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Research Article
Comparison of Uncertainty Between Traditional and Alternative Methods for Food Microbiological Analysis

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Abstract
The objectives highlighted in the present study were to determine the estimates of measurement uncertainty associated with microbiological analysis in food samples performing a gage repeatability and reproducibility study on three different microbiological methods of analysis for the detection and quantification of total coliforms; Plate count method, 3M Petrifilm™ count plates method and the MBS method. For all three methods the contribution the total gage R and R is less than 10%, demonstrating the ability to accurately evaluate the concentration of total coliforms in food samples. However, the repeatability and reproducibility follows the tendency; Plate count method<3M Petrifilm™ count plates method<MBS method. The study also highlights that a significant variation is due to the interaction between the operators and the analyzed samples for plate count and 3M Petrifilm™ count plates methods while for the MBS method it does not significantly affect the measure. Together these results demonstrate that greater repeatability and reproducibility are connected to a more simple analytical procedure demonstrating that uncertainty is certainly related to the amount of manual work and the individual interpretation of results. Results confirm that it is important to diminish these sources of error reducing labor and simplifying procedures and increasing automation.

Key words: Measurement uncertainty, total gage R and R, plate count method, 3M Petrifilm™ count plates method, MBS method

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The aim in microbiological analysis is usually to detect and enumerate a known species or a group of microorganisms in a measured amount of sample. If measurement means counting and identification, quantitative microbiological analysis belong to the sphere of metrology (Niemi and Niemela, 2001).

Measurement of uncertainty has been a common place requirement in physical and chemical analyses for many years but it is only recently that the subject has been addressed by microbiologists. Whilst the accepted concept is the measurement of the "Level of uncertainty" associated with a microbiological test, the recipient really wants to know the "Level of confidence" which the microbiologist can put on the particular result (Dereani and Saric, 2010).

In microbiological laboratory practice many causes of variability can be identified, for instance: The ability of an isolate to give typical reactions on a diagnostic medium, equipment and human errors in weighing, dispensing, pipetting and other laboratory activities, the relative skill levels of different technicians (Jarvis, 2008).

To interpret properly the results obtained using any analytical procedure requires careful consideration of the diverse sources of actual or potential error associated with the results obtained.

Measurement uncertainty is a quantitative indication of the quality of the test result produced. It reflects how well the result represents the value of the quantity being measured and indicates the level of confidence that the value actually lies within the range defined by the uncertainty interval. It allows the data users to assess the reliability of the result and have confidence in the comparability of results generated elsewhere on the same sample or same population of the samples (JCGM., 2008).

The estimation of uncertainty in microbiological measurements is based mainly on the repeatability and reproducibility (Yong *et al*., 2012).

Gage Repeatability and Reproducibility (Gage R and R) study estimates the repeatability and reproducibility components of measurement system variation using an analysis of variance (ANOVA) evaluating how much of the total process variation is caused by the measurement system (Louka and Besseris, 2010). Total process variation consists of measurement system variation plus part-to-part variation. Measurement system variation consists of repeatability and reproducibility.

Repeatability is the variation due to the measuring device, or the variation observed when the same operator measures the same part repeatedly with the same device (Senvar and Firat, 2010).

Reproducibility is the variation due to the measuring system, or the variation observed when different operators measure the same part using the same device (Senvar and Firat, 2010).

Part-to-part variation indicates the ability for the gage to identify variation within products.

The study is designed to ensure stability and consistency of measurements made on an instrument by one or more operators (Smith *et al*., 2007).

Total coliforms are referred to as "Indicator organisms". The detection and quantification of coliforms is used in fact to obtain information regarding sanitary conditions of food and food-processing representing a simple way to provide a measure of food quality and spoilage potential.

Almost all the methods used to detect total coliforms are enumeration methods based on lactose fermentation (ISO 4832, 2006).

The Plate count method on Violet Red Bile Lactose Agar represents the reference method for the detection and enumeration of total coliforms in food. This medium meets the formulation and performance criteria laid down in ISO 4832 (2006). Coliforms rapidly ferment the lactose in VRBL agar and so reduce the pH of the medium, producing red-purple colonies, due to the inclusion of neutral red and crystal violet. These colonies are usually surrounded by red-purple halos of precipitated bile salts.

The 3M Petrifilm™ Coliform Count Plate (CCP), developed by 3M Laboratories, are commercially available agar plate substitutes. The 3M Petrifilm™ plates are composed of two dry rehydratable films coated with nutrients, a cold-soluble gel and a tetrazolium indicator dye to facilitate colony enumeration. Colonies growing on CCP Plates appear pink due to the reduction of the dye. Coliform colonies produce gas by the fermentation of lactose, which obviates the need for confirmatory tests (Gracias and McKillip, 2004).

Micro Biological Survey (MBS) method is a rapid method for selective counting of bacteria developed and patented by MBS srl (spin-off of Roma Tre University, Rome, Italy). It is based on a colorimetric survey performed in mono-use disposable reaction vials in which samples can be inoculated without any preliminary treatment. The operating principle of the MBS method is based on the capability to measure the catalytic activity of the redox enzymes in the

main metabolic pathways of bacteria through a redox indicator that changes color according to the oxidative state of the medium. The time required for the color change is inversely related to the logarithm of bacterial concentration and this allows to obtain an unequivocal correlation between the observed enzymatic activity and the number of viable cells present in the samples. A thermostatic optical reader automatically detects the color change providing an estimate of the number of bacteria in the sample (Bottini *et al*., 2011; Losito *et al*., 2012). The MBS method has been already tested in food samples demonstrating its reliability (Losito *et al*., 2014). For detection and enumeration of total coliforms in food samples, specific MBS vials (MBS coll vials) are available. The presence of coliforms causes a color change of the MBS reagent from red to yellow.

The aim of this study was to perform a Gage Repeatability and Reproducibility (Gage R and R) study to evaluate measurement uncertainty associated to three different microbiological methods of analysis for the detection and quantification of total coliforms in food samples: Plate count method, 3M Petrifilm™ count plates method and the MBS method.

MATERIALS AND METHODS

Samples: Naturally contaminated food samples were randomly selected. Ten samples were chosen among three different food matrices: Raw meat products, raw vegetables and dairy products. Table 1 shows the sample chosen for the analysis.

Preparation of naturally contaminated food samples: Naturally contaminated food samples were homogenized (2 min at high speed) by means of a stomacher (Laboratory Blender Stomacher 400, Seward, London, UK). Homogenized samples were then serially diluted in peptone water. Dilutions were used for the enumeration of total coliforms with the traditional plate count method and the 3M Petrifilm™ count plates. No sample preparation and dilution was required for the enumeration of bacteria using MBS method.

Plate count method: Serial dilutions of each homogenized sample were plated on Violet Red Bile Lactose Agar (VRBLA;Liofilchem, Roseto degli Abbruzzi, Italy) and incubated for 24 h at 37°C according to ISO 4832 (2006). Two operators performed in parallel six replicates per dilution. The bacterial concentration of the sample was calculated on the basis of the first countable dilution, or final suspension (colony forming units from 30-300). Results were obtained as CFU mL^{-1} or g calculated as follows:

$$y = Fc$$

where, y is the estimated particle concentration of the sample; F is the dilution factor (e.g., 1E4), the reciprocal of dilution (1:1E-4); c is the estimated particle concentration of the suspension.

3M Petrifilm™ count plates: Serial dilutions of each homogenized sample were plated on 3M Petrifilm™ *E. coli*/coliform count plates (3M Petrifilm™, St. Paul, USA) and incubated for 24 h at 37°C. Two operators performed in parallel six replicates per dilution. The bacterial concentration of the sample was calculated on the basis of the first countable dilution, or final suspension (colony forming units from 30-300). Results were calculated as described for plate count method.

Colorimetric MBS method procedure: For the analysis by the MBS method, ready-to-use MBS vials, for the detection of total coliforms (coli vials), already sterilized and containing the reagent for the analysis were used. To start the analysis, 10 mL of sterile distilled water were added to the vials that were shaken until all the reagents were dissolved and then inoculated with 1 mL or 1 g of samples. No serial dilutions were necessary. Vials were incubated at 37°C for 26 h in the MBS Multireader, a thermostated colorimeter that automatically looks at the color change and provides the bacterial concentration in the sample. The positive result corresponds to a color change from red to yellow. The color change occurred at different times after inocula in times that were inversely related to the bacterial concentration of the analyzed sample. The persistence of the starting color indicated a negative result, which is absence of the microorganisms of interest. Two operators performed in parallel six replicates of the analysis. Results were obtained as log CFU mL^{-1} or g of sample under the form of a printable report containing all the information concerning the sample and the performed analysis.

Table 1: Food samples chosen as "Parts" for the gage R and R study

Parts	1	2	3	4	5	6	7	8	9	10
Food sample	Rabbit	Chicory	Turkey	Salad	Mozzarella cheese	Calf	Raw milk	Ricotta cheese	Pork	Spinach

Gage R and R study: The SPC for Excel Software (www.spcforexcel.com) was used to perform a gauge R and R study for the three different measurement methods used for the quantification of total coliforms in food samples (AIAG., 2010). All results used for the statistical analysis were expressed as the logarithm of the bacterial concentration (log CFU mL^{-1} or g).

RESULTS

A gage repeatability and reproducibility (Gage R and R) study was carried out on three different methods of analysis used to for the quantification of total coliforms in food samples: Plate count method, 3M Petrifilm™ count plates method and the MBS method. Ten samples chosen among four food matrices were analyzed by two operators in parallel using the three different methods.

Gage R and R study: The ANOVA Table with interaction. Gage Repeatability and Reproducibility (Gage R and R) is the amount of measurement variation introduced by a measurement system, which consists of the measuring instrument itself and the individuals using the instrument. The crossed gage R and R study estimates how much total process variation is caused by the measurement system. Total process variation consists of measurement system variation plus part-to-part variation.

The analysis of variance (ANOVA) calculates variance components and then uses those components to estimate the percent variation due to the measuring system. The percent variation appears in the gage R and R Table. The ANOVA Table with interaction includes terms for the part (Part), operator (Operator) and operator-by-part interaction (Part*Operator). This table allows to verify the significance of the factors that characterize the analysis identified by a p-value <0.25.

The ANOVA table with interaction results show that, excluding the significance of "part" for all three the methods a significant variation is due to the "operator*part" for Plate Count method and 3M Petrifilm™ count plates method while for the MBS method the "Operator*Part" does not significantly affect the measure (Table 2-4).

Gage R and R study

Total variation report: Total Variation (TV) report analyzes the gage study data as a percentage of total variation. The report is divided into sections presenting the total gage R and R divided in equipment variation and appraiser variation and part variation.

Table 2: Analysis of variance of the components contribution to the variation of the measuring system of the plate count method

	ANOVA table with Interaction				
Sources	Df	SS	MS	F	p-value
Part	9	274.052	30.4500	86.830	0.000
Operator	1	0.774	0.7740	2.208	0.171
Operator*Part	9	3.156	0.3510	3.517	0.001
Repeatability	100	9.971	0.0997		
Total	119	287.954			

Table 3: Analysis of variance of the components contribution to the variation of the measuring system of the 3M Petrifilm™ count plates method

	ANOVA table with Interaction				
Sources	df	SS	MS	F	p-value
Part	9	276.076	30.6750	118.217	0.000
Operator	1	0.375	0.3750	1.446	0.260
Operator*Part	9	2.335	0.2590	5.070	0.000
Repeatability	100	5.118	0.0512		
Total	119	283.905			

Table 4: Analysis of variance of the components contribution to the variation of the measuring system of the MBS method

	ANOVA table with Interaction				
Sources	df	SS	MS	F	p-value
Part	9	248.8930	27.6550	613.762	0.000
Operator	1	0.0875	0.0875	1.942	0.197
Operator*Part	9	0.4060	0.0451	1.176	0.318
Repeatability	100	3.8300	0.0383		
Total	119	253.2150			

The total gage repeatability and reproducibility (Gage R and R) represent both the equipment variation and the appraiser variation. The gage R and R% returned in the TV report is the percentage of the total variation due to the measurement system. The total gage R and R percentage is 5.59% for the plate count method, 3.35% for the 3M Petrifilm™ count plates method and 1.69% for the MBS method. For all three methods the contribution the total gage R and R is less than 10% demonstrating that all three methods are considered acceptable.

Equipment Variation (EV) represents the repeatability of the equipment or measurement device. The equipment variation percentage is 3.57% for the plate count method, 1.95% for the 3M Petrifilm™ count plates method and 1.66% for the MBS method. For all three methods the contribution of EV is less than 10% demonstrating that there are no significant issues with the measurement equipment itself.

Appraiser Variation (AV) represents the reproducibility of the system. The appraiser variation percentage is 1.84% for the plate count method, 1.40% for the 3M Petrifilm™ count plates method and 0.03% for the MBS method. Also in this case for all three methods the contribution of EV is less than 10%

demonstrating that there is no significant operator-to-operator difference in measurements.

Part Variation (PV) represents the variation of the products or parts used to conduct the gage study. The part variation percentage is 94.41% for the plate count method, 96.65% for the 3M Petrifilm™ Count Plates method and 98.31% for the MBS method. The high percentage of the total variations from the parts demonstrates that all methods are considered adequate systems because the parts truly represent the range of the process variation. If the PV% is low, less than 30%, the parts selected do not represent the full variation of the process.

Total Variation (TV) report is graphically represented in the Components of variation chart (Fig. 1a-c).

Gage R and R study

Standard deviation report: The Standard Deviation (SD) report analyzes the gage study data as a percentage of total variation based on the standard deviation of each term. The report is divided into sections presenting the total gage R and R divided in equipment variation and appraiser variation and part variation.

Because the standard deviation uses the same measure units as the part measurements it allows for meaningful comparison between the methods under study. The standard deviation percentage of the measurement system is 0.38 for the plate count method, 0.29 for the 3M Petrifilm™ count plates method and 0.19 for the MBS method (Table 5-7).

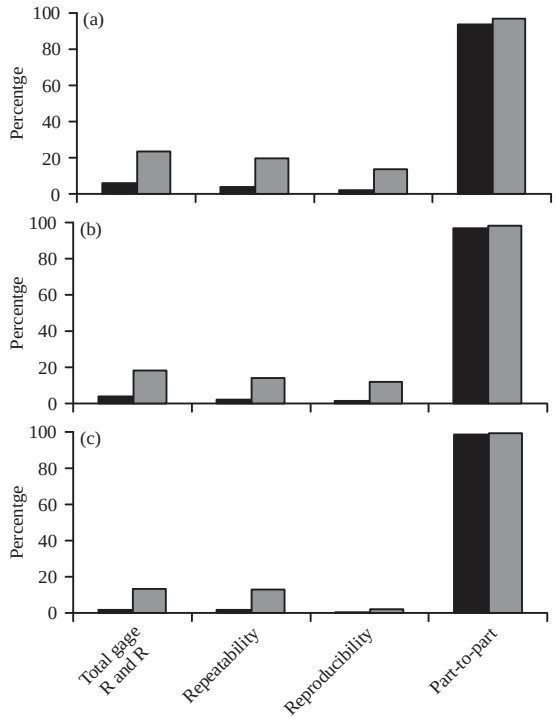

Fig. 1(a-c): Components of variation chart, graphical presentation of the total variation report for the (a) Plate count method, (b) 3M Petrifilm™ count plates method and (c) MBS method. The black bar chart represents the contribution of variance while the grey bar chart represents the contribution of standard deviation

Gage R and R study

Range control chart: Range control chart shows the average ranges obtained for each part and operator. The range is the difference between the maximum and minimum value obtained in a set of measurements and is used to verify the

Table 5: Percentages of total variation based on the standard deviation contribution of each component for the plate count method

	Standard deviation report		
Sources	SD	6*SD	6*SD (%)
Total gage R and R	0.385	2.313	23.65
Repeatability	0.316	1.895	19.37
Reproducibility	0.221	1.327	13.57
Operator	0.0840	0.504	5.16
Operator*part	0.205	1.227	12.55
Part-to-part	1.584	9.503	97.16
Total variation	1.630	9.780	100.00

Table 6: Percentages of total variation based on the standard deviation contribution of each component for the 3M Petrifilm™ count plates method

	Standard deviation report		
Sources	SD	6*SD	6*SD (%)
Total gage R and R	0.296	1.778	18.30
Repeatability	0.226	1.357	13.97
Reproducibility	0.191	1.149	11.82
Operator	0.0439	0.264	2.71
Operator*part	0.186	1.118	11.51
Part-to-part	1.592	9.552	98.31
Total variation	1.619	9.716	100.00

Table 7: Percentages of total variation based on the standard deviation contribution of each component for the MBS method

	Standard deviation report		
Sources	SD	6*SD	6*SD (%)
Total gage R and R	0.199	1.195	13.02
Repeatability	0.197	1.183	12.88
Reproducibility	0.0285	0.171	1.86
Operator	0.0285	0.171	1.86
Operator*part	1.517	9.102	99.15
Part-to-part	1.530	9.180	100.00
Total variation	0.199	1.195	13.02

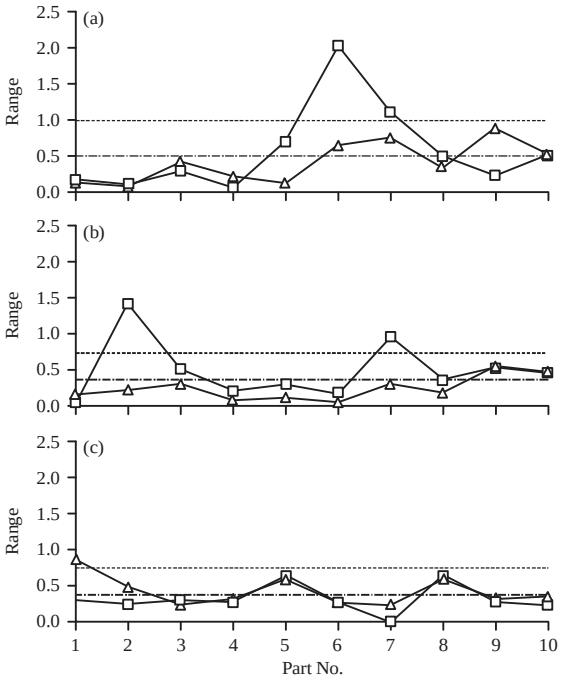

Fig. 2(a-c): Range control chart, average ranges obtained for each part and operator for the (a) Plate count method, (b) 3M Petrifilm™ count plates method and (c) MBS method. Each point is a mean of the ranges for operator A Δ and operator B □. The dashed line represents the Upper Control Limit (UCL) while the dash-dot line represents the average range

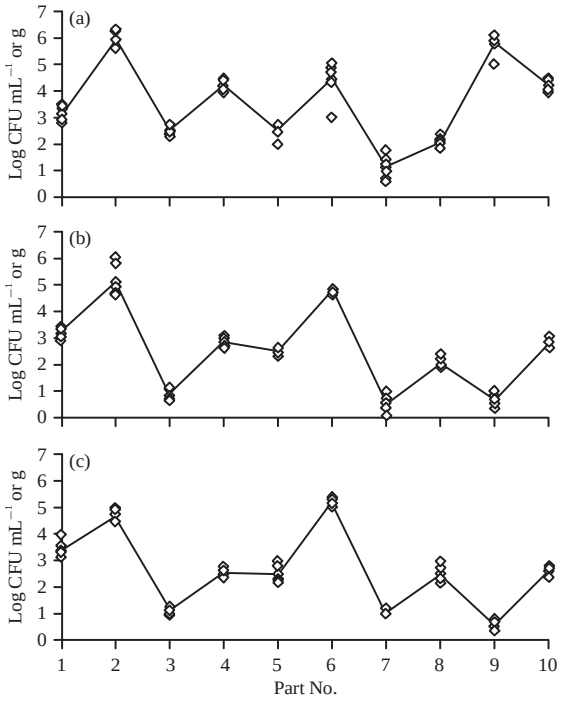

Fig. 3(a-c): Measurement by part chart, all measurements in the study arranged by part for the (a) Plate count method, (b) 3M Petrifilm™ count plates method and (c) MBS method. The line connects the average measurements for each part

repeatability of the measurement system. Control limits (UCL and LCL) are calculated on the basis of the within subgroups variations. If the majority of values falls in of the control limits this means that the measurement system is repeatable.

The range control charts (Fig. 2a-c) show that the repeatability of the measurement system is regular for all three methods except for two cases (B-6 and B-7 for the plate count method, B-2 and B-7 for the 3M Petrifilm™ count plates method, B-2 and B-7 for the MBS method).

Gage R and R study

Measurement by part chart: Measurement by part chart shows all of the measurements in the study arranged by part and shows the variation in each set of measurements. The line connects the average measurements for each part (Fig. 3a-c). It shows that variation is quite constant for all three methods. However, for the plate count method it is possible to observe a great variability in part 6.

DISCUSSION

During the past few years, considerable attention has been paid to the estimation of measurement uncertainty for reference and alternative microbiological methods.

The quality of a measurement system in terms of repeatability and reproducibility can be determined by a gage repeatability and reproducibility study. In this context a gage R and R study was carried out on three different methods of analysis used to for the quantification of total coliforms in food samples: Plate count method, 3M Petrifilm™ count plates method and the MBS method.

The plate count and the 3M Petrifilm™ count plates methods are based on observation and count of the number of colonies and reaction sites deemed typical of the target species that are able to grow forming discrete units on specific growth media. This basic observation depends on the operator and the reading of the results that are always more or less uncertain because of the human factor. The plate count requires several steps as: Sample preparation, media preparation and dispensing, plating out of sample dilutions, plate reading and colony counting. The 3M Petrifilm™ count plates method

instead does not require media preparation and dispensing simplifying the analytical procedure.

The MBS method measures the catalytic activity of the main metabolic pathways of bacteria thanks to redox indicators that are able to change color according to the oxidative state of the medium. The time required for the color change is inversely related to the logarithm of bacterial concentration and this allows to obtain an unequivocal correlation between the observed enzymatic activity and the number of viable cells present in the samples. A thermostatic optical reader automatically detects the color change providing an estimate of the number of bacteria in the sample. In addition analyses do not require sample preparation and 1 g or 1 mL of the samples can be directly inserted in the analysis vial. These characteristics drastically reduce the complexity of procedures.

The substantial differences between the three methods are reflected in the results of the gage R and R study. The ANOVA study of the measurement system variation shows that a significant variation is due to the interaction between the operators and the parts for plate count method and 3M Petrifilm™ count plates method while for the MBS method it does not significantly affect the measure. The variation in each set of measurements confirms that the operator does not significantly affect measurements.

For all three methods the contribution the total gage R and R is less than 10%, demonstrating the ability to accurately evaluate the concentration of total coliforms in food samples. However, the contribution decreases following the tendency: plate count method>3M Petrifilm™ count plates method>MBS method. This trend is confirmed also by the analysis of standard deviation.

Together these results demonstrate that greater repeatability and reproducibility are connected to a more simple analytical procedure. Tests requiring a series of manual steps and individual interpretation of results are more vulnerable to human error.

Previous studies confirm this hypothesis pointing out that, for the reference plate count method, the estimates of uncertainty can in fact be significantly influenced by plating procedures and the choice of medium that can mislead interpretation of results (Munsch-Alatossava *et al*., 2007; Habib *et al*., 2008; Yong *et al*., 2012). Also the role of homogenization, required for sample pretreatment with plate count methods, deserves more attention considering that different studies have led to conflicting results. Rohde *et al*. (2015) pointed out that homogenization can be responsible for considerable of interlaboratory differences in pathogen detection in meat while, Ingham *et al*. (2009) demonstrated that the sample preparation method had no significant effect on the results of any analytical test, although counts tended to be higher after mechanical stomaching instead of manual squeezing. Previous studies have demonstrated that alternative microbiological methods that are more efficient in terms of preparation time and labor, perform similarly or better than the reference methods. Fedio *et al*. (2008) demonstrated that Petrifilm™ staph express count system (STX) revealed no significant difference compared to the bacteriological analytical manual direct-plating method for enumeration of *Staphylococcus aureus* in artificially contaminated hard cheese. De Souza *et al*. (2015) showed good significance between conventional methodologies and Petrifilm™ plates in count of indicators of sanitary-hygienic quality and pathogenic microorganisms in sheep milk and further the Petrifilm™ STX had even higher recoverability of bacteria compared with the conventional methodology.

Reduced labor and automation appears to take out much of the potential error and deliver more reliable results. Interestingly, operator B seems always to encounter more difficulties and the raw milk (sample 7) seems to produce the highest variability for all methods.

CONCLUSION

Evaluation of measurement uncertainty is an integral part of metrology and no measurement result can be interpreted correctly without at least some knowledge of the associated uncertainty. To give some measure of confidence to the measured value in fact measurement errors must be identified and their probable effect on the result estimated. Assessing uncertainties is necessary to better quantify and to improve the quality of measurements. In particular the valid identification and count of microorganisms in food samples can be regarded as a rather complex measurement involving human labor and interpretation. This study is an attempt to evaluate the uncertainty associated to traditional and alternative methods of microbiological analysis of food samples. Results demonstrate that uncertainty is certainly related to the amount of manual work and the individual interpretation of results, indicating that it is important to aim to reduce these sources of error in order to obtain more reliable results.

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