratable Film Methods and 989.10 Bacterial and Coliforms Counts in Dairy Products, Dry Rehydratable Film Methods)

Incubate Petrifilm CC plates 24 h  $\pm$  2 h at  $32^{\circ}\text{C} \pm 1^{\circ}\text{C}$ .

AOAC® Official Methods™ (991.14 Coliform and Escherichia coli Counts in Foods, Dry Rehydratable Film Methods)

Incubate Petrifilm CC plates 24 h  $\pm$  2 h at  $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$ .

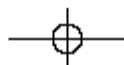
#### AFNOR validated methods

**Total coliforms:** in comparison to ISO 4832 (3M-01/2-09/89 A) and in comparison to ISO 4831 (3M-01/2-09/89 B)

All food except raw shellfish.

- Incubate Petrifilm CC plates 24 h  $\pm$  2 h at  $30^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ,  $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$  or  $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ .

4



- As recommended in ISO 4831 and ISO 4832, these temperatures should be agreed upon between concerned parties and mentioned in the analysis certificate.

**Thermotolerant coliforms:** in comparison to NF V08-060, VRBL incubated at 44.5°C ± 1°C (3M-01/2-09/89 C).  
All food.

- Incubate Petrifilm CC plates 24 h ± 2 h at 44°C ± 1°C.

Nordic System for validation of alternative microbiological methods, NordVal Validation (Ref. No.: 2003-20-5408-00011)

Refer to NordVal validation for Petrifilm CC plate method details.

#### Interpretation

1. Petrifilm CC plates can be counted using a standard colony counter or other illuminated magnifier. Gas produced by coliform may disrupt the colony so that the colony "outlines" the bubble. This should be counted as a single coliform. Do not count colonies on the foam dam since they are removed from the selective influence of the medium. Do not count artifact bubbles that may be present.

The interpretation of coliform colonies on the Petrifilm CC plate varies by method. For example:

#### AOAC Official Methods

Coliform colonies are red and closely associated (within one colony diameter) with entrapped gas. Colonies not associated with gas (a distance greater than one colony diameter between colony and gas bubble) are not counted as coliforms.

#### OR AFNOR validated methods

- As compared to ISO method 4831 (MPN method), coliform colonies are red and closely associated (within one colony diameter) with entrapped gas (see figure e).

Note: a high ratio of non gas-producing gram-negative rods may require a higher dilution in order to enumerate gas producing coliforms within the recommended counting range.

- As compared to ISO method 4832 and NF V08-060 (VRBL methods), count all red colonies with or without gas.

2. The circular growth area is approximately 20 cm<sup>2</sup>. Estimates can be made on plates containing greater than 150 colonies by counting the number of colonies in one or more representative squares and determining the average number per square. Multiply the average number by 20 to determine the estimated count per plate (see figure f); estimations are outside of the AFNOR validation scope.

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3. When present in large numbers, Petrifilm CC plates may have a deepening of the gel color and either or both of the following characteristics: many small, indistinct colonies and/or many gas bubbles. High concentrations of coliforms will cause the growth area to turn dark red (see figure g). When this occurs, record results as too numerous to count (TNTC). When an actual count is required, plate at a higher dilution.
4. Where necessary, colonies may be isolated for further identification. Lift the top film and pick the colony from the gel (see figure h). Test using standard procedures.
5. If the plates cannot be counted within 1 hour of removal from the incubator, they may be stored for later enumeration by freezing in a sealable container at temperatures ≤ minus 15°C for no longer than one week.

For further information refer to the appropriate Petrifilm plate "Interpretation Guide." If you have questions about specific applications or procedures, please contact your official 3M Microbiology representative nearest you.

#### References

1. FDA. 1998. Bacteriological Analytical Manual, 8th ed., Revision A, Appendix 3.64.
2. International Standards Organization, ISO 6887-1:1999. Microbiology of food and animal feeding stuffs – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination
3. International Standards Organization, ISO 8261:2001. Milk and milk products – General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination

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### Explanation of symbols



- Attention, see instructions for use



- The lot in a box and the hourglass symbols are symbols that represent lot number and expiration date. The hourglass is followed by a year and month which represents the expiration date (year and month: 2010-10). The entire line after the hourglass represents the lot number. (2010 - 10 AZ).



- Store below given temperature.

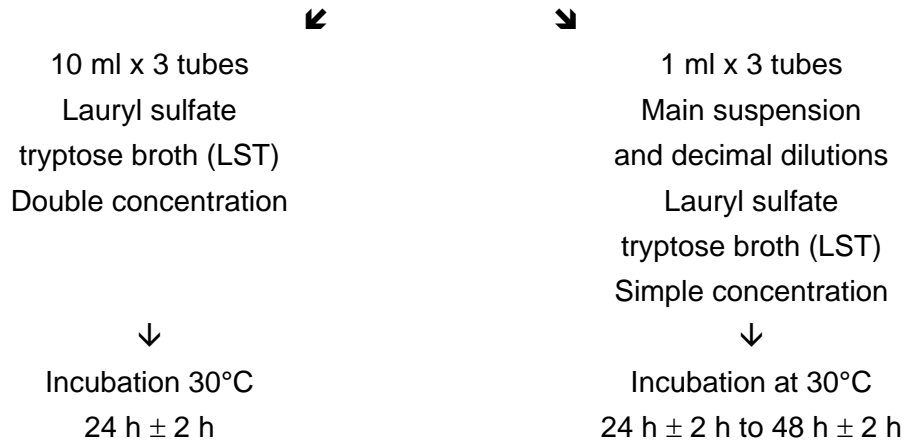
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**Annex 2 – ISO 4831 standard**

Solid sample (1/10 dilution)

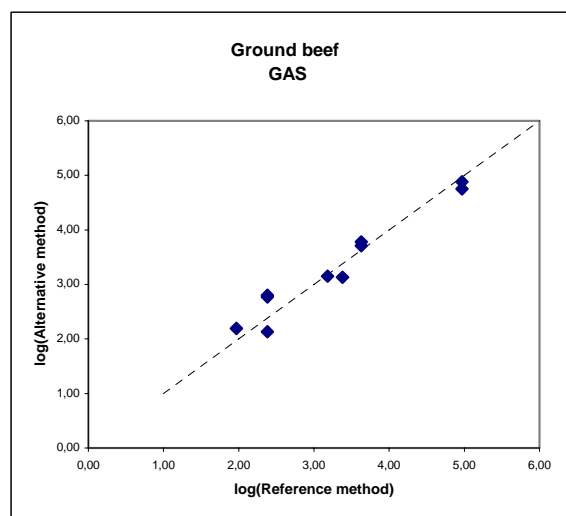
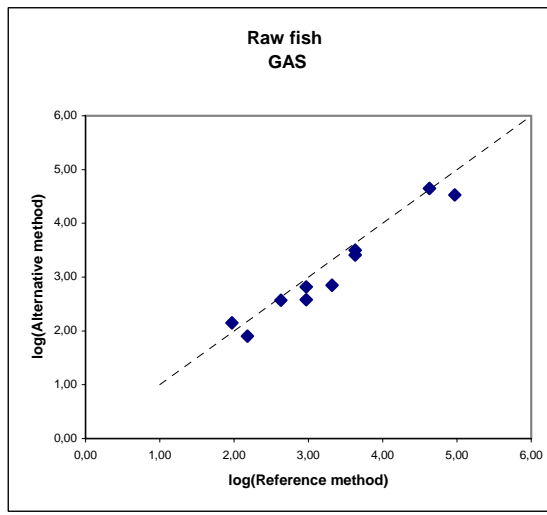
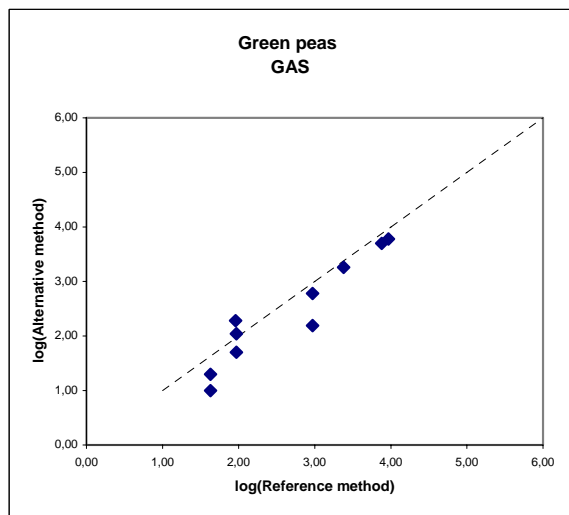
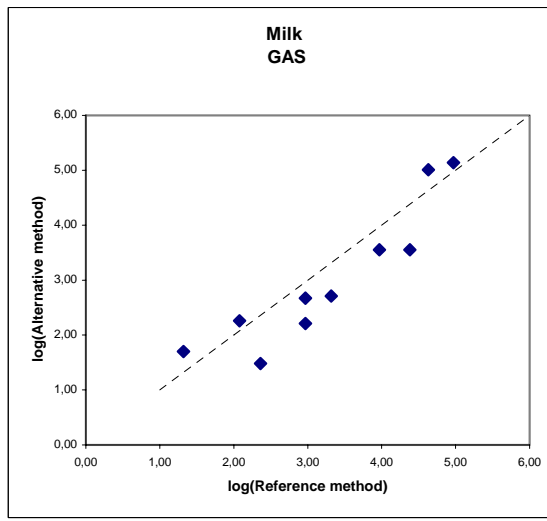
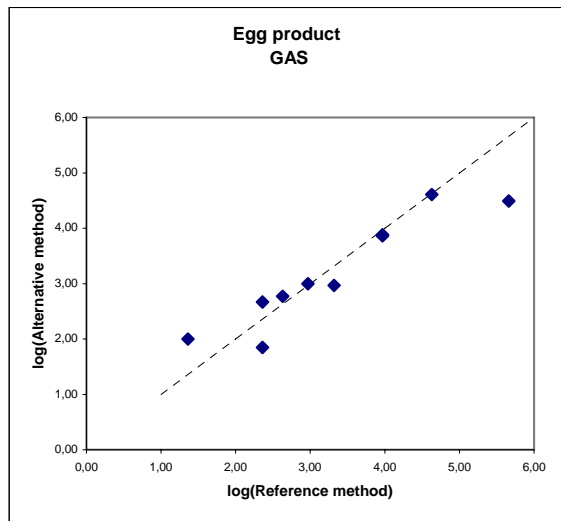
Liquid sample



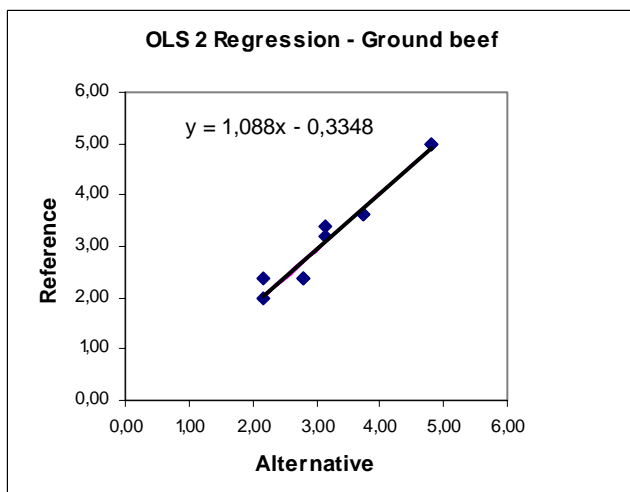
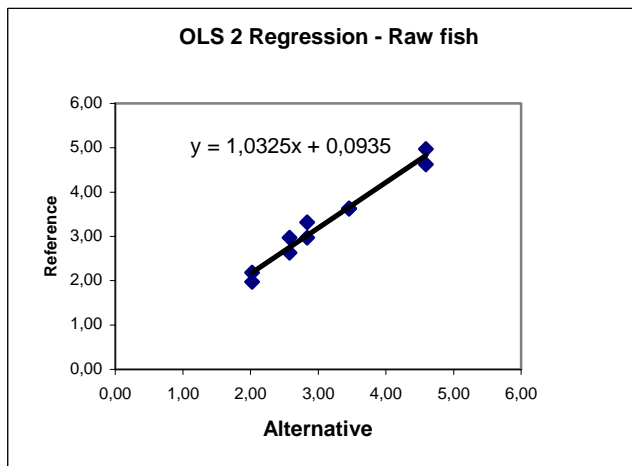
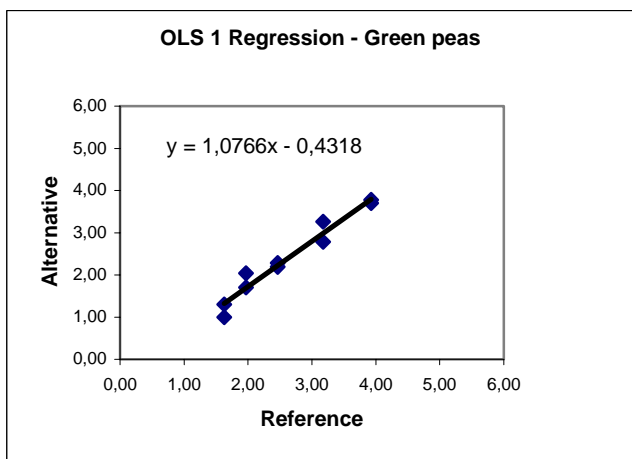
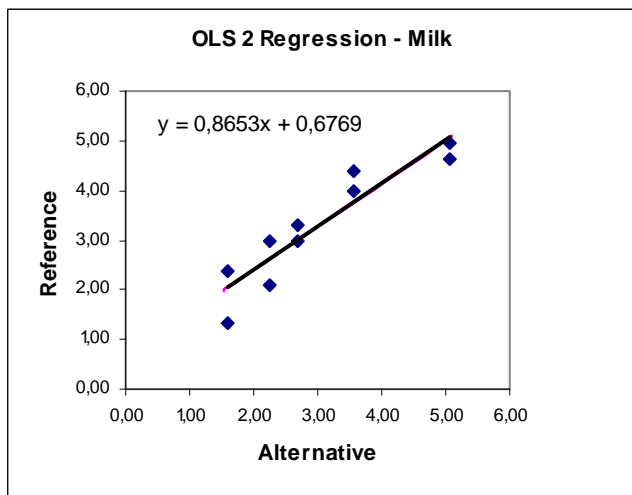
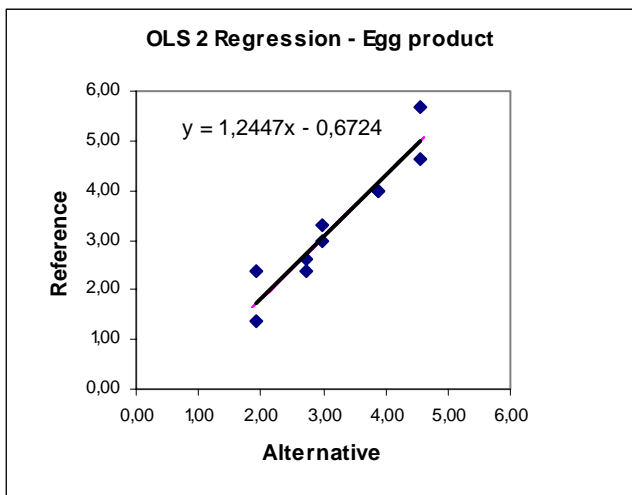
Gas production confirmation	Transfer 10 µl of positive tubes into a brilliant green lactose broth (BLBVB) Incubation at 30°C ± 1 ; 24 h ± 2 h or 48 h ± 2 h
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### Annex 3 – Linearity study

#### Bi-dimensional graphs

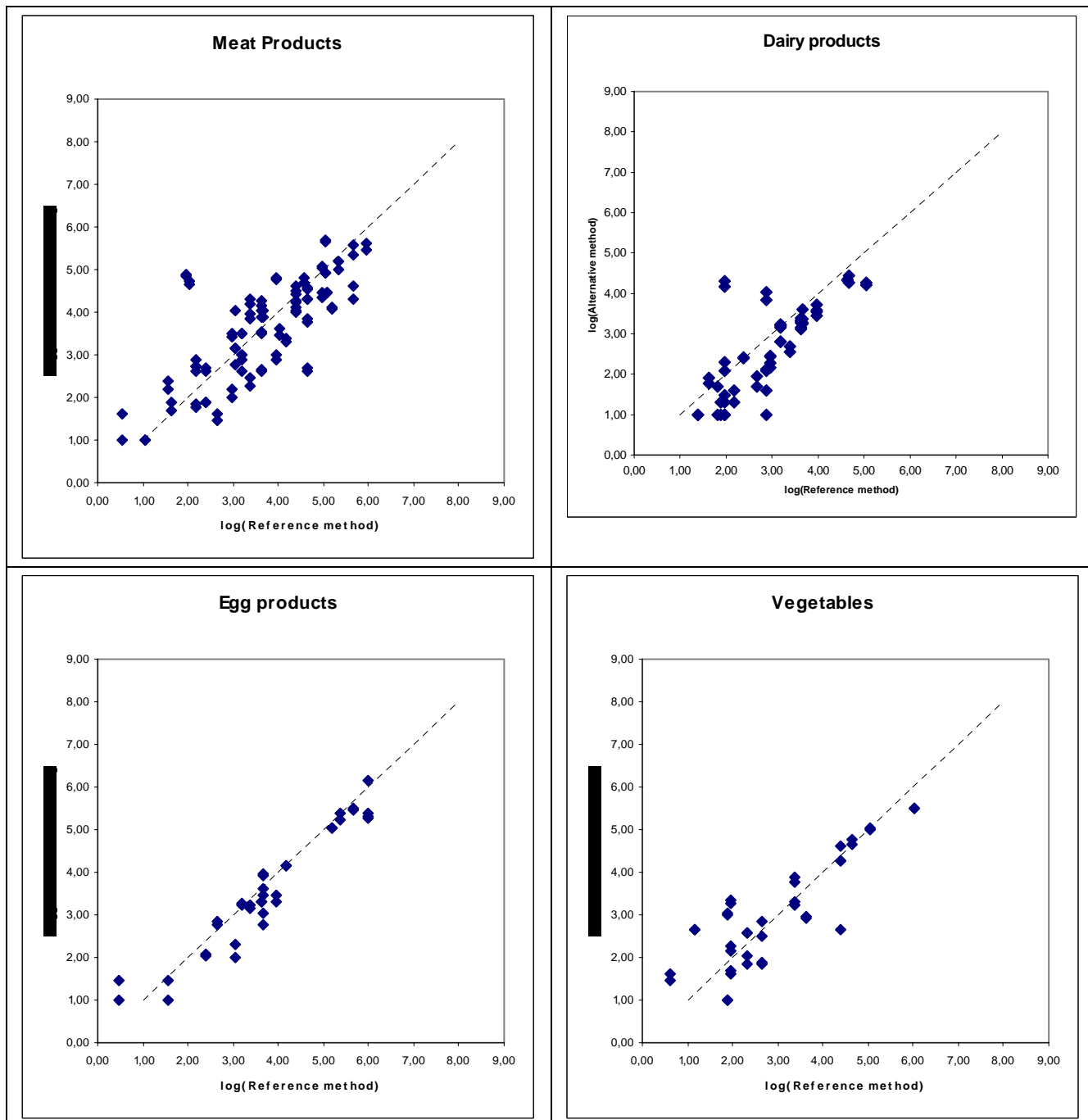


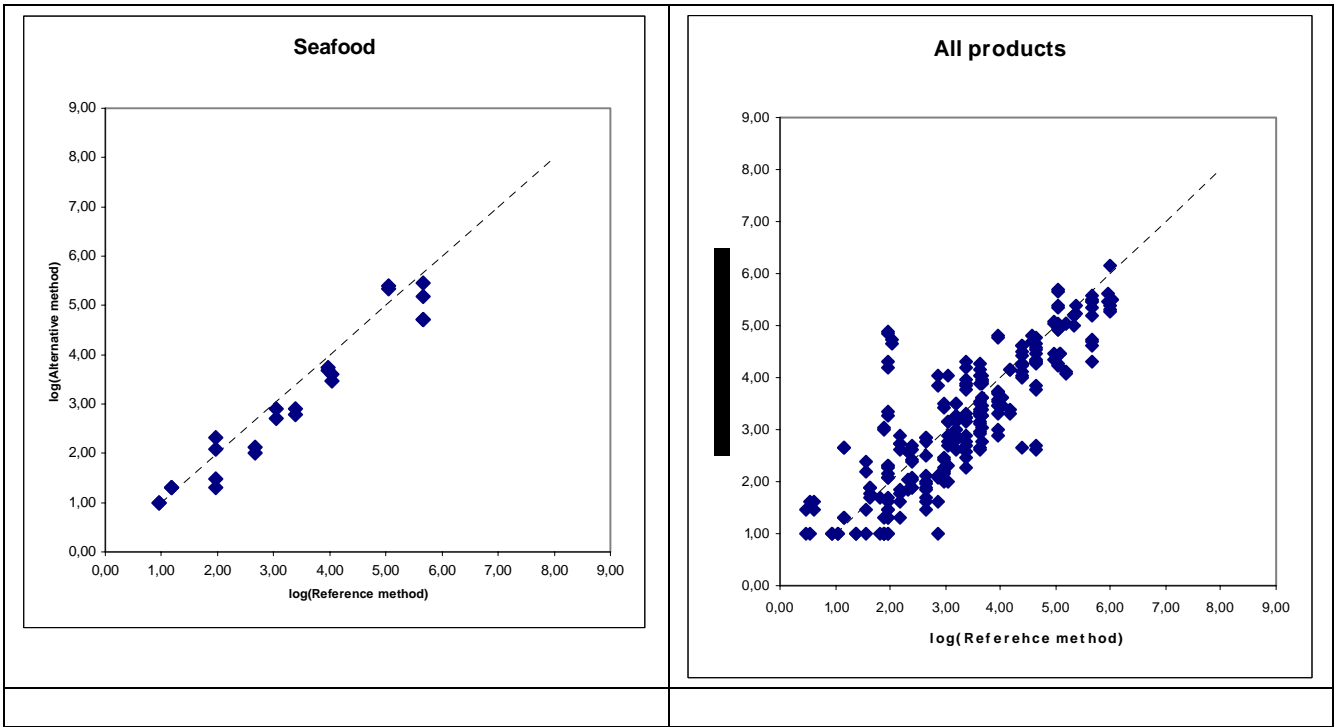
**Regression straight lines**



Annex 4 – Relative accuracy

Bi-dimensional graphs





**Regression straight lines for each food category and for all matrices**