Method Comparison Study Report for the ISO 16140-2:2016 validation of Compact Dry YM, for the enumeration of yeasts and moulds in a broad range of foods

MicroVal study number: 2008LR10

Method/Kit name: Compact Dry YM

Report version:MCS ILS Summary report 03/09/2019

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Foreword

This report is prepared in accordance with ISO 16140-2:2016 and MicroVal Technical Committee interpretation of ISO 16140-2 v.1.0

Company: Nissui Pharmaceutical company Ltd

Expert Laboratory: Campden BRI

Method/Kit name: Compact Dry YM

Validation standard: ISO 16140-2:2016 Microbiology of the food chain —Method validation —Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method

Reference method: ISO 21527-1:2008. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of yeasts and moulds. Colony count technique in products with water activity greater than 0.95.

Scope of validation: A broad range of foods based on categories

1. Dairy products
2. Confectionery bakery and eggs
3. Fruits and vegetables
4. Ready to eat foods
5. Multi component foods or meal components

Certification organisation: Lloyd's Register

List of abbreviations

-	AL	Acceptability Limit
-	AP	Accuracy Profile
-	Art. Cont.	Artificial contamination
-	CFU	Colony Forming Units
-	CL	confidence limit (usually 95%)
-	EL	Expert Laboratory
-	$\bar{D}$	Average difference
-	g	Gram
-	h	Hour
-	ILS	Interlaboratory Study
-	Inc/Ex	Inclusivity and Exclusivity
-	LOQ	Level of Quantification
-	MCS	Method Comparison Study
-	min	minute
-	ml	Millilitre
-	MR	(MicroVal) Method Reviewer
-	MVTC	MicroVal Technical Committee
-	EL	Expert Laboratory
-	n	number of samples
-	na	not applicable
-	neg	negative (target not detected)
-	NG	no growth
-	nt	not tested
-	RT	Relative Trueness
-	SD	standard deviation of differences
-	10^{-1} dilution	10-fold dilution of original food
-	10^{-2} dilution	100-fold dilution of original food
-	VRBA	Violet Red Bile Lactose Agar
-	PSD	Peptone salt diluent

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1 Introduction

In this project a MicroVal validation study, based on ISO 16140-2:2016, of alternative method(s) for the enumeration yeast and moulds in five different food categories was carried out by Campden BRI as the MicroVal Expert Laboratory.

The alternative method used was:

- Enumeration of yeasts and moulds on Compact Dry YM, incubated at 25±1°C for 3-7 days. Both time points were evaluated in this study

The reference method used was:

- ISO 21527-1: 2008. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of yeasts and moulds. Colony count technique in products with water activity greater than 0.95.

Scope of the validation study is: A broad range of foods

Categories included:

- Dairy products
- Confectionery bakery and eggs
- Fruits and vegetables
- Ready to eat foods
- Multi component foods or meal components

Criteria evaluated during the study have been:

- Relative trueness study;
- Accuracy profiles;
- Limits of quantification (LOQ);
- Inclusivity and exclusivity
- Interlaboratory Study

The final conclusion on the Method Comparison Study and ILS is summarized below:

The alternative method Compact Dry YM shows comparable performance to the reference methods (ISO 21527-1:2008) for the enumeration of yeasts and moulds in a broad range of foods.

2 Method protocols

The Method Comparison Study was carried out using 10g gram portions of sample material.

According to ISO 16140-2 the reference method and alternative method were performed with the same sample. The study was therefore a paired study design.

2.1 Reference method

See the flow diagram in Annex A.

Sample preparations used in the reference method were done according to ISO 6887-series parts 1, 2, 3, 4 and 5. Plating was done according to ISO 7218:2007+A1:2013 section 10.2.2 which says at least one plate per dilution shall be used with at least two successive dilutions. Two plates per dilution may also be used to improve reliability. If only one dilution is used, then two plates of this dilution shall be used to improve reliability of the results. Depending on the sample being tested and the expected contamination level, single or multiple dilutions were used with single or duplicate plates if considered necessary to improve the reliability of the calculated result and ensure at least two relevant plates were available for use in calculations.

2.2 Alternative method

See the flow diagram of the alternative method in Annex A.

The alternative method principle is based on chromogenic media

Compact Dry YM are ready-to-use dry media sheets comprising culture medium and a cold-soluble gelling agent, rehydrated by inoculating 1 ml diluted sample into the centre of the self-diffusible medium. The Compact Dry YM method contains chromogenic medium and selective agents for the detection and enumeration of yeasts and moulds. Yeasts grow as blue colonies and moulds form cottony colonies with characteristic colours.

It is intended to be comparable to ISO 21527-1:2008. It would therefore be subject to the same inclusions and restrictions as the ISO method. i.e. it is for enumeration of viable yeasts and moulds in foods with an aw of >0.95 which are capable of growth at 25±1°C within 3-7 days. It does not claim to be able to detect mould spores xerophilic mould species or heat resistant moulds species, which will be associated with foods of water activity<0.95

2.3 Study design

Samples of product containing the target organism were diluted 1 in 10 with an appropriate diluent according to ISO 6887 and homogenised in a stomacher.

Appropriate serial dilutions were made, and all relevant dilutions were analysed using the reference method and alternative method.

3 Method comparison study

3.1 Relative trueness study

The trueness study is a comparative study between the results obtained by the reference method and the results of the alternative method. This study was conducted using naturally or artificially contaminated samples. Different categories, types and items were tested for this.

A total of 5 categories were included in this validation study. A minimum of 15 items for each category were tested by both the reference method and the alternative method in the relative trueness study, with a minimum of 15 interpretable results per category.

Each category was made up of 3 types, with at least 5 items representative for each type.

3.1.1 Number of samples

The categories, the types and the number of samples analyzed are presented in Table 1.

Table 1 – Categories, types and number of samples analysed

Categories	Types	No of samples analysed and interpreted
Dairy products	Cheese e.g. grated cheese, soft cheese, blue	13
	Yogurts with fruit	5
	Fermented milk drinks	4
	Total	**22**
Confectionary, bakery and eggs	Bakery products with custard	5
	Egg products without additives e.g. whole liquid egg	5
	Par baked egg products	5
	Total	**15**
Fruits and vegetables	Fresh fruit salad and fruit purees	6
	Chilled fruit juices	4
	Fermented vegetables e.g. sauerkraut , olives	4
	Total	**14**
Ready to eat foods	Ready to eat meat and poultry e.g. turkey fillet, pate	5
	Cooked and cured fish products e.g. roll herring, seafood terrine	5
	Cured meats e.g. salami, ham	5
	Total	**15**
Multi component foods	Composite foods with raw ingredients e.g. sandwiches, pasta salads.	6
	Mayonnaise based chilled salads	4
	Ambient stable acidified foods e.g. ketchup	4
	Total	**14**
TOTAL		80

80 samples were analyzed, leading to 80 exploitable results.

3.1.2 Test sample preparation
This study was conducted using naturally contaminated samples only.

3.1.3 Protocols applied during the validation study

A single protocol was applied for the study.

Reference method plates were incubated at 25±1ºC for 2 – 5days. Final count was taken after 5 days

Alternative method plates were incubated at 25±1ºC for 3-7 days. Counts were taken at both time points.

3.1.4 Test results
The samples were analysed by the reference and the alternative method in order to have 15 interpretable results per incubation protocol, and 5 interpretable results per tested type by the two methods.

3.1.5 Calculation and interpretation of relative trueness study
The obtained data were analysed using the scatter plot. The graphs are provided with the line of identity (y = x).

Calculations were done for the alternative method at 3 days versus the reference method and for the alternative method at 7 days versus the reference method. The data for 3d is shown in Figures 1 to 6 and the data for 7 days in Figures 7 to 12.

Figure 1 :

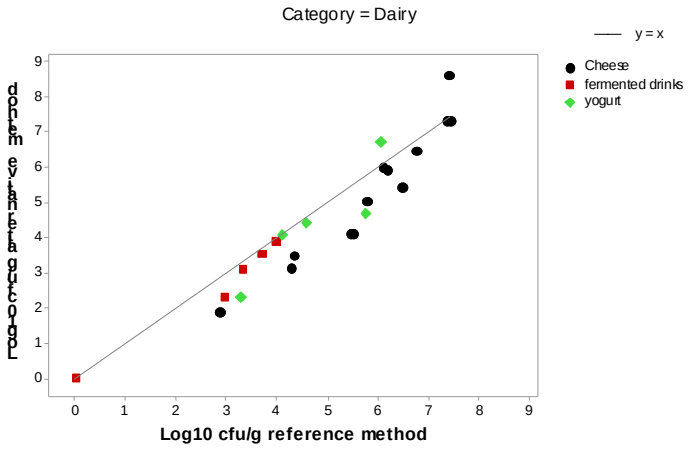


Figure 2:

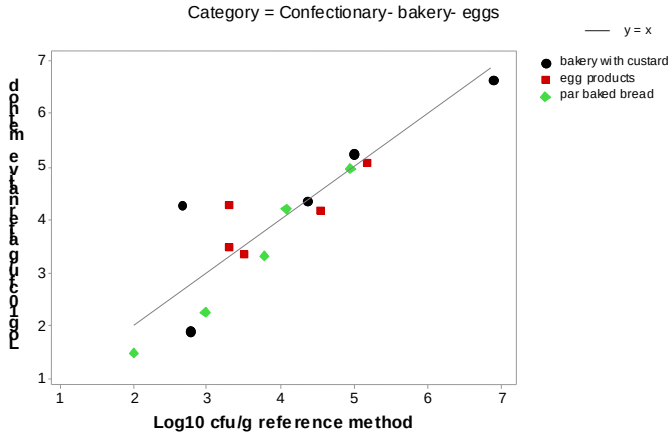


Figure 3:

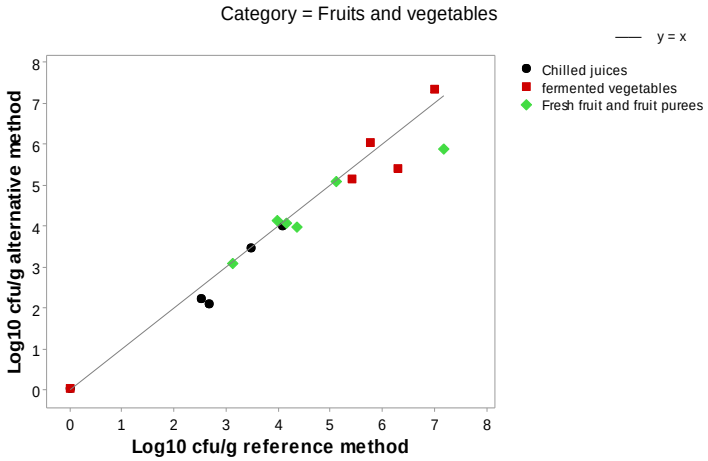


Figure 4:

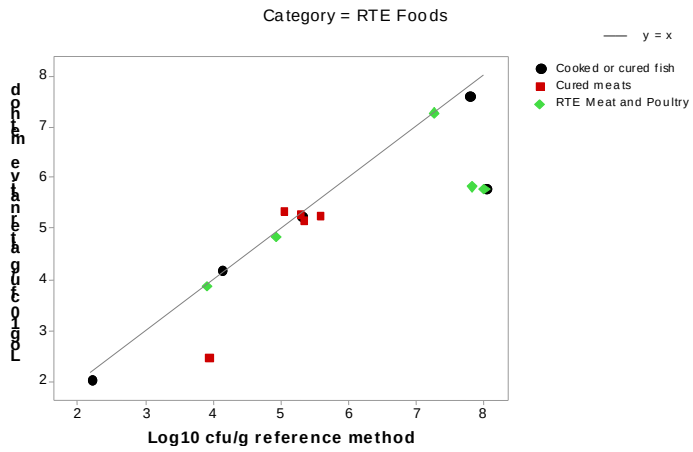


Figure 5:

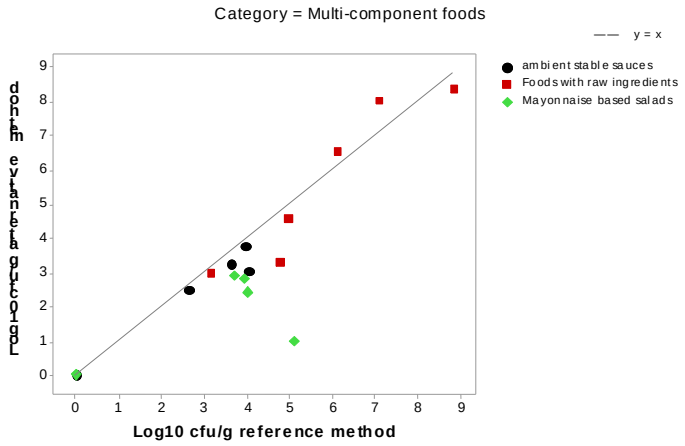


Figure 6:

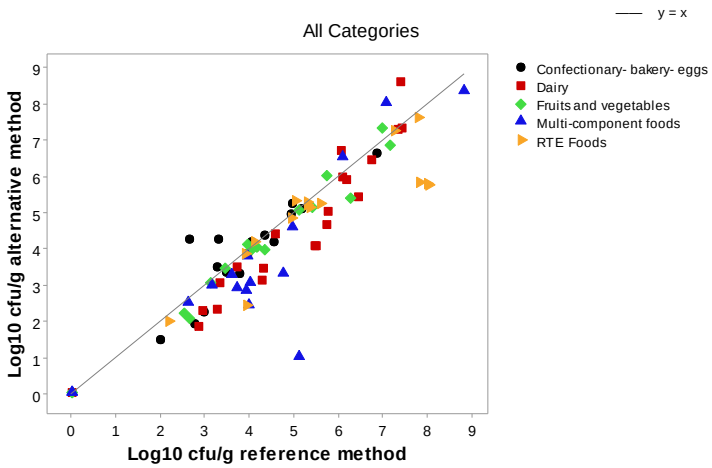


Figure 7 :

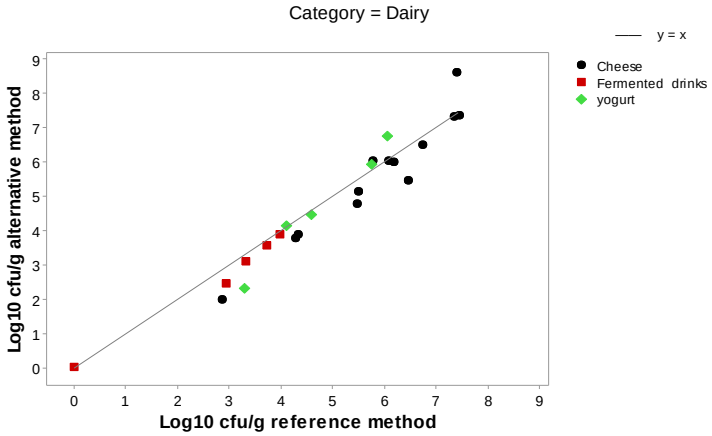


Figure 8:

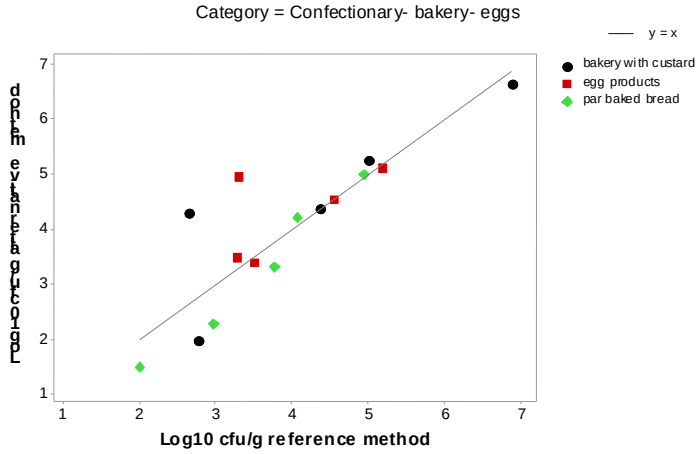
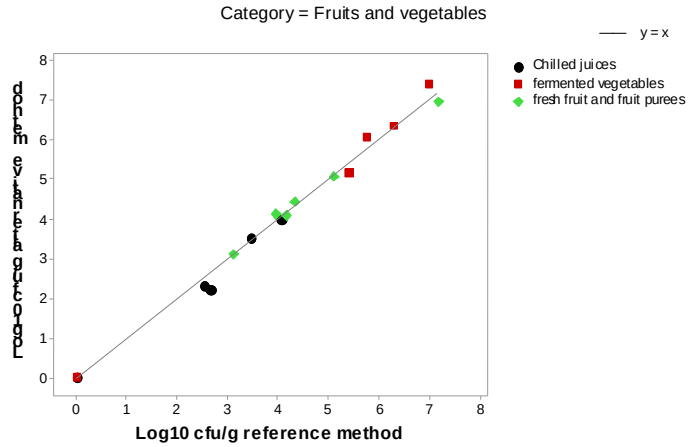


Figure 9:



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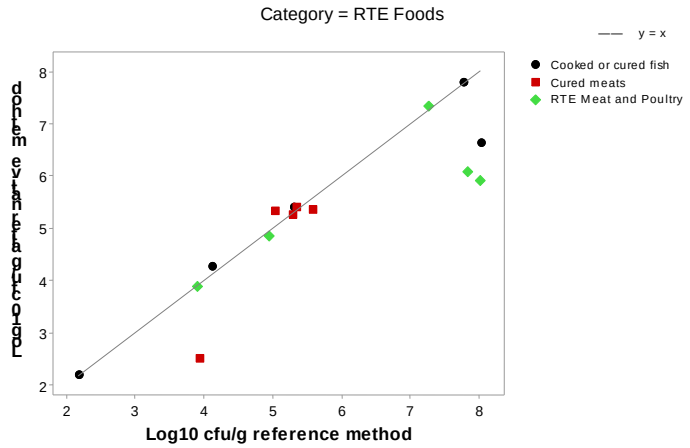


Figure 11:

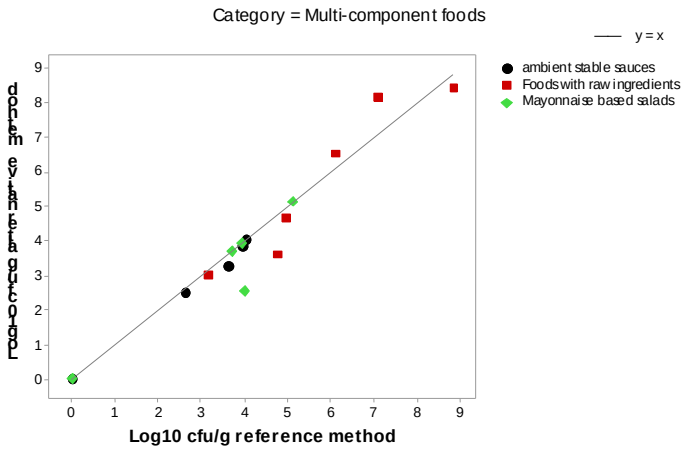
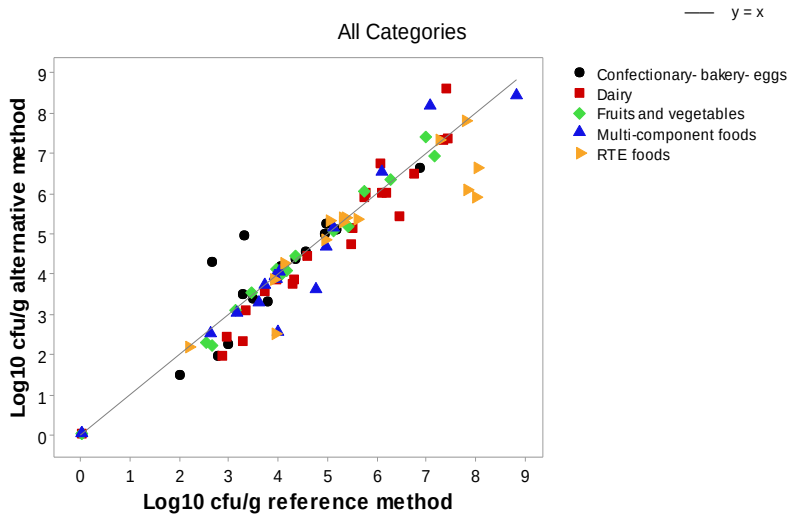


Figure 12:



According to ISO 16140-2:2016 6.1.2.3 the results of the scatter plot are interpreted based on a visual observation on the amount of bias and extreme results.

A summary of the calculated values per category is provided in Table 2 for the 3 day data and Table 3 for the 7 day data. The Bland-Altman difference plot for all the samples is given Figures 13 and 14.

Table 2 - Summary of the calculated values per category – 3 day data

Category.	n	$\overline{D}$	s_D	95% Lower limit	95% Upper limit
Dairy	22	-0.470	0.643	-0.899	0.565
Fruits and vegetables	14	-0.239	0.450	-1.246	0.769
Multi-component foods	14	-0.738	1.184	-3.387	1.910
Confectionary- bakery- eggs	15	-0.016	0.635	-1.422	1.390
RTE Foods	15	-0.584	0.908	-2.595	1.426
All Categories	80	-0.413	0.808	-2.004	1.204

$\overline{D}$: Average difference SD: standard deviation of differences n: number of samples

Table 3 - Summary of the calculated values per category – 7 day data

Category.	n	$\overline{D}$	s_D	95% Lower limit	95% Upper limit
Dairy	22	-0.211	0.511	-1.297	0.876
Fruits and vegetables	14	-0.025	0.229	-0.536	0.487
Multi-component foods	14	-0.177	0.609	-1.540	1.185
Confectionary- bakery- eggs	15	0.064	0.714	-1.518	1.646
RTE Foods	15	-0.428	0.799	-2.198	1.343
All Categories	80	-0.162	0.608	-1.379	1.056

Analysis of the data in Tables 2 and 3 shows that after 3 days incubation there was an overall bias of -0.413 which means that on average the alternative method may underestimate the number of yeasts and moulds present at the 3 day point. By 7 days this bias has reduced significantly so that the overall bias is much lower at -0.162. The same can be seen for the individual food categories. In all cases there was a negative bias at 3 days which was particularly large for the Multi-component foods and the RTE foods. These biases had also reduced by 7 days.)

End-users of the alternative method should conduct in-house verification trials to demonstrate which incubation period is best suited to their individual product types as the agreement between reference and alternate method varies between food items in the same category. However, once this is established, the agreement between replicate test portions of the same food type is very good as shown in the accuracy profile studies.

Although there is an underlying negative bias. the Bland Altman plots show a high dispersion of the data around the line of identity showing both positive and negative deviations. Most of the samples tested contained both yeast and mould colonies although there were generally more yeasts present. The reference method states that '*enumeration methods for yeasts and especially moulds are imprecise because they consist of a mixture of mycelium and asexual and sexual spores. Numbers of colony-forming units depend on the degree of fragmentation of mycelium and the proportion of spores able to grow on the plating medium'*

so it is perhaps not surprising to find a high level of variability based on the fact that the samples contained naturally present yeasts and moulds. In addition, there are differences in the size of the plates used for the reference method and the alternate method and in the volumes analysed, 0.1ml for reference and 1ml for alternate. In addition, the alternate method relies on a chromogenic medium for detection of yeasts and moulds. Considering all these aspects, the agreement between the alternate method and the reference method is acceptable.

Figure 13 – Bland-Altman difference plot for all the samples – 3 days

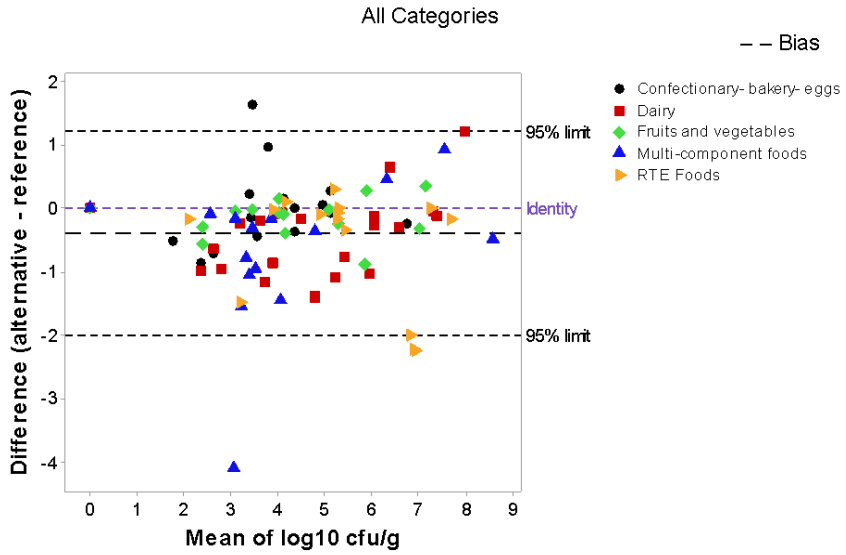
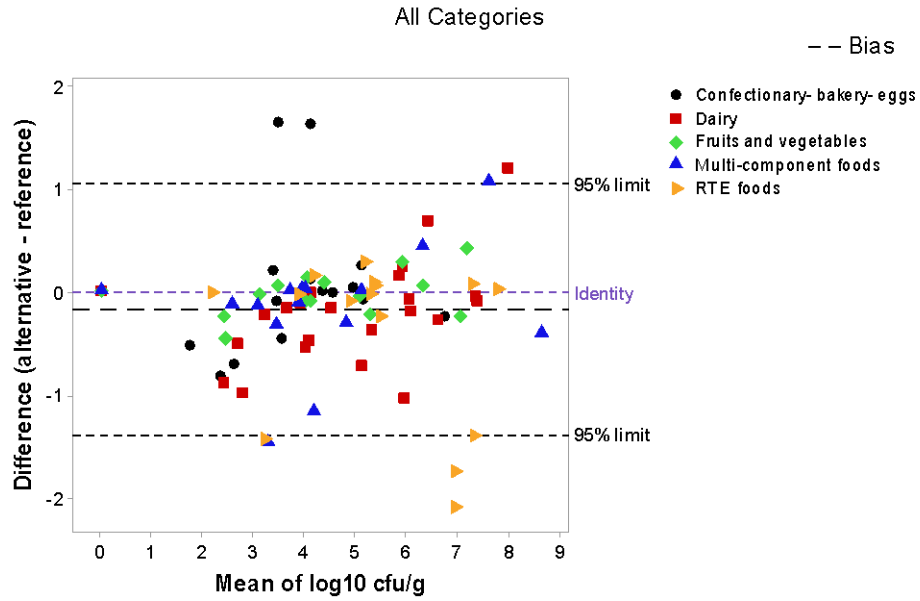


Figure 14 – Bland-Altman difference plot for all the samples –7 days



It is expected that not more than one in 20 data values will lie outside the CLs.

For 'All Categories' for the 3d data there are four in 80 values which lie outside the CLs. This is in agreement with the expectation of less than one in 20, although it is noted that the CL's for this data are large with a negative CL of -2.004 and a positive CL of 1.204. For 'All Categories' the 7d data there are 7 in 80 values which lie outside the CLs. This is slightly more than the expectation of less than one in 20. The points which were outside of the CLs are shown below in Tables 4 and 5. There were no identifiable trends in these data, and they covered 4 different food categories. For the 7d data, the data points have been examined and there are no obvious reasons for the disagreement, all the colony count data are within the target counting range of the methods and appear accurate. Three of the data points (sample 61, 206 and 87) are very close to the lower confidence limit which leaves 4 points which are true outliers. Two of these are above the upper limit with an average difference of 1.635 and two are below the lower limit with an average difference of -1.61. It is concluded that these samples are just individual cases where there is disagreement between the agars with no identifiable explanation. These differences are not unexpected as this data is for a total count of naturally present yeast and moulds which may vary considerably between samples.

Table 4 - Data which are outside of the accepted limits – 3 days

Category	Types	Code	Food item	Difference
Confectionary/eggs	Bakery with custard	156	Egg custard tarts	1.616
Multi component foods	Mayonnaise based salads	306	Jalopena coleslaw	-4.114
RTE Foods	Cooked or cured fish	107	Hot smoked salmon	-2.256
RTE Foods	Meat and poultry	75	Breaded chicken strips	-2.228

Table 5 - Data which are outside of the accepted limits – 7 days

Category	Types	Code	Food item	Difference
Confectionary/eggs	Bakery with custard	156	Egg custard tarts	1.637
Confectionary/eggs	Products with eggs	145	Egg Fried Rice	1.633
Multi component foods	Mayonnaise based salads	61	Potato Salad	-1.474
RTE Foods	Meat and poultry	29	Cooked cocktail sausage	-1.747
RTE Foods	Cooked or cured fish	75	Breaded chicken strips	-2.102
Dairy	Cheese	87	Grated mozarrella	1.191
RTE Foods	Meat and poultry	206	Honey roast ham	-1.473

3.1.6 Conclusion (RT study)

Although there were some discordant results between the alternative method and the reference method, these are potentially caused by over enumerating on the reference method as it is known be less selective than the alternate method. None the less it is noted that for some raw milk and fermented dairy products the alternative method may give a lower count than that obtained on the reference method after 3 days incubation.

Taking into account the overall Bland Altman analysis and the original study analysis it is concluded that the relative trueness study of the ALTERNATIVE method is satisfied.

Although there were some discordant results between the alternative method and the reference method, these are potentially caused by over enumerating on the reference method as it is known be less selective than the alternate method. None the less it is noted that for some raw milk and fermented dairy products the alternative method may give a lower count than that obtained on the reference method after 3 days incubation.

Taking into account the overall Bland Altman analysis and the original study analysis it is concluded that the relative trueness study of the ALTERNATIVE method is satisfied.

For total plate count methods especially yeast and mould methods which are aimed at enumeration of a wide range of mycological groups, this level of outliers is not unreasonable, however end users should perform verification studies to show comparable results with their usual reference method

3.2 Accuracy profile study

The accuracy profile study is a comparative study between the results obtained by the reference method and the results of the alternative method. This study is conducted using artificially contaminated samples, using one type per category.

3.2.1 Categories, sample types and strains

In this study five food categories were tested with a single batch of two different food types using 6 samples per type. Two samples were contaminated at a low level, 2 at intermediate level, 2 at a high level. For each sample, 5 replicates (5 different test portions) were tested. A total of 30 samples were analysed per food type. Each sample was bulk inoculated and five replicate test portions examined from the bulk sample.

Table 6 - Categories, types, items, strains and inoculation levels for accuracy profile study

Category	Types	Strain	Item	Target Level	Test portions
Dairy products	Pasteurised dairy products	*S.cerevisiae* CRA 15968	Fermented yogurt drink	Low 300cf/g	5
				Medium : 5.000cfu/g	5
				High : 100.000cfu/g	5
			Cream cheese	Low 300cf/g	5
				Medium : 5.000cfu/g	5
				High : 100.000cfu/g	5

Fruits and vegetables	Blanched or pasteurised products	*D.hansenii* CRA 15969	Vegetable Juice	Low: 500cf/g	5
				Medium : 5000cfu/g	5
				High : 50.000cfu/g	5
			Beetroot salad	Low 300cf/g	5
				Medium : 5.000cfu/g	5
				High : 100.000cfu/g	5
Confectionary, bakery and eggs	Chilled RTE foods	*A.niger* CRA 16667	Quiche	Low: 100cf/g	5
				Medium : 1000cfu/g	5
				High : 50.000cfu/g	5
			Egg custard tarts	Low 300cf/g	5
				Medium : 5.000cfu/g	5
				High : 100.000cfu/g	5
Ready to eat foods	Fish products	*P. chrysogeum* DSM 848	Cooked prawns	Low: 100cf/g	5
				Medium : 10000cfu/g	5
				High : 100.000cfu/g	5
			Fish pate	Low 300cf/g	5
				Medium : 5.000cfu/g	5
				High : 100.000cfu/g	5
Multi component foods	Composite foods with raw ingredients	*G. candidum* CRA 14398	Sandwiches	Low 500cf/g	5
				Medium : 5000cfu/g	5
				High : 10.000cfu/g	5
			Pasta salad with protein	Low 300cf/g	5
				Medium : 5.000cfu/g	5
				High : 100.000cfu/g	5

Total number of samples tested= 150

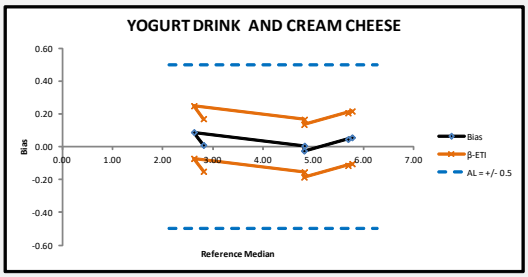
3.2.2 Calculations and interpretation of accuracy profile study

The statistical results and the accuracy profiles are provided in Figures 8 to 12

The calculations were done using the AP Calculation Tool MCS (Clause 6-1-3-3 calculation and interpretation of accuracy profile study) available on http://standards.iso.org/iso/16140

Figure15: Dairy inoculated with S.cerevisiae

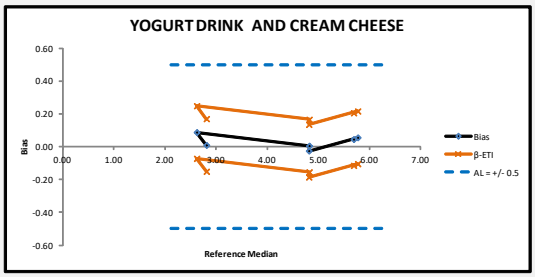
(Food) Category	DAIRY 3-day
(Food) Type	YOGURT DRINK AND CREAM



Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
88 a-e	2.81	0.010	-0.150	0.170	YES	YES
147 a-e	2.62	0.089	-0.072	0.249	YES	YES
84 a-e	4.82	0.006	-0.154	0.166	YES	YES
160 a-e	4.82	-0.025	-0.185	0.135	YES	YES
10 a-e	5.77	0.056	-0.104	0.217	YES	YES
15 a-e	5.69	0.046	-0.114	0.206	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.069	0.111	YES	+/- 0.500

(Food) Category	DAIRY 7-day
(Food) Type	YOGURT DRINK AND CREAM

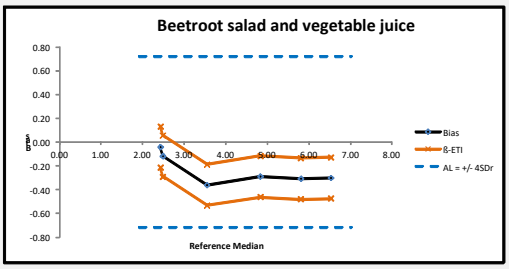


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
88 a-e	2.81	0.010	-0.150	0.170	YES	YES
147 a-e	2.62	0.089	-0.072	0.249	YES	YES
84 a-e	4.82	0.006	-0.154	0.166	YES	YES
160 a-e	4.82	-0.025	-0.185	0.135	YES	YES
10 a-e	5.77	0.056	-0.104	0.217	YES	YES
15 a-e	5.69	0.046	-0.114	0.206	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.069	0.111	YES	+/- 0.500

Figure 16: Fruits and vegetables inoculated with D.hansenii

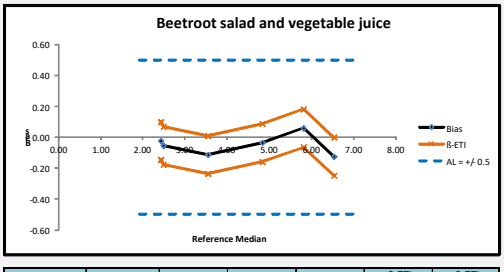
(Food) Category	PRODUCE -3d
(Food) Type	Beetroot salad and vegetable juice



Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
31 a-e	2.43	-0.042	-0.215	0.131	YES	YES
25 a-e	2.49	-0.118	-0.292	0.055	YES	YES
133 a-e	3.55	-0.361	-0.534	-0.187	NO	YES
85 a-e	4.83	-0.290	-0.463	-0.116	YES	YES
190 a-e	5.81	-0.307	-0.480	-0.134	YES	YES
13 a-e	6.53	-0.301	-0.474	-0.128	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.180	0.120	NO	+/- 0.720

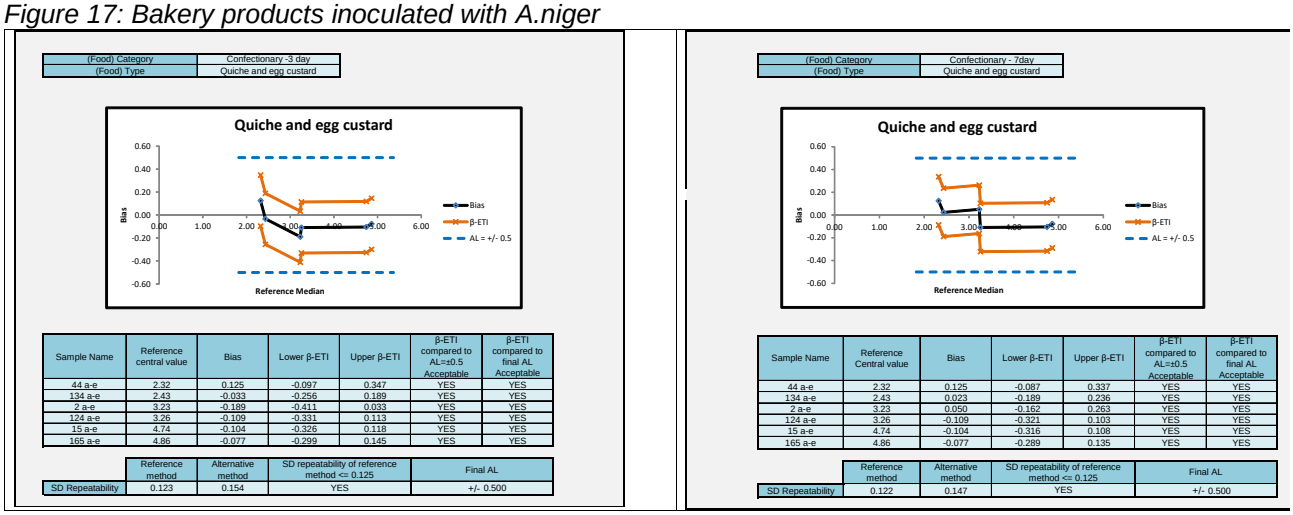
(Food) Category	PRODUCE -7d
(Food) Type	Beetroot salad and vegetable juice



Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
31 a-e	2.43	-0.025	-0.148	0.098	YES	YES
25 a-e	2.49	-0.055	-0.178	0.068	YES	YES
133 a-e	3.55	-0.114	-0.237	0.009	YES	YES
85 a-e	4.83	-0.036	-0.159	0.086	YES	YES
190a-e	5.81	0.057	-0.065	0.180	YES	YES
13 a-e	6.53	-0.126	-0.248	-0.003	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.180	0.085	NO	+/- 0.500

Figure 17: Bakery products inoculated with A.niger



(Food) Category	Confectionary -3 day
(Food) Type	Quiche and egg custard

Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
44 a-e	2.32	0.125	-0.097	0.347	YES	YES
134 a-e	2.43	-0.033	-0.256	0.189	YES	YES
2 a-e	3.23	-0.189	-0.411	0.033	YES	YES
124 a-e	3.26	-0.109	-0.331	0.113	YES	YES
15 a-e	4.74	-0.104	-0.326	0.118	YES	YES
165 a-e	4.86	-0.077	-0.299	0.145	YES	YES

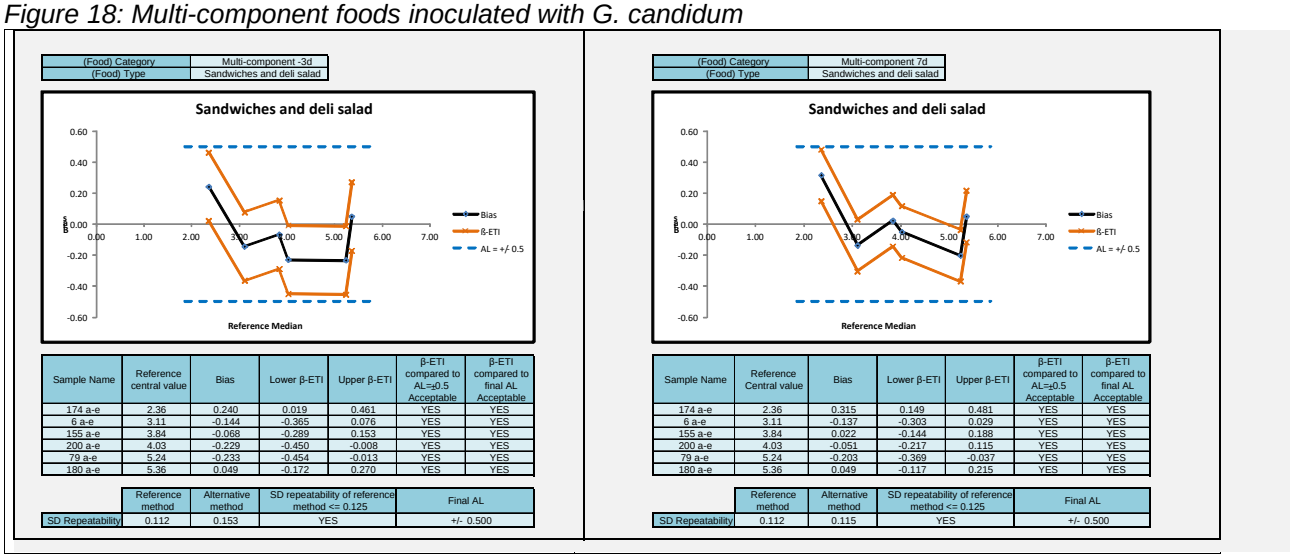
	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.123	0.154	YES	+/- 0.500

(Food) Category	Confectionary - 7day
(Food) Type	Quiche and egg custard

Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
44 a-e	2.32	0.125	-0.087	0.337	YES	YES
134 a-e	2.43	0.023	-0.189	0.236	YES	YES
2 a-e	3.23	0.050	-0.162	0.263	YES	YES
124 a-e	3.26	-0.109	-0.321	0.103	YES	YES
15 a-e	4.74	-0.104	-0.316	0.108	YES	YES
165 a-e	4.86	-0.077	-0.289	0.135	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.122	0.147	YES	+/- 0.500

Figure 18: Multi-component foods inoculated with G. candidum



(Food) Category	Multi-component -3d
(Food) Type	Sandwiches and deli salad

Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=0.5 Acceptable	β-ETI compared to final AL Acceptable
174 a-e	2.36	0.240	0.019	0.461	YES	YES
6 a-e	3.11	-0.144	-0.365	0.076	YES	YES
155 a-e	3.84	-0.068	-0.289	0.153	YES	YES
200 a-e	4.03	-0.229	-0.450	-0.008	YES	YES
79 a-e	5.24	-0.233	-0.454	-0.013	YES	YES
180 a-e	5.36	0.049	-0.172	0.270	YES	YES

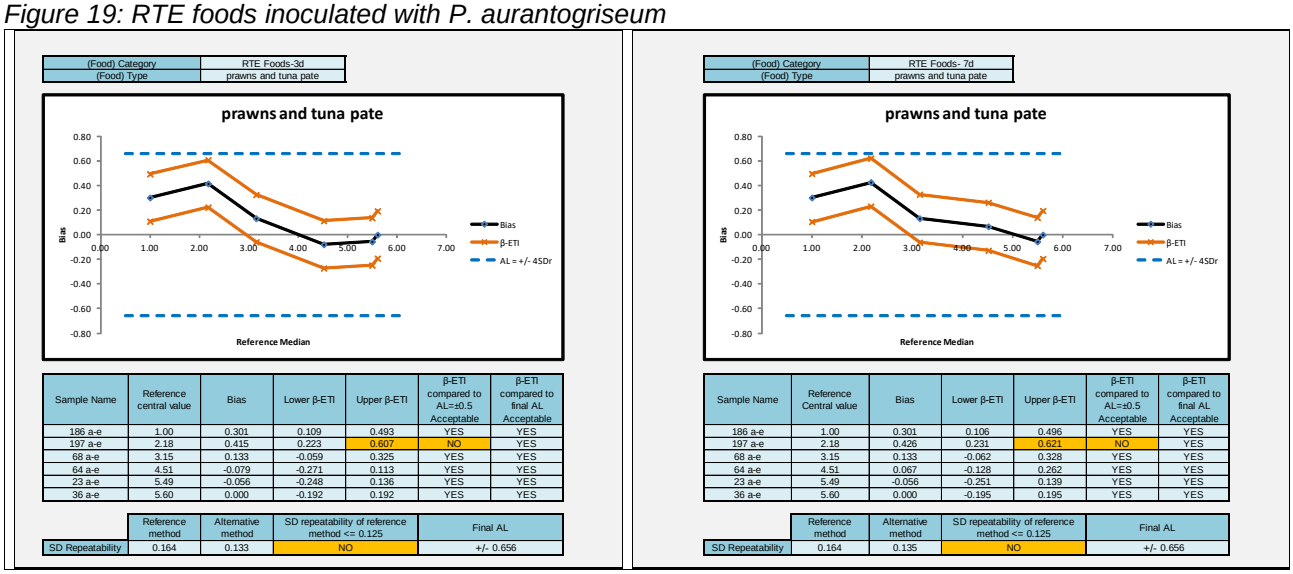
	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.112	0.153	YES	+/- 0.500

(Food) Category	Multi-component 7d
(Food) Type	Sandwiches and deli salad

Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=0.5 Acceptable	β-ETI compared to final AL Acceptable
174 a-e	2.36	0.315	0.149	0.481	YES	YES
6 a-e	3.11	-0.137	-0.303	0.029	YES	YES
155 a-e	3.84	0.022	-0.144	0.188	YES	YES
200 a-e	4.03	-0.051	-0.217	0.115	YES	YES
79 a-e	5.24	-0.203	-0.369	-0.037	YES	YES
180 a-e	5.36	0.049	-0.117	0.215	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.112	0.115	YES	+/- 0.500

Figure 19: RTE foods inoculated with P. aurantogriseum



Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
186 a-e	1.00	0.301	0.109	0.493	YES	YES
197 a-e	2.18	0.415	0.223	0.607	NO	YES
68 a-e	3.15	0.133	-0.059	0.325	YES	YES
64 a-e	4.51	-0.079	-0.271	0.113	YES	YES
23 a-e	5.49	-0.056	-0.248	0.136	YES	YES
36 a-e	5.60	0.000	-0.192	0.192	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.164	0.133	NO	+/- 0.656

Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
186 a-e	1.00	0.301	0.106	0.496	YES	YES
197 a-e	2.18	0.426	0.231	0.621	NO	YES
68 a-e	3.15	0.133	-0.062	0.328	YES	YES
64 a-e	4.51	0.067	-0.128	0.262	YES	YES
23 a-e	5.49	-0.056	-0.251	0.139	YES	YES
36 a-e	5.60	0.000	-0.195	0.195	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.164	0.135	NO	+/- 0.656

If any of the upper or lower limits exceeded the 0.5log AP limits and the standard deviation of the reference method was >0.125, additional evaluation procedures are required, as described in ISO 16140-2:2016 and the new acceptability limits are calculated

For two of the food categories the additional AL calculation was required. This was for the fresh produce where the medium level of beetroot salad just exceeded the lower limit on the 3d incubation data (Figure 3b) and RTE products where the low level for tuna pate just exceeded the upper limit on the 3 and 7d incubation data (Figure 3d). However, the re-calculated AL's were achieved for all food categories

3.3 Inclusivity / exclusivity

The inclusivity study is a study involving pure target strains to be detected or enumerated by the alternative method. According to ISO/FDIS 16140-2:2015 6.1.5, this test is not required for general enumeration methods such as yeast and mould counts. Therefore, it has not been done in this study.

3.4 Limit of quantification (LOQ)

The limit of Quantification (LOQ) is only required for instrumental measurements. It was not done in this study

3.5 Conclusion (MCS)

Overall, the conclusions for the Method Comparison are:

- The alternative method Compact Dry YM for enumeration of yeasts and moulds shows satisfactory results for relative trueness after 3 day and 7 days

- The alternative method Compact Dry YM for enumeration of yeasts and moulds shows satisfactory results for accuracy profile after 3 day and 7 days

4 Interlaboratory study

The inter-laboratory study is a study performed by multiple laboratories testing identical samples at the same time, the results of which are used to estimate alternative-method performance parameters.

4.1 Study organisation

4.1.1 Collaborators
There were 9 collaborative laboratories used in this study representing 6 different countries.

4.1.2 Matrix and strain used

A single strain of the yeast *Debaromyces hansenii* (Campden code 15969) and a single strain of mould *Penicillium chrysogenum* (IMI 1394016) were grown in Malt extract broth and mixed together to inoculate 8 samples of orange juice.
Two samples of UHT orange juice remained uninoculated. For the remaining six samples, appropriate dilutions of the yeasts and moulds culture were used to individually inoculate 2 x 20ml juice samples at the lower (10^3 cfu/ml), middle (10^4 cfu/ml) and higher (10^5 cfu/ml) contamination levels. The samples were blind-coded and stored at 0-4°C prior to despatch.
The study was done in November 2010.

4.1.3 Sample preparation
Samples were prepared and inoculated and despatched as described below:

For each of the 14 collaborators participating in the interlaboratory study 7 x 10g samples of salmon pâté were weighed into sterile stomach bags. One sample of salmon pâté remained uninoculated. For the remaining six samples, appropriate dilutions of the yeast and mould cocktail were used to individually inoculate 2 x 10g samples at the low (~10^2 cfu/ml), middle (~10^4cfu/ml) and high (~10^6cfu/ml) contamination levels.

4.1.4 Labelling and shipping

Prior to despatch, each set of samples was removed from the freezer and packed into plastic containers (Air-Sea Containers Limited, code 490). These plastic containers were then placed inside a thermal control unit (Air-Sea Containers Limited, TC-20 code 802) with cool packs (Air-Sea Containers Limited, CP-20 code 405). The samples were packaged frozen so as de-frost occurred during transportation. Each laboratory also received an additional vial containing water “temperature control sample” which was packed with the test samples.

This was used to enable the laboratory to take a temperature measurement, representative of the samples, upon receipt. In addition to this a continuous electronic temperature monitor (Thermochron iButton) was placed in the sample packages. The laboratories were requested to return the ibuttons to the expert

laboratory upon receipt. The target storage conditions were for the temperature to stay lower or equal to 8°C during transport, and between 0°C – 8°C in the labs.

Shipping was arranged so that each laboratory would receive their samples within 72-96h dependent on location and speed of the International courier service. The samples to be sent to Europe were dispatched Thursday, and the samples sent to the UK were dispatched Monday.

All the samples were delivered on time and in appropriate conditions.

4.2 Calculation and summary of data

The data from the collaborative trial were calculated and interpreted according to section 6.2.3 of ISO 16140-2:2016 using the freely available Excel® spreadsheet (http://standards.iso.org/iso/16140). Version 14-03-2016 was used for these calculations.

The results obtained by the collaborators are shown in Tables 7 and 8.

Table 7: Summary of the results of the interlaboratory study per analyte level (k)- 3days

		Reference method x_{ijk}		**Alternative method k_{ijk}**	
Collaborators (i)	**Level (k)**				
1	Blank	<10		<10	
2	Blank	<10		<10	
3	Blank	<10		<10	
4	Blank	<10		<10	
5	Blank	<10		<10	
6	Blank	<10		<10	
7	Blank	<10		<10	
8	Blank	<10		<10	
Collaborators (i)	**Level (k)**	**Duplicate 1**	**Duplicate 2**	**Duplicate 1**	**Duplicate 2**
1	Low	4.210	4.030	4.330	3.910
2	Low	3.980	4.060	3.960	3.990
3	Low	3.820	3.900	3.680	3.680
4	Low	4.090	3.980	4.210	4.020
5	Low	3.980	4.010	3.860	3.910
6	Low	4.180	4.110	4.160	3.840
7	Low	4.040	3.920	4.130	3.920
8	Low	3.960	3.900	3.920	3.910
Collaborators (i)	**Level (k)**	**Duplicate 1**	**Duplicate 2**	**Duplicate 1**	**Duplicate 2**
1	Medium	4.780	4.970	4.790	4.910
2	Medium	5.000	4.720	4.920	4.760
3	Medium	4.650	4.460	4.500	4.310
4	Medium	5.080	4.930	5.030	5.030
5	Medium	4.730	4.690	4.730	4.700
6	Medium	4.880	4.850	4.640	4.650
7	Medium	4.930	4.790	4.750	4.670
8	Medium	4.700	4.810	4.680	4.850

		Reference method x_{ijk}		Alternative method k_{ijk}	
Collaborators (i)	**Level (k)**				
Collaborators (i)	**Level (k)**	**Duplicate 1**	**Duplicate 2**	**Duplicate 1**	**Duplicate 2**
1	High	5.850	5.850	5.860	5.790
2	High	5.840	5.860	5.760	5.740
3	High	5.660	5.390	5.430	5.300
4	High	5.960	6.100	6.130	6.270
5	High	5.680	5.520	5.630	5.480
6	High	5.700	5.720	5.560	5.540
7	High	5.730	5.650	5.580	5.530
8	High	5.710	5.670	5.660	5.580

Table 8: Summary of the results of the interlaboratory study per analyte level (k)- 3days

		Reference method x_{ijk}		Alternative method k_{ijk}	
Collaborators (i)	**Level (k)**				
1	Blank	<10		<10	
2	Blank	<10		<10	
3	Blank	<10		<10	
4	Blank	<10		<10	
5	Blank	<10		<10	
6	Blank	<10		<10	
7	Blank	<10		<10	
8	Blank	<10		<10	
0	Blank	<10		<10	
Collaborators (i)	**Level (k)**	**Duplicate 1**	**Duplicate 2**	**Duplicate 1**	**Duplicate 2**
1	Low	4.210	4.030	4.330	3.920
2	Low	3.980	4.060	3.960	3.990
3	Low	3.820	3.900	3.730	3.680
4	Low	4.090	3.980	4.210	4.020
5	Low	3.980	4.010	3.860	3.910
6	Low	4.180	4.110	4.160	3.840
7	Low	4.040	3.920	4.120	3.920
8	Low	3.960	3.900	3.920	3.910
9	Low	4.090	4.120	4.070	4.080
Collaborators (i)	**Level (k)**	**Duplicate 1**	**Duplicate 2**	**Duplicate 1**	**Duplicate 2**
1	Medium	4.780	4.970	4.800	4.910
2	Medium	5.000	4.720	4.760	4.920
3	Medium	4.650	4.460	4.370	4.530
4	Medium	5.080	4.930	5.030	5.030
5	Medium	4.730	4.690	4.700	4.730
6	Medium	4.880	4.850	4.650	4.640
7	Medium	4.930	4.790	4.690	4.760
8	Medium	4.700	4.810	4.680	4.850
9	Medium	4.830	5.160	4.850	5.100
Collaborators (i)	**Level (k)**	**Duplicate 1**	**Duplicate 2**	**Duplicate 1**	**Duplicate 2**
1	High	5.850	5.850	5.860	5.790

		Reference method x_{ijk}		Alternative method k_{ijk}	
Collaborators (i)	**Level (k)**				
2	High	5.840	5.860	5.760	5.740
3	High	5.660	5.390	5.640	5.330
4	High	5.960	6.100	6.130	6.270
5	High	5.680	5.520	5.630	5.480
6	High	5.700	5.720	5.560	5.540
7	High	5.730	5.650	5.580	5.560
8	High	5.710	5.670	5.660	5.580
9	High	5.830	6.090	5.780	6.030

The accuracy profile plot is shown in Figures 20 and 21 and the statistical analysis of the data shown in Tables 9 and 10

Figure 20. Accuracy profile of Compact Dry YM from the ILS – 3 days

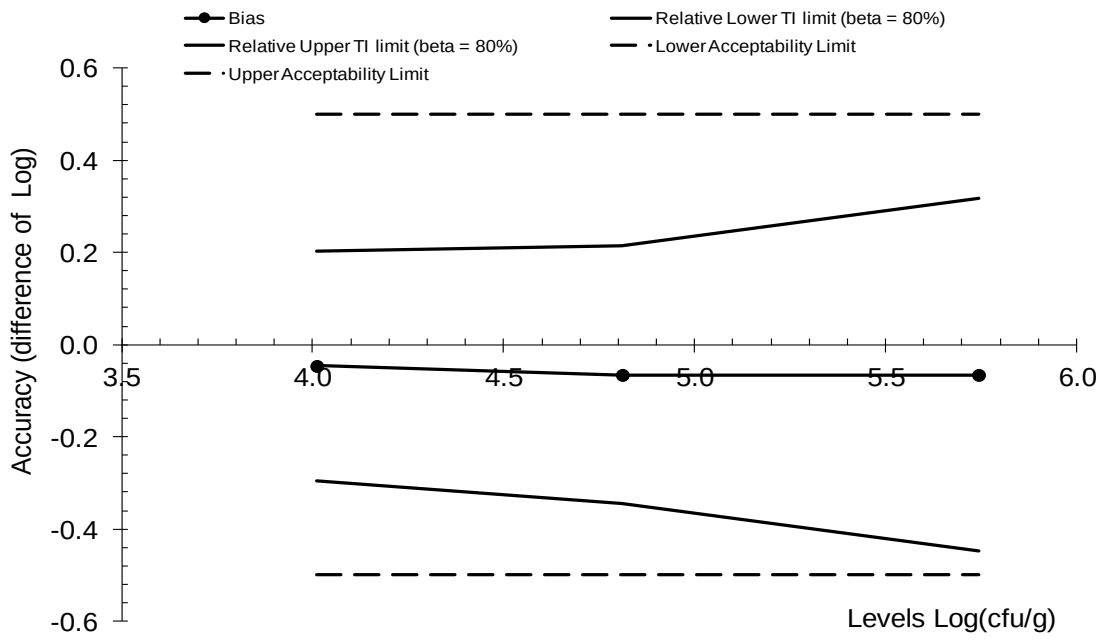
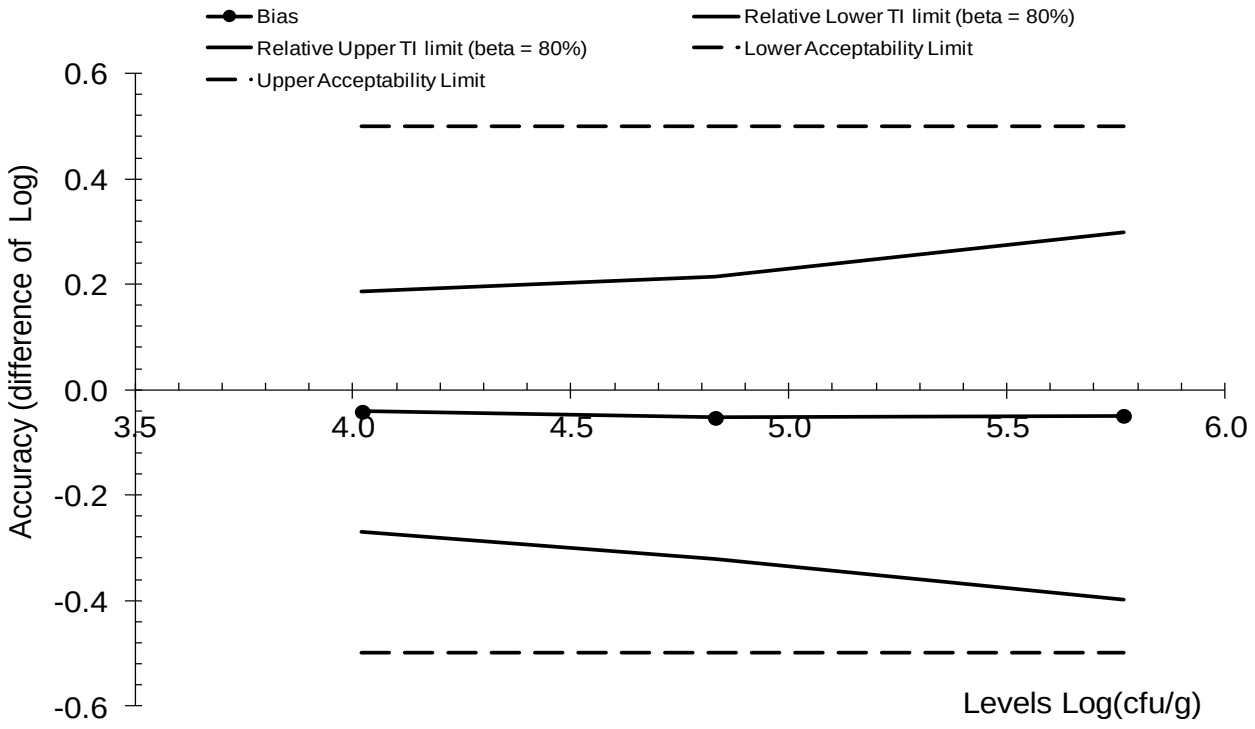


Figure 21 . Accuracy profile of Compact Dry YM from the ILS – 7 days



The statistical analysis of the ILS data is shown in Table 6 below. It can be seen that the repeatability standard deviation (S_r) was similar for the alternative and reference method ranging from 0.095 to 0.127 for Compact Dry YM and 0.077 to 0.132 for the reference method.

The between-labs standard deviation (S_L) was also of a similar microbiological magnitude for the alternative method (0.131 to 0.197) and the reference method (0.106 to 0.138).

The mean $\log_{10}$ count from the 14 samples at each level were very similar for the two methods with low, medium and high average counts of 2.561. 3.911 and 5.623 for the alternative method and 2.652, 3.964 and 5.621 for the reference.

According to the ISO 16140-2:2016 standard, if any of the values of the β-ETI fall outside of the Acceptability Limits AL (±0.5log units)then a further calculation is done to calculate the pooled average SR of the reference method. There was no requirement for this as all values met the AL's.

Table 9. Statistical analysis of the ILS data according to the ISO spreadsheet – 3 days

Accuracy profile	0.5		
Study Name	YM ILS analysis		
Date	03/03/2017		
Coordinator	Campden BRI		
Tolerance probability (beta)	80%	80%	80%
Acceptability limit in log (lambda)	0.50	0.50	0.50

FALSE

Application of clause 6.2.3
Step 8: If any of the values for the β-ETI fall
the acceptability limits, calculate the pooled
reproducibility standard deviation of the re
method.
Step 9: Calculate new acceptability limits
function of this standard deviation.

	Alternative method			Reference method		
Levels	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
Target value	4.011	4.811	5.743			
Number of participants (K)	8	8	8	8	8	8
Average for alternative method	3.964	4.745	5.677	4.011	4.811	5.743
Repeatability standard deviation (sr)	0.151	0.084	0.068	0.071	0.114	0.089
Between-labs standard deviation (sL)	0.094	0.171	0.248	0.079	0.109	0.147
Reproducibility standard deviation (sR)	0.178	0.190	0.257	0.106	0.158	0.172
Corrected number of dof	13.368	8.496	7.505	10.856	11.558	9.136
Coverage factor	1.401	1.466	1.488			
Interpolated Student t	1.348	1.390	1.406			
Tolerance interval standard deviation	0.1846	0.2009	0.2724			
Lower TI limit	3.715	4.466	5.295			
Upper TI limit	4.213	5.024	6.060			
Bias	**-0.046**	**-0.066**	**-0.066**			
Relative Lower TI limit (beta = 80%)	**-0.295**	**-0.345**	**-0.449**			
Relative Upper TI limit (beta = 80%)	**0.203**	**0.214**	**0.317**			
Lower Acceptability Limit	**-0.50**	**-0.50**	**-0.50**			
Upper Acceptability Limit	**0.50**	**0.50**	**0.50**			
New acceptability limits may be based on reference method pooled variance						
Pooled repro standard dev of reference	0.148					

FALSE
FALSE

Select ALL blue lines to draw the accuracy profile as illustrated in the worksheet "Graph Profile"

Table 10. Statistical analysis of the ILS data according to the ISO spreadsheet – 7 days

Accuracy profile	0.5		
Study Name	YM ILS Analysis		
Date	03/03/2017		
Coordinator	Campden BRI		
Tolerance probability (beta)	80%	80%	80%
Acceptability limit in log (lambda)	0.50	0.50	0.50

FALSE

App
Step 8: If any of t
the acceptability l
reproducibility st

Step 9: Calcula
function

	Alternative method			Reference method		
Levels	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
Target value	4.021	4.831	5.767			
Number of participants (K)	9	9	9	9	9	
Average for alternative method	3.979	4.778	5.718	4.021	4.831	5
Repeatability standard deviation (sr)	0.140	0.094	0.109	0.067	0.133	0
Between-labs standard deviation (sL)	0.087	0.161	0.216	0.080	0.105	0
Reproducibility standard deviation (sR)	0.165	0.187	0.242	0.104	0.169	0
Corrected number of dof	15.248	10.312	9.794	12.038	14.186	11
Coverage factor	1.386	1.434	1.441			
Interpolated Student t	1.340	1.369	1.374			
Tolerance interval standard deviation	0.1707	0.1959	0.2536			
Lower TI limit	3.751	4.509	5.369			
Upper TI limit	4.208	5.046	6.066			
Bias	**-0.042**	**-0.053**	**-0.049**			
Relative Lower TI limit (beta = 80%)	**-0.270**	**-0.322**	**-0.398**			
Relative Upper TI limit (beta = 80%)	**0.187**	**0.215**	**0.299**			
Lower Acceptability Limit	**-0.50**	**-0.50**	**-0.50**			
Upper Acceptability Limit	**0.50**	**0.50**	**0.50**			
New acceptability limits may be based on reference method pooled variance						

FALSE
FALSE

Select ALL blue lines to draw the accuracy profile as illustrated in the worksheet "Graph Profile"

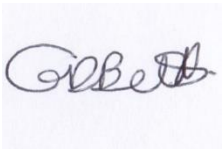
5 Overall conclusions of the validation study

- The alternative method Compact Dry YM for enumeration of yeasts and moulds shows satisfactory results for relative trueness;
- The alternative Compact Dry YM for enumeration of yeasts and moulds shows satisfactory results for accuracy profile;
- The alternative Compact Dry YM for enumeration of yeasts and moulds shows satisfactory performance in the ILS

The alternative Compact Dry YM shows comparable performance to the reference method ISO 21527-1:2008 for enumeration of yeasts and moulds in a broad range of foods.

Date 03/09/2019

Signature

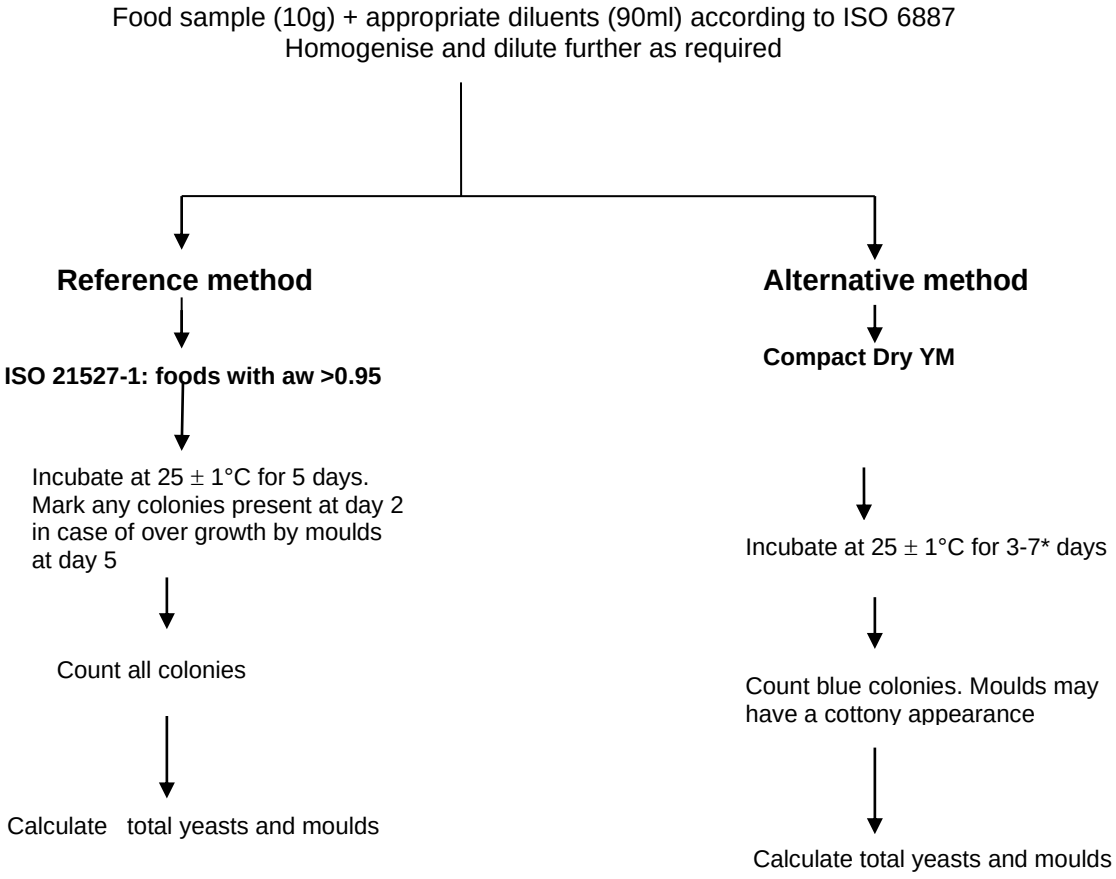


Annexes

A. Flow diagram of the reference and alternative method

ANNEX A: Typical colony morphology and Flow diagram of the alternative method and reference methods

Comparison of Reference method (ISO21527-1) and Alternative method: Compact Dry YM



*Both times were evaluated