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"VALIDATION AND STABILITY STUDIES ON THE MBS METHOD FOR MICROBIOLOGICAL CONTROL OF FOOD SAMPLES"

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CHAPTER I
Introduction

1.1 Food borne diseases

1.1.1 Major food borne pathogens

Food and water represent important vehicles in the spread of food borne infectious diseases that constitute a major public health problem not only in underdeveloped countries but also in those with high socio-economic development (in the U.S., for example, there are an average of 15 million cases of food borne illness each year, of which about one third are due to bacterial etiologic agents). Food borne diseases are disease states that occur as a result of consumption of food or drinks that have been contaminated by a pathogenic microorganism or by a bacterial toxin (Barbuti *et al*., 2002). They are distinguishable in three categories depending on the pathogenicity:

- Food borne infections: they are caused by the ingestion of live pathogens, with subsequent invasion and multiplication within the gastrointestinal tract or in other tissues; a classic example is the food borne infection caused by *Listeria monocytogenes*.
- Food borne intoxications: they are caused by the ingestion of bacterial toxins. They are pathological events resulting from the consumption of food containing toxins produced by microorganisms that have multiplied in food before its consumption. Thus for intoxication to manifest itself microorganisms must not be necessarily present; instead the presence of the toxin is necessary and sufficient. Examples of food borne intoxications are the diseases caused by the toxins of *Clostridium botulinum* and *Staphylococcus aureus* (Murray *et al*., 2003).
- Food toxicoinfections: they are caused by the ingestion of food containing both bacteria and their toxins. In this case, toxicity is doubled and is given both by the toxins and by living microorganisms within the food. A widespread food borne toxicoinfection that can be used as an example is the one caused by bacteria of the genus *Salmonella* (Cevenini, 2000).

The pathogenic patterns of infections and intoxications, while substantially different, are generally included in the name of "food toxicoinfections". This term identifies a group of

syndromes that are different from food borne infections that are characterized by the epidemiological specificity of being transmitted only after that a bacterial multiplication has occurred in food. Poisonous chemical compounds, such as pesticides used in agriculture can also cause food contamination: to avoid this problem use and distribution of these substances are strictly regulated, with an approach based on prevention more than research of the substances themselves. Food borne toxicoinfections usually affect the gastrointestinal tract, appearing suddenly after a short period of incubation (from a few hours to 1-2 days) and developing into an epidemic among those who consumed the contaminated food.

Factors affecting the occurrence of toxicoinfection, disturbing or favouring bacterial multiplication, are: temperature, pH, osmotic pressure, saline concentration, amount of oxygen and food characteristics. In order to induce toxicoinfections the presence of bacteria in food is not sufficient, it is in fact necessary for the bacteria to reach a certain concentration, the so-called minimum infectious dose, high enough to cause infection; this is typically in the order of ~ 10^4 cells / gram, even though this value can be either higher, as for *V. cholerae*, or lower and even down to a single unit, as for some species of *Shigella* (Barbuti *et al*., 2002).

One of the factors which make food borne illnesses so important is the tendency, in the last years, to a significant increase of their incidence. The reasons for the increase of these diseases are different:

1) The demographic changes occurring in industrialized countries that have led to an increase of the amount of population susceptible to such infections.

2) The increased consumption of food that can be easily contaminated (such as vegetables and fresh fruit) or not properly cleaned, and the increased tendency to eat in restaurants (Jones & Angulo, 2006), are also secondary causes of the increase of these diseases.

3) The expansion of food industry itself plays an important role, since the wide distribution of products derived from industrial processes can represent an important risk of widespread epidemics (Angelillo *et al*., 2001).

Currently, they exist more than 250 types of food borne toxicoinfections. The most common, even in developed countries, are those caused by bacteria belonging to *Salmonella* spp. and *Escherichia coli (E.coli)*; regarding diseases that are caused by the production of toxins, the most hazardous pathogens are *Staphylococcus aureus*, *Clostridium botulinum* and *Clostridium perfringens*.

Furthermore emerging pathogens such as *E.coli* O157: H7, *Yersinia enterocolitica* and *Campylobacter jejuni* that in the U.S. are responsible for more cases than those previously

mentioned, are second only to Norwalk-like viruses as agents of food borne illnesses (Tauxe, 2002).

1.2 Main indicators of food microbial contamination

1.2.1 Hygienic indicators: Total Viable Count (TVC)

The Total Viable Count TVC is a parameter that allows to obtain a general picture of the hygienic state of the sample, whether it is a food sample or a surface, and to establish if the procedures adopted during production (in case of a food sample) have been able to control successfully contamination and bacterial growth.

Given that the incubation temperature is 30°C, the TVC parameter allows to determine the presence of mesophilic microorganisms (able to grow in warm-blooded animals as human beings) in a food sample; however it does not provide any information on the type of microorganisms present. This information is particularly important in relation to the shelf-life of the product; in good preservation conditions in fact it is clear that the shelf life of a product is in inverse proportion to the level of bacteria initially present.

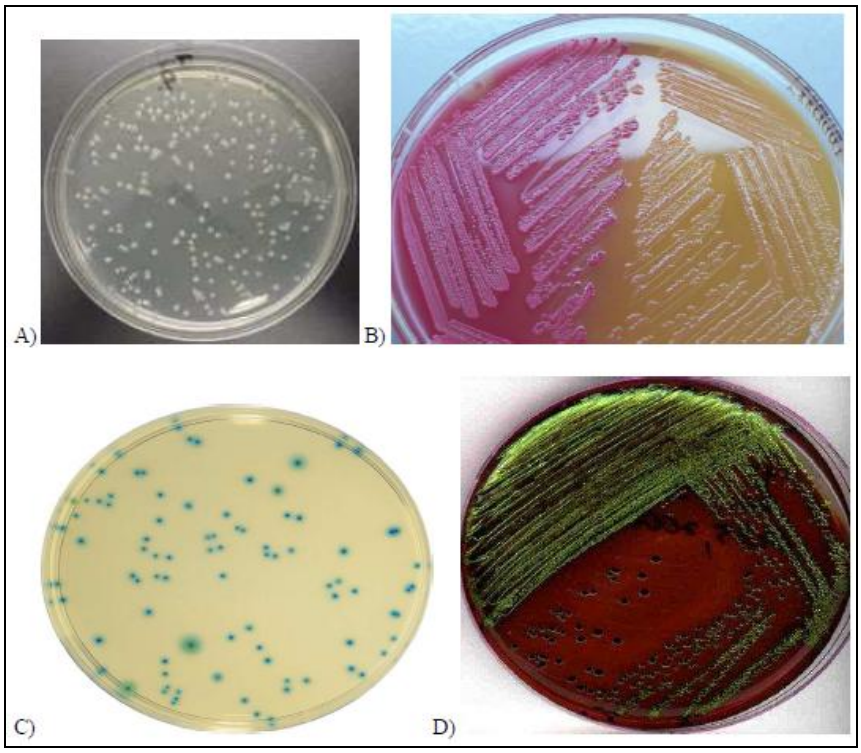


Figure 1: A) Total Viable Count on PCA; B) Growth on MacConkey agar of lactose negative (transparent colonies) and lactose positive (coliforms, pink/red colonies) Enterobacteriaceae; C) Growth of *E. coli* on selective TBX agar; D)Growth of coliforms (metallic green) and lactose negative Enterobacteriacea on EMB agar

TVC is frequently higher than zero in food products and on surfaces with acceptable limits that vary and depend from type of product (for example the upper limit acceptable for dairy products is quite high, as for raw meat); higher is the amount of bacteria present higher will be the possibility that among these they will be pathogens potentially harmful for humans (Tiecco, 1997).

1.2.2 Indicators of faecal contamination: coliforms and *Escherichia coli*

At the end of XIX century Schardinger (1892) introduced the use of gram negative bacteria taxonomically related to *Salmonella typhi* as possible markers to detect water safety; a few years later, in 1904, Eiijkman set up a test for the isolation of *E.coli* by increasing the temperature of incubation at 46°C.

The survey techniques available at the beginning of the century for the detection of this bacterial species weren't able to grant a routine monitoring and a rapid verification of fecal contamination; for this reason a larger group of similar microorganism (that included *E.coli*) were subject of research as surrogate indicators.

This group was named "of coliforms" by Blachstein in 1893 (Tallon *et al*., 2005; Kornacki & Johnson, 2001), and it has been subjected to many taxonomical revisions especially with the advent of extremely precise molecular methods of classification: *Bacterium coli* is now known as *Escherichia coli*, *Bacterium Aerogenes* as *Enterobacteraerogenes* and the different species of minor importance have all been reclassified correctly.

More than a real taxonomic entity, the coliform group is substantially an operating definition useful "on field", that includes all the lactose-fermenting Enterobacteria. These are present in the intestinal tract of warm blooded vertebrates and have an average concentration of 10^9 CFU/g in feces; fecal coliforms instead reach a concentration of 10^7 CFU/g. To this group belong Enterobacteria that inhabit exclusively the gastro-intestinal tract but also of environmental origin. Apart from *E.coli*, in most cases microorganism defined as coliforms have essentially a saprophyte life cycle; therefore their presence in foodstuff is not necessarily index of fecal contamination or of the presence of pathogens (Jay *et al*., 2005).

The definition of "fecal coliforms" was therefore revised due to their not strict fecal origin: it results in the more actual and precise form of address of thermo tolerant coliforms. Depending on some physiological and biochemical characteristics three groups of coliforms can be distinguished:

- Total coliforms: gram negative rods, non-sporing, facultative anaerobic, catalase +, oxidase -, belonging to the family of Enterobacteriaceae; they are able to grow in the presence biliary salts and ferment lactose producing acid and gas in 24-48 h at 30-37°C.
- Fecal coliforms or thermo tolerant: they have the same properties of the other coliforms but that grow also at 44,5°C.

Escherichia coli is a species taxonomically defined of Enterobacteriaceae with all the characteristics to be an excellent indicator. *E.coli* belongs to the group of thermo tolerant coliforms, it is non-sporing, facultative anaerobic, lactose-fermenting; it is normally present as non-pathogenic commensal of the intestinal tract of humans and other warm-blooded animals, that is colonized a few hours after birth (Tallon *et al*., 2005; Alonso *et al.*, 1999; Leclerc *et al*., 2001). The 93% of *E.coli* isolates are lactose-fermenting, and only some diarrheagenic isolates are lactose negative; the test for the production of indole from tryptophan is positive for the 99% of isolates; it is very precise and utilized also as confirmation test through the Kovacs reagent.
In anaerobiosis all ferment glucose producing acid and gas, they are catalase + (differing from the other thermo tolerant coliforms) and the 97% of isolates have β-glucuronidase activity; this property is highly selective and it is used in the more recent laws for the search of *E.coli* in food microbiology. A series of biochemical tests, called IMVC, is based on the multiple assay for the production of indole from tryptophan (I), the reaction of methyl red (M), the Voges-Proskauer test (V, production of acetoin) and the ability to grow on Citrate agar (C): the biochemical profile of *E.coli* I(+); M(+); V(-); C(-).

1.2.3 Other relevant pathogenic microorganism

Unlike *E.coli, Staphylococcus aureus* cannot be considered as an index of contamination but rather as an indicator of hygiene of the process and, at the same time, as a potential pathogen.
The microorganisms of the genus *Staphylococcus* belong to the family of *Micrococcaceae*, Gram-positive cocci, non-sporing, facultative anaerobic, catalase +, and capable of producing a variety of toxins and enzymes (among which coagulase). They can be isolated from many animal products such as meat and dairy products and come from raw material, but usually their presence is related to poor manipulation. Food is hardly ever suitable for bacteria replication, but if during the storage the conditions are established toxin production and consequent intoxication may occur. Among bacteria researched for food safety it is important to mention the group of microorganism belonging to *Salmonella* spp. and *Listeria monocytogenes* (Figure 2 A and B). *Salmonella* is a widespread enteric pathogen, particularly in food of animal origin (it can, for

example, infect the ovaries of hens and contaminate the egg before the shell is formed, and subsequently develop if the egg is eaten raw); *Listeria* is a particularly insidious species both for the severity of clinical manifestations that is able to establish (the heterogeneous, from a clinical point of view, listeriosis) and for the extreme psicrofilia (Tiecco, 1997).

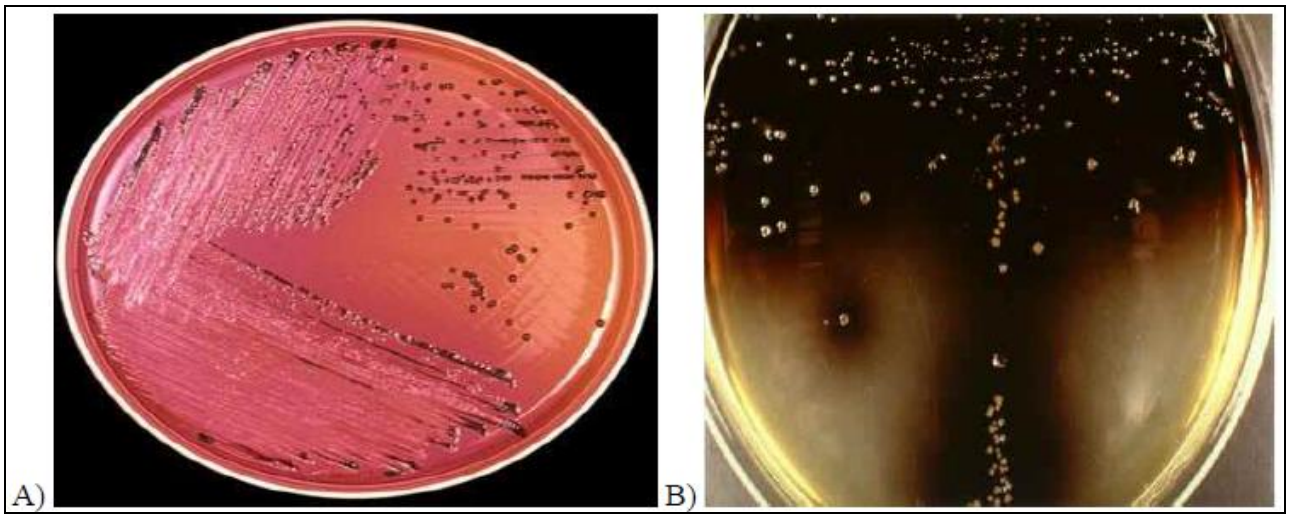


Figure 2: A) *Salmonella* on XLD agar; B) *Listeria monocytogenes* on Oxford agar

1.3 Food law evolution

Since the '90s, the food processing sector has been involved in several crises that pointed out to Government and public opinion all the issues concerning food safety.

In particular, the Bovine Spongiform Encephalopathy epidemic, the spread of avian flu and contaminations by dioxins, PCBs and mycotoxins, demonstrated the weakness of the European Community food policy, bringing to light several critical points:

- The ineffectiveness of food legislation and regulations, due to an excessively fragmented structure that controls only the finished products and provides pointless details for food safety;
- The disorganization and disparity between official controls, due to marked organizational differences between Union members;
- The insufficient controls on animal feed that have shown to be crucial to prevent many food borne diseases;

In this context apart from the difficulty to foresee and manage public health crises it is also important to consider some cultural, social and economic transformations that concerned the food industry, leading to an increased demand for controls to assure food safety. Among these:

- The globalization of markets of food products and raw materials: that causes an obvious difficulty in detecting and containing the risk. The trend to minimize food processing in order to obtain more fresh, nutritious and tasty products: this search for authenticity, though commendable, is an additional element of risk because it increases the risk of contamination.
- The growing attention of consumers towards food safety, that led the European Commission to reach for the highest standards possible.

The legislative innovations introduced by EC Regulation 178/2002 and especially by the so-called "Hygiene package" in 2006 (that represents its natural implementation), consider as possible all these points: through a process of awareness and acquired responsibility, all food chain operators, from raw materials producers to consumers, can be involved and have an active role in the control of food safety. The principles of this new legislation result in a radical change in approaching food safety, which translates into a greater awareness of personal rather than company responsibilities (Roggi & Turconi, 2003).

1.3.1 Food safety and quality

Until the mid-'90s, food control in Italy and in the other countries of Western Europe was focused on the finished product. The controls included a series of inspections carried out by the competent authority to verify compliance with good maintenance and hygiene practices (GMP or Good Manufacturing Practices and GHP or Good Hygiene Practices), and random sampling of finished products. The outcome of food borne diseases dramatically revealed the limit of sampling plans especially caused by the uneven distribution of pathogen microorganisms in food. This approach can in fact be totally ineffective when there is a serious risk of contamination at low bacterial concentrations and in case of diseases with low or quite variable prevalence (as, for example, listeriosis).

Since the mid-90s controls are no longer concentrated on the finished product, but they are rather distributed throughout food processing, making food chain operators ever more responsible for food safety. The introduction in food companies of the concept of quality as an added value from the '80s has greatly accelerated this process, which had been already in operation by law.

In this context, the assurance of food quality and safety becomes an essential determinant of the quality system: the evolving legislation has led companies to consider quality as a determining criteria indispensable in a context of choice that has now evolved into a requirement imposed by law (Civille, 1991).

1.3.2 The new microbiological standards: European Directive 2073/2005

EC Regulation 2073/2005, which is complementary to Regulation 852/2004 and in force since 1 January 2006, represents an element of innovation in microbiological food control and, in the coming years it will become the legislative reference model for microbiological food safety policy.

The fundamental principles are the following:

- Food must not contain microorganisms, their toxins or metabolites in quantities sufficient to represent an unacceptable risk for human health.
- Microbiological criteria indicate the acceptability of a product and its processing, handling and distribution.

The application of microbiological criteria is a part of HACCP procedures and must be implemented with the other hygienic control measures. Microbiological criteria are defined in EC Regulation 2073/2005 Article 2 as "criteria defining the acceptability of a product, a batch of foodstuffs or of a process, based on the absence, presence or the number of microorganisms and / or based on the amount of their toxins / metabolites, per unit mass, volume, area or lot" (Villani, 2007; Galli Volonterio, 2005). The implementing Regulation EC 2073/2005 lays down microbiological criteria for the major microorganisms able to cause food borne diseases, through the analysis of the microbiological risk. These criteria are not absolute and they can be modified, if necessary, to take account of the evolution of food safety and food microbiology: advances in science, technology and methodology, changes in the prevalence and levels of contamination, the percentage of vulnerable consumers and any unexpected results emerging from the evaluation of risks. Setting safety criteria for food pathogens has undoubtedly the advantage of providing simple standards for the acceptability of food products: however, it is not possible to assess how public health could be positively influenced by the protection provided by specific food safety criteria. Microbiological tests alone can, in fact, generate false negatives due to the limitation of statistical sampling plans, especially in cases where the danger represents an unacceptable risk even at low concentrations and / or low and variable prevalence (Villani, 2007). Food safety is in fact a result of several factors: microbiological criteria should therefore be respected along with the other aspects of EU food policy, like HACCP and official controls to verify the compliance of operators.

The elements to research as described in the Regulation are:

- *Listeria monocytogenes*
- *Salmonella* spp.

- Staphylococcal enterotoxins
- *Enterobactersakazakii*
- *Escherichia coli*
- Histamine levels

The document describes in detail how to relate these parameters to the different food matrices where they are presumably present (and therefore they have to be researched), and the principles of the sampling plan that have to be activated, referring to ISO standards (International Organization for Standardization) and guidelines of Codex Alimentarius (set of rules and standards developed by the Codex Alimentarius Commission, commission established in 1963 by FAO and WHO). According to these Regulations, food chain operators must ensure the respect of hygienic criteria in food processing and food safety throughout the shelf-life of the products (Art. 3) (Jay *et al*., 2005). In this regard, two distinct categories of microbiological criteria are defined:

- Food safety criteria defines the acceptability of a food product / batch, and it should be applied to products on sale;
- Hygienic criteria of the process defines the acceptability of the process: it cannot be applied to products on sale but to their intermediate (meat and meat products, milk and dairy products, egg products, fishery products, vegetables, fruits and products thereof); it indicates the specific levels of contamination that must be respected and above which corrective measures are needed (Ray, 2004).

As already mentioned, EC Regulation 2073/2005 should be considered an "open" standard, a first list of microbiological limits, scientifically determined, that should be updated depending on epidemiological evidence leading to new criteria that should be followed with the same strictness. Modifications have already been performed, for example, through L.ve Decree n. 193 and EC Regulation 1441/2007 that modified, in a more or less relevant way, the analysis procedures and the microbiological limits of acceptability of some food products. The following table (Table 1) lists the basic microbiological limits provided in Regulation No 2073/2005, including the updated 1441/2007.

Food category	Micro-organisms/their toxins, metabolites	Sampling-plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies
		n	c	m	M		
1.1. Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes (4)	*Listeria monocytogenes*	10	0	Absence in 25 g		EN/ISO 11290-1	Products placed on the market during their shelf-life
1.2. Ready-to-eat foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes	*Listeria monocytogenes*	5	0	100 cfu/g (5)		EN/ISO 11290-2 (6)	Products placed on the market during their shelf-life
		5	0	Absence in 25 g (7)		EN/ISO 11290-1	Before the food has left the immediate control of the food business operator, who has produced it
1.3. Ready-to-eat foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes (4) (8)	*Listeria monocytogenes*	5	0	100 cfu/g		EN/ISO 11290-2 (6)	Products placed on the market during their shelf-life
1.4. Minced meat and meat preparations intended to be eaten raw	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.5. Minced meat and meat preparations made from poultry meat intended to be eaten cooked	*Salmonella*	5	0	From 1.1.2006 Absence in 10 g From 1.1.2010 Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.6. Minced meat and meat preparations made from other species than poultry intended to be eaten cooked	*Salmonella*	5	0	Absence in 10 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.7. Mechanically separated meat (MSM) (9)	*Salmonella*	5	0	Absence in 10 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.8. Meat products intended to be eaten raw, excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life

Food category	Micro-organisms/their toxins, metabolites	Sampling-plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies
		n	c	m	M		
1.9. Meat products made from poultry meat intended to be eaten cooked	*Salmonella*	5	0	From 1.1.2006 Absence in 10 g From 1.1.2010 Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.10. Gelatine and collagen	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.11. Cheeses, butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurisation (10)	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.12. Milk powder and whey powder (10)	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.13. Ice cream (11), excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.14. Egg products, excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk	*Salmonella*	5	0	Absence in 25g		EN/ISO 6579	Products placed on the market during their shelf-life
1.15. Ready-to-eat foods containing raw egg, excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk	*Salmonella*	5	0	Absence in 25 g or ml		EN/ISO 6579	Products placed on the market during their shelf-life
1.16. Cooked crustaceans and molluscan shellfish	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.17. Live bivalve molluscs and live echinoderms, tunicates and gastropods	*Salmonella*	5	0	Absence in 25g		EN/ISO 6579	Products placed on the market during their shelf-life

Food category	Micro-organisms/their toxins, metabolites	Sampling-plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies
		n	c	m	M		
1.18. Sprouted seeds (ready-to-eat) (12)	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.19. Pre-cut fruit and vegetables (ready-to-eat)	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.20. Unpasteurised fruit and vegetable juices (ready-to-eat)	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.21. Cheeses, milk powder and whey powder, as referred to in the coagulase-positive staphylococci criteria in Chapter 2.2 of this Annex	Staphylococcal entero-toxins	5	0	Not detected in 25g		European screening method of the CRL for Milk (13)	Products placed on the market during their shelf-life
1.22. Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age, as referred to in the Enterobacter-iaceae criterion in Chapter 2.2 of this Annex	*Salmonella*	30	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.23. Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age, as referred to in the Enterobacter-iaceae criterion in Chapter 2.2 of this Annex	*Enterobacter sakazakii*	30	0	Absence in 10 g		ISO/DTS 22964	Products placed on the market during their shelf-life
1.24. Live bivalve molluscs and live echino-derms, tunicates and gastropods	*E.coli* (14)	1 (15)	0	230 MPN/100g of flesh and intra-valvular liquid		ISO TS 16649-3	Products placed on the market during their shelf-life
1.25. Fishery products from fish species asso-ciated with a high amount of histidine (16)	Histamine	9 (17)	2	100 mg/kg	200 mg/kg	HPLC (18)	Products placed on the market during their shelf-life

Food category	Micro-organisms/their toxins, metabolites	Sampling-plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies
		n	c	m	M		
1.26. Fishery products which have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine (16)	Histamine	9	2	200 mg/kg	400 mg/kg	HPLC (18)	Products placed on the market during their shelf-life

(1) n = number of units comprising the sample; c = number of sample units giving values over m or between m and M.
(2) For points 1.1-1.24 m=M.
(3) The most recent edition of the standard shall be used.
(4) Regular testing against the criterion is not useful in normal circumstances for the following ready-to-eat foods:
- those which have received heat treatment or other processing effective to eliminate *L. monocytogenes*, when recontamination is not possible after this treatment (e.g. products heat treated in their final package),
- fresh, uncut and unprocessed vegetables and fruits, excluding sprouted seeds,
- bread, biscuits and similar products,
- bottled or packed waters, soft drinks, beer, cider, wine, spirits and similar products,
- sugar, honey and confectionery, including cocoa and chocolate products,
- live bivalve molluscs.

(5) This criterion applies if the manufacturer is able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit 100 cfu/g throughout the shelf-life. The operator may fix intermediate limits during the process that should be low enough to guarantee that the limit of 100 cfu/g is not exceeded at the end of the shelf-life.
(6) 1 ml of inoculum is plated on a Petri dish of 140 mm diameter or on three Petri dishes of 90 mm diameter.
(7) This criterion applies to products before they have left the immediate control of the producing food business operator, when he is not able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 cfu/g throughout the shelf-life.
(8) Products with pH ≤ 4,4 or a_w ≤ 0,92, products with pH ≤ 5,0 and a_w ≤ 0,94, products with a shelf-life of less than five days are automatically considered to belong to this category. Other categories of products can also belong to this category, subject to scientific justification.
(9) This criterion applies to mechanically separated meat (MSM) produced with the techniques referred to in Chapter III, paragraph 3, in section V of Annex III to Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin.
(10) Excluding products when the manufacturer can demonstrate to the satisfaction of the competent authorities that, due to the ripening time and a_w of the product where appropriate, there is no salmonella risk.
(11) Only ice creams containing milk ingredients.
(12) Preliminary testing of the batch of seeds before starting the sprouting process or the sampling to be carried out at the stage where the highest probability of finding *Salmonella* is expected.
(13) Reference: Hennekinne et al., J. AOAC Internat. Vol. 86, No 2, 2003.
(14) *E. coli* is used here as an indicator of faecal contamination.
(15) A pooled sample comprising a minimum of 10 individual animals.
(16) Particularly fish species of the families: *Scombridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae, Scombresosidae.*
(17) Single samples may be taken at retail level. In such a case the presumption laid down in Article 14(6) of Regulation (EC) No 178/2002, according to which the whole batch should be deemed unsafe, shall not apply.
(18) References: 1. Malle P., Valle M., Bouquelet S. Assay of biogenic amines involved in fish decomposition. J. AOAC Internat. 1996, 79, 43-49.
2. Duflos G., Dervin C., Malle P., Bouquelet S. Relevance of matrix effect in determination of biogenic amines in plaice (*Pleuronectes platessa*) and whiting (*Merlangus merlangus*). J. AOAC Internat. 1999, 82, 1097-1101.

Food category	Micro-organisms	Sampling plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.1.1. Carcases of cattle, sheep, goats and horses (4)	Aerobic colony count			3,5 log cfu/cm² daily mean log	5,0 log cfu/cm² daily mean log	ISO 4833	Carcases after dressing but before chilling	Improvements in slaughter hygiene and review of process controls
	Enterobacteriaceae			1,5 log cfu/cm² daily mean log	2,5 log cfu/cm² daily mean log	ISO 21528-2	Carcases after dressing but before chilling	Improvements in slaughter hygiene and review of process controls
2.1.2. Carcases of pigs (4)	Aerobic colony count			4,0 log cfu/cm² daily mean log	5,0 log cfu/cm² daily mean log	ISO 4833	Carcases after dressing but before chilling	Improvements in slaughter hygiene and review of process controls
	Enterobacteriaceae			2,0 log cfu/cm² daily mean log	3,0 log cfu/cm² daily mean log	ISO 21528-2	Carcases after dressing but before chilling	Improvements in slaughter hygiene and review of process controls
2.1.3. Carcases of cattle, sheep, goats and horses	*Salmonella*	50 (5)	2 (6)	Absence in the area tested per carcase		EN/ISO 6579	Carcases after dressing but before chilling	Improvements in slaughter hygiene, review of process controls and of origin of animals
2.1.4. Carcases of pig	*Salmonella*	50 (5)	5 (6)	Absence in the area tested per carcase		EN/ISO 6579	Carcases after dressing but before chilling	Improvements in slaughter hygiene and review of process controls, origin of animals and of the biosecurity measures in the farms of origin
2.1.5. Poultry carcases of broilers and turkeys	*Salmonella*	50 (5)	7 (6)	Absence in 25 g of a pooled sample of neck skin		EN/ISO 6579	Carcases after chilling	Improvements in slaughter hygiene and review of process controls, origin of animals and biosecurity measures in the farms of origin

Food category	Micro-organisms	Sampling plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.1.6. Minced meat	Aerobic colony count (7)	5	2	$5x10^5$ cfu/g	$5x10^6$ cfu/g	ISO 4833	End of the manufacturing process	Improvements in production hygiene and improvements in selection and/or origin of raw materials
	E.coli (8)	5	2	50 cfu/g	500 cfu/g	ISO 16649-1 or 2	End of the manufacturing process	Improvements in production hygiene and improvements in selection and/or origin of raw materials
2.1.7. Mechanically separated meat (MSM) (9)	Aerobic colony count	5	2	$5x10^5$ cfu/g	$5x10^6$ cfu/g	ISO 4833	End of the manufacturing process	Improvements in production hygiene and improvements in selection and/or origin of raw materials
	E.coli (8)	5	2	50 cfu/g	500 cfu/g	ISO 16649-1 or 2	End of the manufacturing process	Improvements in production hygiene and improvements in selection and/or origin of raw materials
2.1.8. Meat preparations	*E.coli* (8)	5	2	500 cfu/g or cm²	5 000 cfu/g or cm²	ISO 16649-1 or 2	End of the manufacturing process	Improvements in production hygiene and improvements in selection and/or origin of raw materials

(1) n = number of units comprising the sample; c = number of sample units giving values between m and M.
(2) For points 2.1.3 — 2.1.5 m=M.
(3) The most recent edition of the standard shall be used.
(4) The limits (m and M) apply only to samples taken by the destructive method. The daily mean log is calculated by first taking a log value of each individual test result and then calculating the mean of these log values.
(5) The 50 samples are derived from 10 consecutive sampling sessions in accordance with the sampling rules and frequencies laid down in this Regulation.
(6) The number of samples where the presence of salmonella is detected. The c value is subject to review in order to take into account the progress made in reducing the salmonella prevalence. Member States or regions having low salmonella prevalence may use lower c values even before the review.
(7) This criterion does not apply to minced meat produced at retail level when the shelf-life of the product is less then 24 hours.
(8) *E. coli* is used here as an indicator of faecal contamination.
(9) These criteria apply to mechanically separated meat (MSM) produced with the techniques referred to in Chapter III, paragraph 3, in section V of Annex III of Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin.

Food category	Micro-organisms	Sampling plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.2.1. Pasteurised milk and other pasteurised liquid dairy products (4)	Enterobacteriaceae	5	2	<1 cfu/ml	5 cfu/ml	ISO 21528-1	End of the manufacturing process	Check on the efficiency of heat- treatment and prevention of recontamination as well as the quality of raw materials
2.2.2. Cheeses made from milk or whey that has undergone heat treatment	*E.coli* (5)	5	2	100 cfu/g	1 000 cfu/g	ISO 16649- 1 or 2	At the time during the manufacturing process when the *E. coli* count is expected to be highest (6)	Improvements in production hygiene and selection of raw materials
2.2.3. Cheeses made from raw milk	Coagulase-positive staphylococci	5	2	10^4 cfu/g	10^5 cfu/g	EN/ISO 6888-2	At the time during the manufacturing process when the number of staphylococci is expected to be highest	Improvements in production hygiene and selection of raw materials. If values >10^5 cfu/g are detected, the cheese batch has to be tested for staphylococcal enterotoxins.
2.2.4. Cheeses made from milk that has undergone a lower heat treatment than pasteurisation (7) and ripened cheeses made from milk or whey that has undergone pasteurisation or a stronger heat treatment (7)	Coagulase-positive staphylococci	5	2	100 cfu/g	1 000 cfu/g	EN/ISO 6888-1 or 2		
2.2.5. Unripened soft cheeses (fresh cheeses) made from milk or whey that has undergone pasteurisation or a stronger heat treatment (7)	Coagulase-positive staphylococci	5	2	10 cfu/g	100 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene. If values > 10^5 cfu/g are detected, the cheese batch has to be tested for staphylococcal enterotoxins.
2.2.6. Butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurisation	*E.coli* (5)	5	2	10 cfu/g	100 cfu/g	ISO 16649- 1 or 2	End of the manufacturing process	Improvements in production hygiene and selection of raw materials

Food category	Micro-organisms	Sampling plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.2.7. Milk powder and whey powder (4)	Enterobacteriaceae	5	0	10 cfu/g		ISO 21528- 1	End of the manufacturing process	Check on the efficiency of heat treatment and prevention of recontamination
	Coagulase-positive staphylococci	5	2	10 cfu/g	100 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene. If values > 10^5 cfu/g are detected, the batch has to be tested for staphylococcal enterotoxins.
2.2.8. Ice cream (8) and frozen dairy desserts	Enterobacteriaceae	5	2	10 cfu/g	100 cfu/g	ISO 21528- 2	End of the manufacturing process	Improvements in production hygiene
2.2.9. Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age	Enterobacteriaceae	10	0	Absence in 10 g		ISO 21528- 1	End of the manufacturing process	Improvements in production hygiene to minimise contamination. If Enterobacteriaceae are detected in any of the sample units, the batch has to be tested for *E. sakazakii* and *Salmonella*

(1) n = number of units comprising the sample; c = number of sample units giving values between m and M.
(2) For point 2.2.7 m=M.
(3) The most recent edition of the standard shall be used.
(4) The criterion does not apply to products intended for further processing in the food industry.
(5) *E. coli* is used here as an indicator for the level of hygiene.
(6) For cheeses which are not able to support the growth of *E. coli*, the *E. coli* count is usually the highest at the beginning of the ripening period, and for cheeses which are able to support the growth of *E. coli*, it is normally at the end of the ripening period.
(7) Excluding cheeses where the manufacturer can demonstrate, to the satisfaction of the competent authorities, that the product does not pose a risk of staphylococcal enterotoxins.
(8) Only ice creams containing milk ingredients.

Food category	Micro-organisms	Sampling plan (1)		Limits		Analytical reference method (2)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.3.1. Egg products	Enterobacteriaceae	5	2	10 cfu/g or ml	100 cfu/g or ml	ISO 21528-2	End of the manufacturing process	Checks on the efficiency of the heat treatment and prevention of recontamination

(1) n = number of units comprising the sample; c = number of sample units giving values between m and M.
(2) The most recent edition of the standard shall be used.

Food category	Micro-organisms	Sampling plan (1)		Limits		Analytical reference method (2)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.4.1. Shelled and shucked products of cooked crustaceans and molluscan shellfish	*E.coli*	5	2	1 cfu/g	10 cfu/g	ISO TS 16649-3	End of the manufacturing process	Improvements in production hygiene
	Coagulase-positive staphylococci	5	2	100 cfu/g	1 000 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene

(1) n = number of units comprising the sample; c = number of sample units giving values between m and M.
(2) The most recent edition of the standard shall be used.

Food category	Micro-organisms	Sampling plan (1)		Limits		Analytical reference method (2)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.5.1. Pre-cut fruit and vegetables (ready-to-eat)	*E.coli*	5	2	100 cfu/g	1 000 cfu/g	ISO 16649- 1 or 2	Manufacturing process	Improvements in production hygiene, selection of raw materials
2.5.2. Unpasteurised fruit and vegetable juices (ready-to-eat)	*E.coli*	5	2	100 cfu/g	1 000 cfu/g	ISO 16649- 1 or 2	Manufacturing process	Improvements in production hygiene, selection of raw materials

(1) n = number of units comprising the sample; c = number of sample units giving values between m and M.
(2) The most recent edition of the standard shall be used.

Table 1: Microbiological limits defined in Regulation No 2073/2005

1.3.3 The HACCP system

To ensure food safety, a method of self-control was introduced years ago, which analyzes the entire food chain identifying the risks associated with each step and possible solutions. This method is called HACCP: Hazard Analysis and Critical Control Point.

HACCP was introduced in Europe by Directive 93/43 and in Italy with Legislative Decree 155 of May 26, 1997, that required the application of HACCP for all the food chain operators at any level of the food chain. This decree was repealed and replaced by CE n.852, 853 and 854 of 2004 (the aforementioned "hygiene package" from 1 January 2006), that define the general hygienic rules of hygiene for all food products and for industries and the policy of official controls. Furthermore EC Regulation n.2073 of 2005, defines the microbiological criteria for foodstuffs and the various standards for sampling. These standards provide control along the entire chain of production and distribution of food, in order to ensure food safety and nutritional quality, in order to protect consumers and the market. Food chain operators must obviously also take into account the "Handbook of Self-control and good hygiene practices for food collective ".

The Manual's aim is to define and make accessible to operators and official organs of control, the procedures underlying the identification and control of critical points throughout the production line, and also the roles and responsibilities of personnel involved in the self-control system. It explains step by step all the operations required to preserve hygiene of premises, equipment, staff and in food preparation, as well as the way to manage unsuitable products or waste, and explains how to organize all the documentation. In addition this manual establishes the frequency of quality checks of the self-control system that help document the effectiveness of prescriptions, and a program of information and training, addressed to the staff, to raise their awareness on the issues related to the hygienic quality of food (Mortimore, 2001; Untermann, 1999). Thus, the term HACCP identifies a system designed for food safety and it is based especially on prevention, allowing operators to intervene before hygienic safety is threatened. This method is defined pro-active and ensures the production of healthy food ready for human consumption (Cullor, 1997).

In essence, companies that adopt such a system must accomplish two important actions: identify within their food production process (industrial, commercial, catering, etc...), the specific hazards that may impair in any way food wholesomeness during all its "stages" (that is until its consumption) and develop more appropriate measures of prevention and control to ensure food safety. HACCP has been built around seven basic principles:

1) analysis of potential hazards and prevention measures;

2) determination of critical control points (CCP);
3) determination of critical limits for CCPs;
4) establishment of a supervising system of the CCP;
5) determination and establishment of corrective actions;
6) establishment of testing procedures;
7) determination of a system that manages all the documentation.

It is therefore important to consider the risks and dangers that foodstuff could encounter at various stages from acceptance to distribution and consumption, and evaluate analytically at each stage of the process, the critical points of Control (CCP), the critical limits and, in case of noncompliance, the corrective actions to adopt. The importance of monitoring critical control points should be therefore emphasized: this is represented by a programmed series of observations or measurements to verify that a particular CCP is in control. The monitoring system will ascertain that, at defined times, the chosen parameter respects the critical limits established. The monitoring control system should provide information in a reasonable time so that, in case a critical point is no longer under control, corrective measures can be undertaken.

In addition, controls must be planned in order to be frequent enough to ensure, in any time, the precise control of the selected parameter. Planning an adequate monitoring system allows therefore to identify whenever a parameter is far from the critical acceptability limits and to intervene rapidly, identifying products obtained in the period where critical limits were exceeded, and destining them to recovery or destruction after submitting them to appropriate controls (Buchanan, 1995).

1.4 Regulation EN ISO/IEC 17025:2005

1.4.1 Generality and main principles of the regulation

The UNI EN ISO/IEC 17025 is a standard that includes technical and managerial requirements and it is widely utilized to achieve the accreditation of testing and calibration by all laboratories. The term “accreditation” is used instead of the well-known “certification” as by the National System for Quality, testing and calibration laboratories are classified as “ specialized operators in evaluation of conformity”, qualified to perform tests and calibrations granting accuracy and adequacy that are performed using “calibrated” instruments.

Unlike ISO 9001, that a company achieves implementing a quality control system to provide goods or services that meet consumers requirements, accreditation is achieved for a test or

calibration that is able to pass an inspection test performed by the competent authority, that attests compliance to the law. For this reason a laboratory that has a Sinal or a Sit accreditation has not acquired an automatic recognition for all its tests and calibrations, but only for those that after a specific request have passed successfully the inspection.

1.4.2 Technical requirements general description

The requirements that a laboratory must attend are technical and administrative. The first are very similar to those indicated in UNI EN ISO 9001, but do not concern the obligation to create a section dedicated to the design and develop of the new method and the related processes. The general technical requirements are the following:

1) Personnel of the laboratory :certification of skills and appropriate qualifications, training, regular contract;
2) Laboratory environment: suitability of laboratory equipment and conditions, with attention to biological sterility, dust, electromagnetic interference, radiations, humidity, electrical supply, temperature, vibrations and noise;
3) Reference methods: using the most up to date, which are scientifically and internationally recognized;
4) Validation methods: exhaustive documentation , including references and data for each test performed.

1.4.3 Test methods validation

Section 5.4.5.1 of UNI CEI EN ISO/IEC 17025 defines validation as "the confirmation by examination and adduction of objective evidence, that the specific requirements for the intended use are fulfilled ", practically it is the demonstration that the efficiency of the testing method is comparable with that required by the reference method for the same type of test. Greater is the degree of comparison between the two methods, greater is the effectiveness of the procedure.
A testing method must be validated in the following cases:

- the method is designed and / or developed by the laboratory;
- the method is standardized, but it is used outside of the purpose and / or field of application originally intended;
- the method is standardized, but there have been substantial changes and extensions;

When the method presents broad-spectrum applications, the validation can be performed in collaboration with other laboratories; instead, when the method cannot be spread or is not widely used it can be performed by one laboratory. In this case, the achievement of a validation can be performed using various techniques that include the use of certificated material, the comparison with results obtained with other methods and so on. The are several parameters that must be evaluated during the validation of a method ; assistance for the identification of the appropriate is provided by general outlines, as those provided by the USPC (United States Pharmacopeial Convention) and the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use), summarily indicated in Table 2.

(USPC) Method	**Category I** (main compounds)	**Category II** (purity tests)		**Category III** (performance tests)	**(ICH) Method**	**Identification tests**	**Purity tests**		**Titles**
Performance parameters		**Quantitative**	**Limit**		**Performance parameters**		**Quantitative**	**Limit**	**Main compounds**
Accuracy	+	+	**(*)**	**(*)**	**Accuracy**	-	+	-	+
Precision	+	+	-	+	**Precision**	-	+	-	+
Specificity	+	+	+	**(*)**	**Specificity**	+	+	+	+
Detection Limit	-	-	+	**(*)**	**Detection Limit**	-	+	+	-
Quantification limit	-	+	-	**(*)**	**Quantification limit**	-	+	-	-
Linearity	+	+	-	**(*)**	**Linearity**	-	+	-	+
Application field	+	+	**(*)**	**(*)**	**Application field**	-	+	-	+
Robustness	+	+	+	+					

Table 2: General criteria of validation provided by USPC e ICH, not specifically intended for food microbiological analysis

According to the criteria established by USPC analytical methods are grouped into three categories: the first one includes all the test methods that are used to quantify a specific component of a material or of a compound; the second groups the methods used for the

determination of secondary components, such as degradation compounds or impurities; the third is representative of all testing methods that analyse specific features of materials or products (also known as "behaviour tests"). The difference between ICH and USPC criteria is that behaviour tests are eliminated and replaced with qualitative test, that aim to detect the presence of a given compound in a material or a product trough specific recognition reactions.

The validation of a method requires a careful planning. This must define the resources that have to be employed, the requirements that have to be accomplished and (if already existent) the criteria used to choose the method itself. The selected method should then be described as clearly as possible, collecting and quoting all the existing information about it; after this it is important to identify the performance parameters to control and, for each one of them, the criteria used to determine the critical limits according to the results obtained for each parameter.

The validation of a method is confirmed by the comparison of all the results obtained for each parameter with the correspondent critical limit. A final evaluation including the reference method considered must then be drawn. These outlines are to be considered indicative, however, as the guidelines of ISO 17025; the choice of the performance parameters depends strictly from the type of test and the class to which it belongs. The parameters needed for the validation of a method for which there is already a specific legislation are in fact different from those needed for a "not regulated" method, as one that has been fully developed by one laboratory. In areas where the advent of alternative methods is frequent specific rules are generally available: these are not limited to a general approach but provide a precise technical protocol to follow during the validation test. Even in the field of food microbiological quality, there is a specifically dedicated legislation: UNI EN ISO 16140 in 2003. This establishes, in a much more specific way compared to the guidelines provided by ISO 17025, all the requirements and procedures of validation for new analytical methods in food microbiology.

1.5 Regulation EN ISO 16140:2003

1.5.1 Main principles of the regulation and validation procedures

The UNI EN ISO 16140 establishes the basic criteria and the technical protocol needed for the validation of alternative methods for microbiological analysis of food, feed and environmental samples. An alternative method is intended as a protocol of analysis that estimates the presence/absence or the quantity of a given analyte in certain products, as well as the related internationally recognized and accepted reference method. The validation consists in the

demonstration that there is an acceptable comparability between the results obtained with both methods ensured by a statistical analysis of data obtained on a given number of samples. The analyte, or better the component detected by the analytical method, is almost always represented by a microorganism or by its metabolites or toxins. Results depend on the analysis and can be of two different types:

1) Qualitative: the results obtained are expressed as presence or absence of the analyte under consideration; these analysis concern important pathogens that have to be necessarily absent, as *Listeria monocytogenes* or *Salmonella spp.*

2) Quantitative: the results obtained indicate the amount of analyte directly (as the enumeration of colonies) or indirectly (as absorbance) measurable, expressed in relation to the amount of sample in weight or volume; these analysis concern parameters as Total Viable Count or the detection of coliforms.

For these tests the food safety policy doesn't require the total absence of the analytes but indicates critical limits of acceptability that can range in relation to the product.

Validation requires two basic steps: the first one is "internal", performed within the laboratory that planned the research, the second one is "external", so called inter-laboratory where the results obtained by different laboratories for the same samples are compared.

Both steps, can easily be performed at the same time because the alternative method is used along with the reference one; in this way the two methods can be compared, as well as the results obtained by different laboratories, in order to establish the efficacy of the analytical system that has to be validated and to obtain an evaluation from technicians and operators apart from those who participated to develop the method.

1.6 Micro Biological Survey Method

The MBS method, used and validated in this thesis, is a rapid colorimetric assay that allows to selectively detect and quantify various microorganisms.

1.6.1 Principles and biochemistry of the method

The analytical base is the measurement of the catalytic activity of enzymes of primary metabolism of bacteria that allows the establishment of a direct correlation between enzymatic activity detected and microbial load in the sample. Detection of enzymatic activity is made possible thanks to the presence of a redox indicator called TMPD (N, N, N ', N'tetrametil-p-

phenylenediamine hydrochloride), capable to change colour as a consequence of the modification of redox potential of the culture medium. TMPD is yellow in its reduced form, and it becomes blue in its oxidized form; its potential, of about 250 mV (Prince *et al*., 1981), is very close to the one of cytochromes belonging to the mitochondrial electron transport chain, which goes from 100 to 300 mV (Moran *et al*, 1996). For this reason, this indicator can be easily reduced by cytochromes and at the same time can be directly oxidized by the oxygen present, which has a potential of about 820 mV (Moran *et al*, 1996). A distinctive characteristic of TMPD is to be an anphipathic molecule, capable to cross the cytoplasmatic and mitochondrial membranes and rapidly reduce cytochrome c (Sarti *et al.*, 1992). The reaction vials include in addition to selective medium for the detection of microorganisms, an appropriate amount of vaseline (1.5 ml) to prevent the passage of atmospheric oxygen in the solution, acting as a "cap". Initially, bacterial growth in is in lag phase and, consequently, the oxygen present in the vial oxidizes the indicator that becomes blue. When bacterial growth passes to the log phase, being in the presence of oxygen, all electrons are transported toward the IV complex of the respiratory chain favouring reduction of oxygen and, consequently, leaving TMPD in its oxidized blue form (Figure 3A). Once bacteria have used all the oxygen present in the vial, respiratory chain will arrive at a point of saturation and electrons will be transferred on TMPD, that will be reduced turning yellow (Figure 3B).

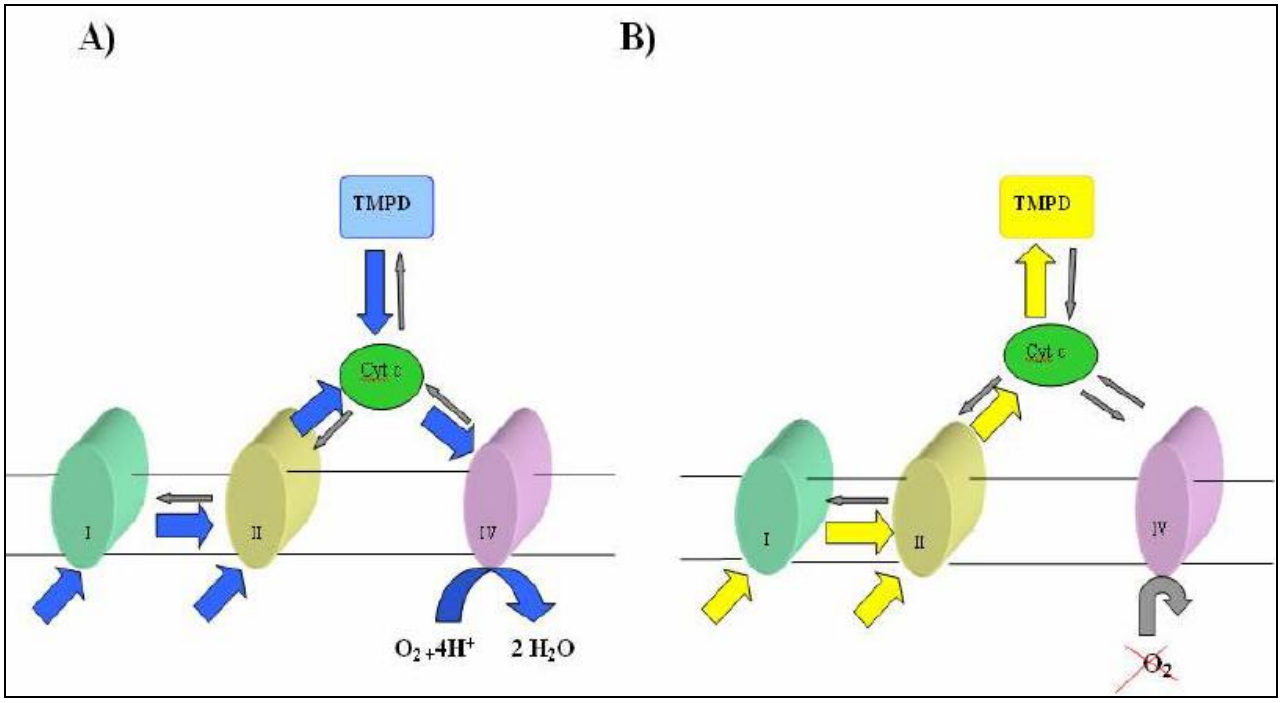


Figure 3: A) Oxidation of TMPD in presence of oxygen; B) Reduction of TMPD in absence of oxygen

The reaction in the medium used for Total Viable Count (Figure 4) goes exactly as described and, given the type of examination, there is no need of selective agents.

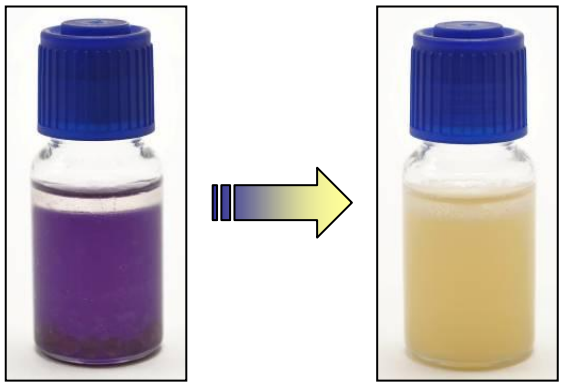


Figure 4: Change of colour of the MBS vials for TVC: the colour change is due to reduction of TMPD in its yellow reduced form by oxidoreductase enzymes of bacterial primary metabolism

In *E.coli* vials, instead, colour changes from red to yellow, thanks to the presence of an indicator called Phenol Red that detects oxygen-independent metabolic processes, as lactose fermentation; in this case there is an acidification of the medium that can be observed colorimetrically by the colour change of Phenol from red to yellow (Figure 5).

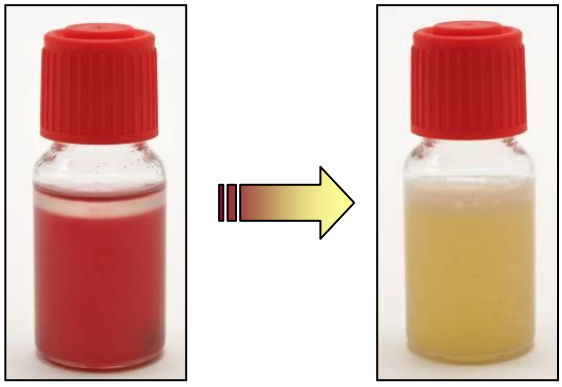


Figure 5: Change of colour of the MBS vials for *E.coli*: the colour change is due to reduction of TMPD in its yellow reduced form by oxidoreductase enzymes of bacterial primary metabolism

In addition to Phenol Red and TMPD, the reagent for *E.coli* detection contains Piperacillin-selective agents (inhibiting the growth of several faecal coliform) and Cycloheximide (against yeasts growth), that along with the incubation at 44° C, ensure the specificity of the medium. It is therefore possible to observe the colour change of Phenol Red, once TMPD is completely reduced due to the consumption of oxygen, only in the presence of Enterobacteriaceae able to

ferment lactose at 44 ° C. At this temperature, and in the presence of these selective compounds, the growth of *E.coli* is predominant compared to that of other thermo-tolerant coliforms (Figure 6).

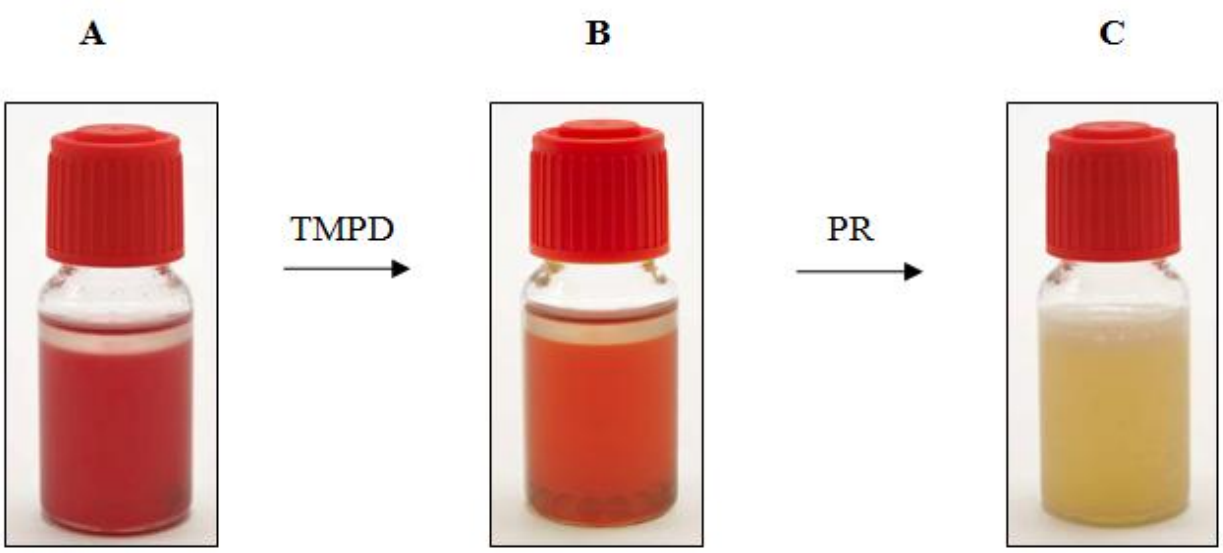


Figure 6: Change of colour of MBS vials for the detection of *E. coli*. A) Initial phase; B) Change of colour due to TMPD; C) Change of colour due to PR

To note is how the analysis of samples containing enteric bacteria other than *E. coli* does not show a colour change to yellow, in fact after 24-48 hours the reagent is red / orange, demonstrating the specificity of MBS method to *E. coli*. The orange colour is due to TMPD and it is typical of all Enterobacteriaceae, while the yellow colour is due to Phenol Red and to the ability to ferment the lactose with high efficiency (Figure 7).

Figure 7: Change of colour of MBS vials for the detection of *E. coli* with lactose negative Enterobacteria without acidification of medium

The presence of *E.coli* in the samples is confirmed through the Indole test with Kovacs reagent. Adding a few drops of this compound in the vials, after colour change is completed, a red ring appears on the surface of the medium thanks to the reaction between indole (Gas produced from tryptophan, through a specific enzyme of *E.coli* and a few other bacteria) and p-dimethylaminobenzaldehyde, of Kovacs reagent (Figure 8). Some strains of *E.coli* (~ 5%), especially among those that can be found in water, however, do not produce indole; instead, other coliforms (~ 3.4%) as certain strains of *Klebsiella* and *Citrobacter freundii oxytocae*, can be indole producers (Anderson & Baird-Parker, 1975).

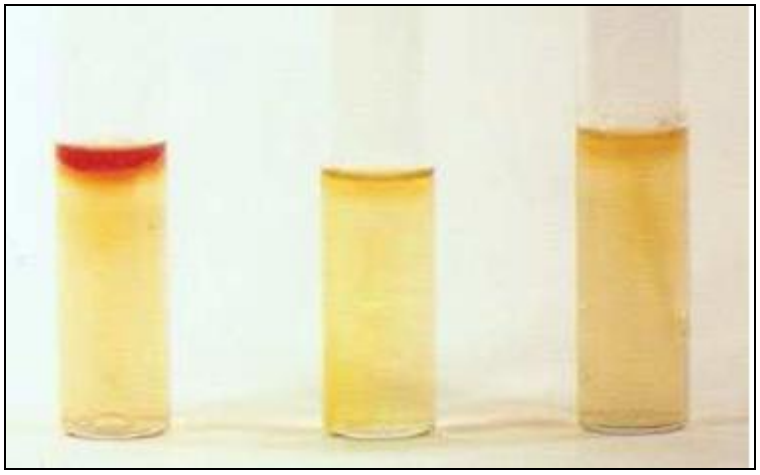


Figure 8: Samples tested with Kovacs reagent. Only the first one results positive with an evident red ring caused by the reaction of reagent compounds and indole produced by microorganisms in the vial (Image modified from MontgomeryCountyCommunity College, Healt Sciences Database)

This apparent limit of the method, however, does not undermine its efficiency, since the reference method ISO 16649 itself doesn't take account of the whole *E.coli* group. There isn't indeed an absolute correspondence between *E.coli* and β-glucuronidase (Chang *et al.*, 1989; Frampton & Restaino, 1993; Hansen & Yourassowsky, 1984; Kilian & Bulow, 1976), in particular for *E. coli* isolated in food, that result β-glucuronidase-positive only for the 85% (Maheux *et al.*, 2008).

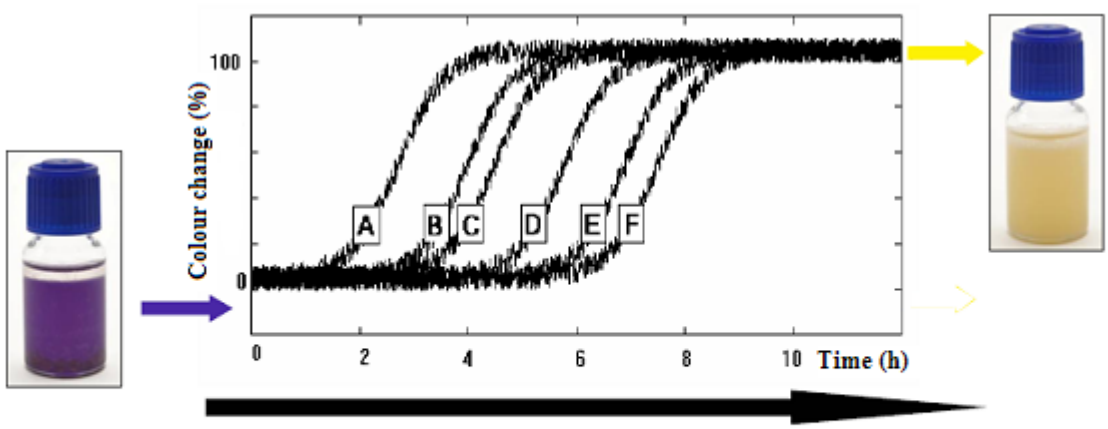
It's also known, as already mentioned in the specifics of TBX medium, that many Coliforms and Enterobacteria other than *E. coli*, such as some strains of *Salmonella* and *Shigella*, are producers of β-glucuronidase (Damare, 1985; Ushiyama & Iwasaki, 2010); furthermore some of the most pathogenic strains, such as verocitotoxin producers (eg *E. coli* O157: H7), result β-glucuronidase

negative (Scotland, 1992) but indole producers, and therefore detectable by the MBS method and not by the reference method ISO 16649.

1.6.2 Performance

The principles of MBS method can be described using for example an experiment conducted on water samples, which are analyzed in the same way of any other type of sample (after homogenization in saline solution or in buffered peptone water for solid food matrixes).
Six sterile reaction vials containing the MBS TVC reagent were inoculated with artificially contaminated water samples. After a certain time the vial changed colour going from blue to yellow (Figure 9); the correlation between bacterial concentration in the samples and the time taken to change colour is clearly linear (Figure 10).
The sensitivity of the method to different microbial genera, as described later, is obtained using specific reagents that contain inhibitory substances against microorganisms other than those researched; these selective agents are usually already used in traditional cultivation mediums. In order to obtain what described, and meet nutritional requirements of different microorganisms, MBS reagents contain different classes of compounds:

- Nutrients: mainly sugars lipids, and proteins;
- Selective agents: organic substances (eg surfactants), antibiotics (egpolymyxin) and salts not ordinarily present (egCsCl) or common but in concentrations different from the physiological one (EgNaCl);
- Reducing agents: organic substances capable to release electrons to the respiratory chain, and that are not easily oxidized by the oxygen dissolved in solution;
- Indicators: organic substances capable of changing colour in result to variations of the chemical and physical state of the medium. In particular the two indicators utilized are: TMPD (N, N, N ', N', tetramethyl benzene-1 ,4-diamine hydrochloride), a redox indicator, and Phenol Red (PR), a pH indicator; the first allows detection oxygen consumption of microorganisms while the second detects acidification of the soil, resulting from fermentation;
- Vaseline: organic substance used to avoid an external input of oxygen into the reaction vial; as highly hydrophobic compared to reagent, it sets at the interface with the air excluding the oxygen input .



Sample	A	B	C	D	E	F
CFU/ml	$>10^8$	10^8	10^7	10^6	10^5	10^4

Figure 9: Change of colour of MBS vials in relation to time. Letters A,B,C,D,E,F indicate different samples which concentrations are reported in the table

The different components of the reagents used in the Micro Biological Survey method (composition indicated in the tables below) are micronized together in a mortar and then distributed in defined amounts, in the single vial.

Before closure, 1.5 ml of vaseline is added. After preparation, the vials are sterilized with γ rays (5kGy) in a specialized company and are then ready for use.

The gamma rays are produced by the decay of radioisotopes, particularly of 137 Cs (caesium) and 60Co (Cobalt). The latter is the main source of radiation used in heating systems for industries. The 60Co is more used then the 137Cs because it does not produce long-term radioactive waste (half-life 5.3 years), and therefore, has a lower environmental impact. It is obtained by neutron bombardment of 59Co, a non-radioactive isotope. Thanks to the high penetrating power, gamma rays can be used to treat voluminous material without leaving a trace of radioactivity on the irradiated material.

The technological properties of gamma-ray are used in different fields: for the sterilization of material and equipment primarily for medical, pharmaceutical and cosmetic use, and for everyday objects. Gamma-ray are also used for sanitizing and conservation of food not adapt to heat treatment

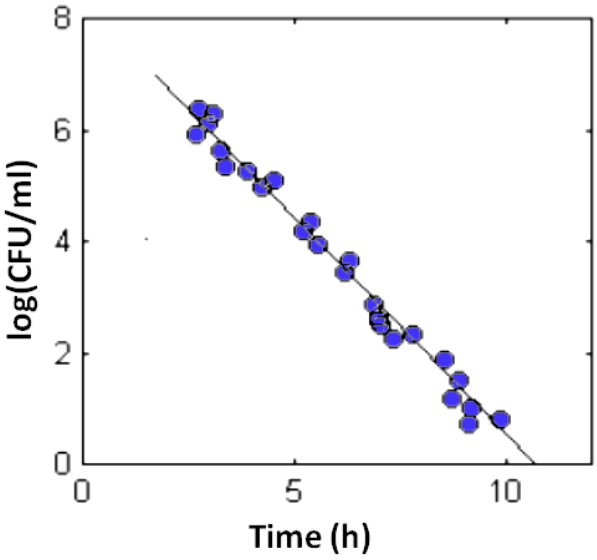


Figure 10: Correlation plot between bacterial concentrations (expressed as log CFU/ml), and time taken for vials to change colour (expressed in hours)

This is an extremely simple method that requires the use of disposable vials, previously sterilized by γ-rays, containing the selective reagent for each microorganism. The vials are made in PET (Polyethylene Terephthalate), provided with a screw cap for a secure closure and a safety cap that includes a tank containing a sterilizing chemical compound that must be manually injected in the vial, after testing, for a proper disposal (Figure 11).

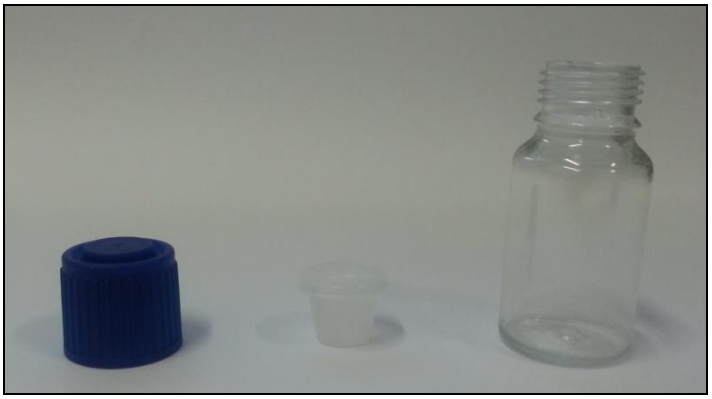


Figure 11: Disposable reaction vial

The sample can be directly introduced in the sterile reaction vial containing a specific reagent (Figure 12A): 1 ml for liquid samples (water or a homogenate of a solid or semi-solid matrix), approximately 1g for solid samples (or directly the tampon for surfaces). After adding 10ml of

sterile water (Figure 12B) the vial must then be tightly closed and shaken until all the reagent is completely dissolved (Figure 12C). The final colour required to start analysis (specific for each type of reagent) is achieved in 5-10 minutes. Vials must then be placed in a thermostatic device, which allows to maintain a given temperature of incubation depending on the type of microorganisms searched (30°, 37° or 44°C). Results are obtained monitoring colour variation of the vials at different times, referring to the colour scale provided with the test kit (Figure 12D). After the analysis the safety cap is pushed down; this opens the tank that releases the sterilizing compound into the vials (Figure 12E); in a few minutes all microorganism are eliminated and vials can be disposed as non-pathogenic waste.

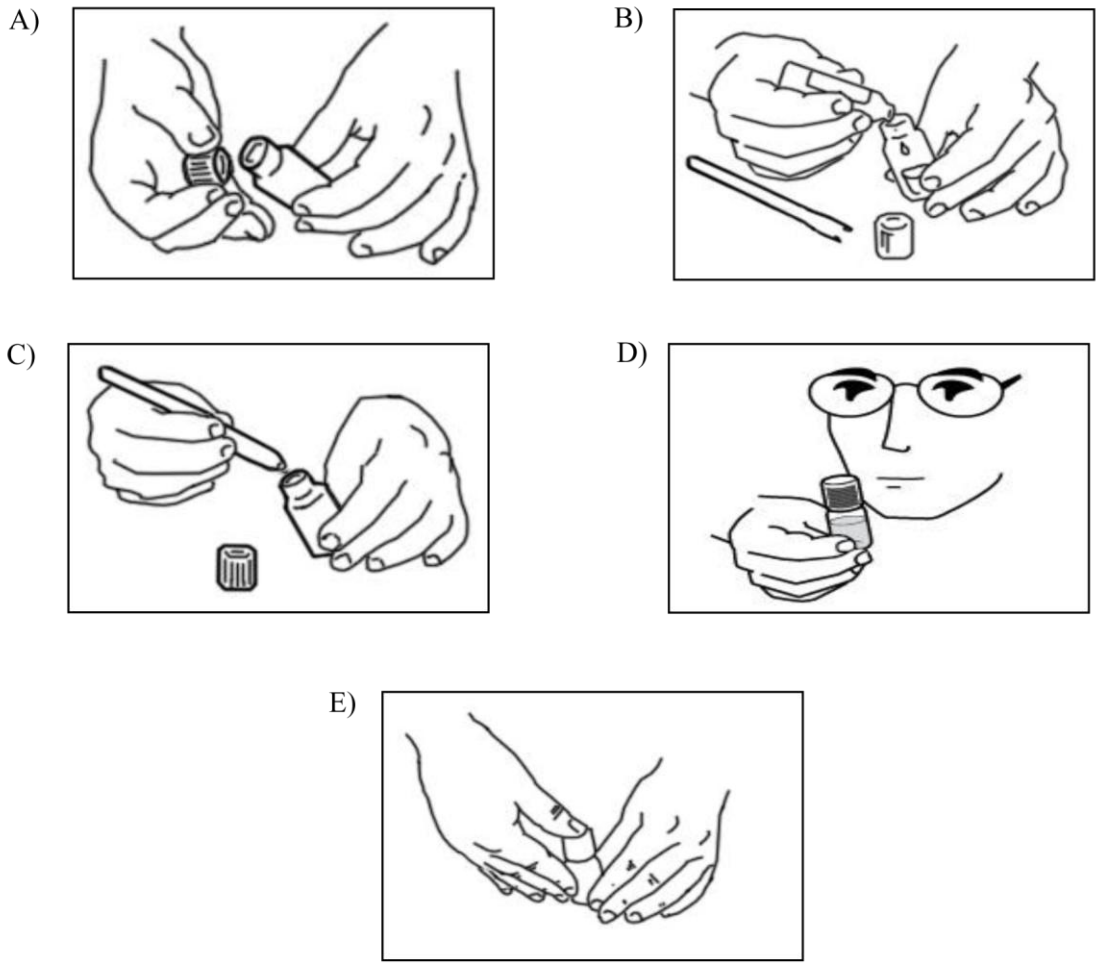


Figure 12: Directions for use of the disposable reaction vial in six steps: introduction of 1 ml or 1 g of sample (A), addition of 10 ml of sterile water (B), closure and shaking of the vial (C), incubation at the specific temperature of analysis and control of re results (D) sterilization and disposal of the vial (E)

To complete the analysis it is necessary to relate the time taken by the vial to change colour to the bacterial concentration of the sample. This can be done using the reference table provided with the kit.

Each MBS reagents shows a slightly different time of reaction (meaning the time taken to change colour in relation to bacterial concentration of the sample), therefore each reagents has its own specific calibration curve, but the time lags indicated in the table are still useful for a realistic valuation of bacterial contamination.

Table 3 was designed according to the curve showed below, whose equation is[Log cfu = -0.5 (t) + 9]: bacterial concentrations are expressed as logarithmic values, in cfu/ml, on the ordinate; the time taken by MBS reagent to change colour is expressed in hours on the abscissa .

TIME OF COLOR CHANGE [hours.minutes]	< 3.30	3.30	6.30	9.30	12.30	16.00	19.00	22.00	> 24.00
CONTAMINATION [CFU/g or ml]	$> 10^6$	10^6	10^5	10^4	10^3	10^2	10	1	0

Table 3: Table of correlation between bacterial concentration and time taken for the vials to change colour

The curve has been developed using experimental points obtained from the analysis of different food products (meat, vegetables, cottage cheese), with the MBS method in accordance to EN/ ISO 16140:2003.These points all fall within the confidence interval determined by previous analysis that used as samples artificially contaminated food (Figure 13).

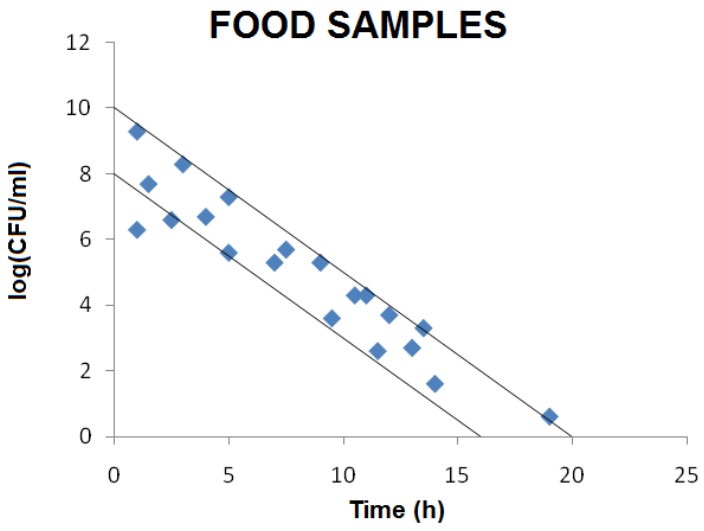


Figure 13: Correlation plot between bacterial concentrations (expressed as log CFU/ml), and time taken for vials to change colour developed on experiments performed on contaminated food samples. Dots represent the experimental values obtained from the analysis of different food products. Lines define a confidence interval of 90%

CHAPTER II

Aim of the work

Food safety and quality are issues of vital importance nowadays. The increased potential threat for human health has led to continuous research for innovative processes and systems for food control, in order to obtain a significant improvement of food quality and, at the same time, a considerable reduction of biological contamination.

For this reason, in recent years it's become necessary to develop faster and more reliable analysis systems that allow one to obtain immediate results making possible a more rigorous prevention of microbiological hazards.

Leg. 31/01 and Regulation EC 2073/2005 specify that in addition to reference methods for food microbiological analysis, alternative analytical methods can be used, after validation. To validate an alternative method means to compare it with the reference one, in order to define specificity, sensitivity and reproducibility of the two methods in comparison.

The innovative MBS method meets these requirements: the MBS method has the prerequisite of being a fast method that can detect bacterial contamination and provide results within the same day.

This thesis is an industrial project in which the sperimentation essentially aims to the creation of microbiological analysis kits and their commercialization. It is therefore important to underline the impact of the study and formulation of reagents, with particular attention to the issues related to their stability for it is a parameter that surely influences the possibility to successfully market this method.

The work in this thesis concerns all the studies made on the stability of MBS reagents, used for food microbiological analysis. From the data provided by these studies it will be possible to obtain information on the best storage conditions of reagents, on their shelf-life, and as an ultimate goal, on how to increase and improve stability. The various reagents were subjected to physio-chemical and microbiological aging tests and in some cases accelerated tests were also conducted .

Tests were carried out on several food samples using a Multi reader, which is a modular optical device made by the Department of Physics of the University of Tor Vergata. The Multi reader is the only device used for the analysis: vials can be incubated, the time taken for them to change colour is automatically detected and the amount of microorganisms present in the sample is calculated. This thesis describes the work concerning the set-up of biological parameters of the Multi reader.

The last part of the work the validation of the Micro Biological Survey (MBS) methodfor food microbiological analysis has been carried out. The MBS method for Total Viable Count (TVC) and *Escherichia coli* (*E.coli*) was validated following the requirements defined in ISO 16140:2003.

This alternative method was validated in comparison to the reference method. The general estimates of precision, reliability, uncertainty, linearity, accuracy and selectivity were determined. For detection and count of TVC and *E. coli* both in artificially contaminated and in naturally contaminated samples, all performance parameters have demonstrated total correlation with the reference method, demonstrating that the MBS method can be used as a rapid and user friendly screening method for detection of TVC and *E. coli* in the food industry.

CHAPTER III

MBS reagents stability

3.1 Introduction

3.1.1 ICH guidelines and shelf life of medicines

The "International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use", or ICH, had place in 1990 in Brussels. It is a project conceived for all authorities involved in drugs regulation and for pharmaceutical industries of Europe, Japan and USA, for the evaluation of scientific and technical aspects related to the registration of new products. ICH guidelines provide the experimental protocols that must be followed to carry out studies on the stability of a "new molecular entity"(Dwan *et al.*, 2008). These guidelines dictate that stability studies have to be performed on the pharmacologically active compound and on the finished product. Thanks to the data collected during such studies, it is possible to determine not only the period of validity, but also the best storage conditions so to allow registration and application (ICH Harmonised Tripartite Guideline, 2003).
ICH guidelines, therefore, deal in particular with pharmaceutical products, but they can also be taken as a reference to perform stability studies on any chemical or biological compound.
Stability studies are critical to assess the quality of chemical, biological or pharmaceutical product. The stability of a product can be defined as the ability to maintain its characteristics of identity, quality, effectiveness and purity; the period of time in which a product remains stable in certain conditions of storage is indicated as shelf life (validity/effectiveness period).

3.1.2 Stability studies

Stability studies evaluate how the quality of a specific product is influenced by time and environmental factors such as temperature, humidity and light. Stability studies can be performed in two ways: real-time testing, with a long term evaluation plan and accelerated testing, with a short term evaluation plan (ICH Harmonised Tripartite Guideline, 2003, Gaudiano *et al*, 2006).

In real-time stability tests the product is stored in the packaging conditions recommended for commercialization, and monitored until it loses its stability (meaning throughout its shelf life): this is called natural aging.
In accelerated stability test, instead, the product is subjected to high stress conditions in order to accelerate the processes of chemical and physical degradation, or its aging process. Accelerated studies are important because they predict the results of long-term stability tests, which can take several years to be completed. This means that the validity/effectiveness period (shelf life) of a compound, can be predicted from the degradation rate under stress condition knowing the relationships between the acceleration factor and the degradation rate (ICH Topic Q5C, 1996). The real-time stability test is still preferable because accelerated studies do not always predict all of the physical or chemical modifications that can eventually occur.

3.1.3 Accelerated aging

Accelerated aging can be achieved varying the conditions of storage of a chemical, pharmaceutical or biological product. For example, we can modify factors as temperature, humidity and pH, in order to make the product less stable and accelerate its degradation.
Most of the times accelerated tests are performed at high temperatures; the degradation rate in fact increases with increasing of temperature. Chemical reactions in fact increase their velocity depending on temperature because higher temperatures increase the number of effective collisions between the molecules, which is the number of collisions that enable molecules to chemically react. Only molecules that overcome a certain threshold energy, called activation energy "Ea", take part to effective collisions (Manotti Lanfredi & Tiripicchio, 2001).
The relationship between degradation rate and temperature is described by the Arrhenius equation:

$$k = A \cdot e^{-\frac{E_a}{RT}}$$

Where:

- **k** → velocity constant of the reaction (or specific velocity)
- **A** → proportionality constant, called frequency factor, which represents the total frequency of collision

- **E_a** → activation energy, the energy threshold that molecules must overcome to make effective collisions
- **R** → gas constant (8,31 $J \cdot mol^{-1} \cdot K^{-1}$)
- **T** → temperature in Kelvin degrees (K)

Furthermore, the fraction of molecules that possess higher energy than Ea is represented by the Boltzmann factor, described by the following expression:

$$e^{-\frac{E_a}{RT}}$$

According to the Arrhenius equation the degradation rate is proportional to the Boltzmann factor, via the constant of proportionality A, and depends on both temperature and the activation energy: the degradation rate in fact increases with increasing of temperature, and decreases with the increase of activation energy. Thus temperature is the accelerator factor most commonly used for chemical, pharmaceutical and biological products, because its relation to the degradation rate is characterized by the Arrhenius equation. Temperatures used in accelerated aging test should stimulate degradation and speed up tests, without destroying the basic features of the product (Magari, 2003). It would not make sense to test products with very high temperatures for a short period of time, because the degradation mechanisms at high temperatures can be very different from those that occur at the storage temperature recommended. Summarizing temperature variations affect critically the stability of a product and increasing temperature increases the reaction velocity. In general reaction velocity increases from two to four times for each increase of temperature of 10°C. This means that it doubles or quadruples the time in which a substance decomposes and loses its properties. Also very low temperatures can affect the stability of a product for example freezing the aqueous phases of emulsions; substances incorrectly stored in the refrigerator, can absorb humidity or increase their viscosity in case of substances with a pasty consistency. ICH guidelines explain how to perform real-time and accelerated tests.

They dictate that stability studies must be performed both on the active principle and on the final product, under the following conditions of temperature and relative humidity (RH):

- Long-term studies: 25°C ± 2°C/60% RH ± 5% RH
- Accelerated studies: 40°C ± 2°C/75% RH ± 5% RH

In addition we must consider special cases, as active principles or products that must be stored in the refrigerator or in the freezer.

For products that must be stored in the refrigerator long-term stability studies are performed at 5°C ± 3°C and the accelerated ones at 25°C ± 2°C/60% RH ± 5% RH for a minimum of six months. For products that must be stored in the freezer the long-term studies are carried out at -20°C ± 5°C, but a batch must be tested at 5°C ± 3°C or 25°C ± 2°C, for an appropriate period of time, in order to evaluate the effect of short thermal excursions.

3.1.4 The importance of chemical composition and storage conditions

The instability of a compound may alter its characteristics and impair its activity (Magari, 2003). It is therefore very important to ensure that a given product, in the dose and form used, is certainly functional; this means that it is important to know how the compound is influenced by several factors. The factors that influence stability are pH, light, packaging, humidity, and, as previously described, temperature. pH is a key determinant of stability. Each substance has an optimum pH at which its stability is maximum: when the maximum stability is reached at pH = 7 this means that the effect of H + and OH- ions is equivalent. Instead, when the maximum stability is reached in acid conditions, pH <7, as for most of the compounds subject to hydrolytic degradation, this means that OH-ions are more harmful and vice versa. It is therefore essential to maintain, choosing carefully the buffer solutions, an optimal range of pH, in order to ensure a greater stability and, at the same time, the best features of the compound (Some *et al.*, 2001).
Essential is also the type of packaging used for storage, which must ensure protection from external agents such as humidity, gas, solvents and light (ICH Harmonised Tripartite Guideline, 2003). The interaction of a compound with the environment can in fact cause oxidative degradations due to atmospheric oxygen, photolysis or polymerization triggered by light and hydrolysis by the action of air humidity. The packaging material must also be chemically and physically inert towards the product in order to avoid phenomena of container-contained migration and absorption of molecular structures. Semi-permeable or impermeable containers can be used. Impermeable containers (e.g., metal containers, cans or bottles sealed with glass) provide a permanent barrier to the passage of gases or solvents and protect from humidity. Therefore they can be used for storage in humid environments. Semi-permeable containers allow the passage of solvents, usually water, while preventing the loss of solute. The transport mechanism of the solvent occurs through absorption by a side of the container, diffusion, and desorption from the other side. The transport is driven by a partial pressure gradient. Examples of semi-permeable containers are plastic bags, bottles and low density polyethylene (LDPE) vials. Also humidity can promote deterioration of the compounds, shortening their shelf life. The

ideal is to keep them cool and dry places. Another possible cause of instability can be found in the incompatibilities that occurs between the various components, such interactions lead to unpredictable and, of course, undesirable changes in the nature of the substances. Even light can cause alterations in the structure of the compounds. Particular materials (chemical or biochemical), characterized by photosensitivity, can chemically react when exposed to light. Such compounds, for example many drugs, must be kept in dark or opaque containers (as brown bottles, opaque aluminium blisters) until their use to prevent photo-degradation. In conclusion, instability is the limiting factor for the shelf life of a product: shelf life is limited to the period within which the characteristics of the particular compound remain the same or, however, change within established limits.

3.2 Materials and methods

3.2.1 MBS reagents stability tests

The experiments in this work consist in the evaluation of the stability of the reagents used in the MBS method. For this purpose, tests were performed at different storage conditions and aging times. First of all we analysed all the reagents in vials kept at room temperature; these tests were performed after 3 and 6 months. Accelerated aging tests on the TVC (Total Viable Count) and COLI (*E.coli*) reagents were also performed keeping the vials at high temperatures and in different storage conditions.

Initially we tested two different formulations of the TVC reagent, starting from a medium previously formulated, that was subsequently modified. At first we performed accelerated aging tests at 60°C on both formulations in order to compare them and select the one that would display the best results. The vials were kept in plastic bag and tests were carried out after 1, 2 and 3 weeks. The new recipe appeared more stable, so we tested it also after 1, 2 and 3 months of accelerated aging. The new TVC reagent was also tested keeping vials at 60°C in a glass container and in a metal container and analysing them after 1 week, 2 weeks, 1 month, 2 and 3 months. For the COLI reagent, accelerated aging tests have been performed at 45°C, after that we had verified that the reagent lost its stability at 60°C.

The tests were conducted on the original COLI formulation that was then modified in a new COLI formulation. The vials were kept at 45°C in the following ways: the vials were kept in plastic bag and put directly in the oven without packaging; in addition the vials were kept in a metal envelope and put directly in the oven without packaging. The tests were performed after 1

week and 3 weeks for the original COLI vials and after 3, 5 and 8 weeks for the new COLI vials. Summarizing accelerated aging test were always performed in two different packaging conditions both for COLI and TVC:

- Without humidity protective packaging: plastic bags and put directly in the oven.
- With Humidity protective packaging: metal envelope, glass and metal containers.

In both COLI and TVC accelerated tests we always performed a control test with vials kept at room temperature. In the ovens humidity was ensured with beakers filled with water. Bags of dehydrated salts were placed in each envelope and container used as packaging. These tests demonstrated the importance of the storage condition: the stability was higher when vials were stored in humidity protective containers.

3.2.2 Methods of analysis of reagent stability

All the aging tests performed on the MBS reagents consist in physical-chemical and microbiological analysis that were subsequently reported on a data sheet (attached in Figure 14).

MBS
MICRO BIOLOGICAL SURVEY

QUALITY CONTROL SHEET

Part 1 General features

Reagent …… **(Composition)**……..
(Batch) ……**Production date** ……..
Date of sending to sterilization ……..
Date of return from sterilization……..
Date of quality control (production bath) ……..

NOTES ……

Part 2 Chemical and physical analyses

Addition of ……..
Dissolution time of the reagent (manually)……..
Colour after 5 minutes ……
Colour after 10 minutes ……
Colour after 20 minutes ……
Colour after 40 minutes ……
Expected colour after 40 minutes ……
pH after 40 minutes ……..

Optical spectra after 40 minutes
References: water, 400-750 nm, dil 1:10, cuvette 3 ml, 1 cm l.p.

Spectrophotometer ……..
(Attached spectra)

NOTES ……

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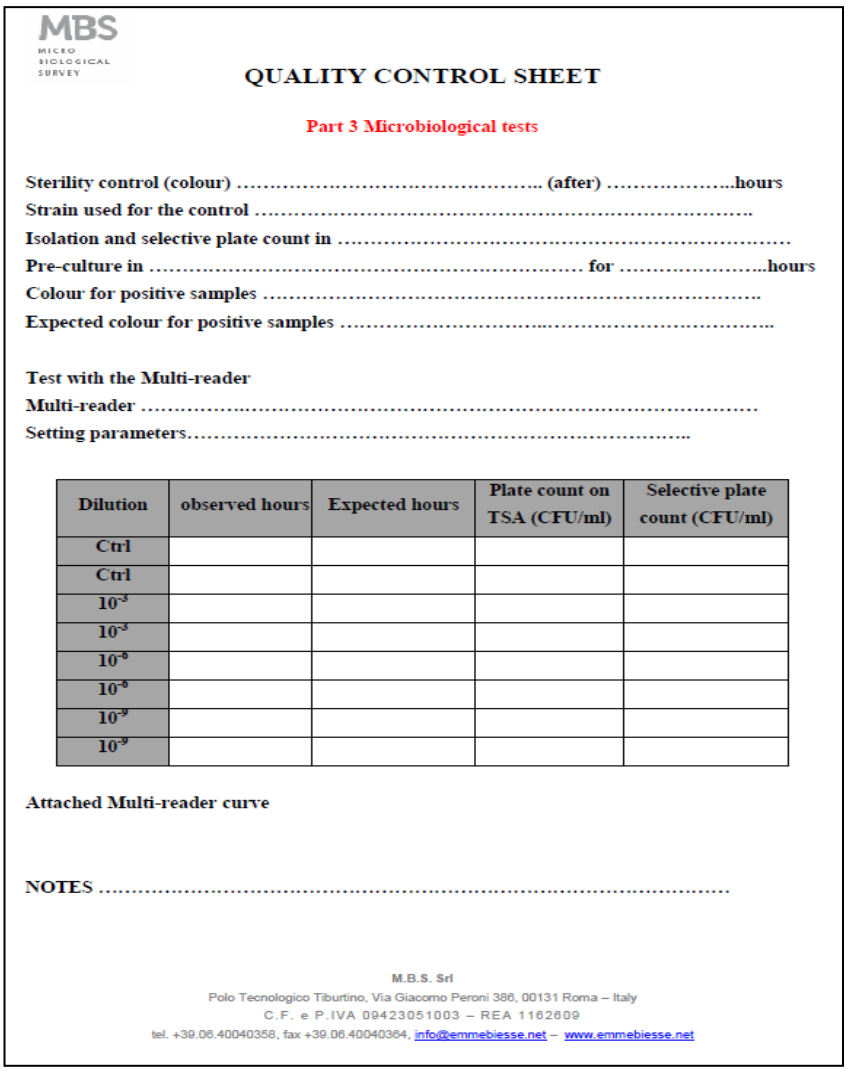

MBS
MICRO BIOLOGICAL SURVEY

QUALITY CONTROL SHEET

Part 3 Microbiological tests

Sterility control (colour) …….. (after) ……..hours
Strain used for the control ……..
Isolation and selective plate count in ……
Pre-culture in …… for ……..hours
Colour for positive samples ……..
Expected colour for positive samples ……..

Test with the Multi-reader
Multi-reader ……
Setting parameters……..

Dilution	observed hours	Expected hours	Plate count on TSA (CFU/ml)	Selective plate count (CFU/ml)
Ctrl				
Ctrl				
10^{-3}				
10^{-3}				
10^{-6}				
10^{-6}				
10^{-9}				
10^{-9}				

Attached Multi-reader curve

NOTES ……

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Figure 14: Quality control sheet

3.2.2.1 Chemical test and physical stability

Physical-chemical tests are performed to evaluate the intrinsic properties of reagents, without microorganisms. To perform these tests we filled vials containing reagents with 10 ml of sterile water, observed the time of dissolution of the reagent (by hand, without using the vortex), the colour immediately after dissolution and the colour after 5, 10, 20 and 40 minutes. The colours at 40 minutes were compared with those expected (Table 4).

Reagent	Observed colour				Expected colour
	5 min	10 min	20 min	40 min	40 min
TVC	grey	grey-blue	light blue	Blue	violet-blue
E. coli	orange	dark orange	light red	Red	Red

Table 4: Colours expected after 40 minutes for the two different reagents

Subsequently, we measured with a pH-meter the pH of each reagent, transferring 5 ml of the vials content in tubes of 15 ml. The pH values expected are all near neutrality.

Finally, we analysed reagents with a spectrophotometer: we diluted the vials content 1:10 preparing solutions made of 2.7 ml of water and 300 µl of sample for a total of 3 ml that is the appropriate volume for the spectrophotometers cuvette.

The following figures are examples of TVC and COLI spectra performed at room temperature (Figure 15 and Figure 16)

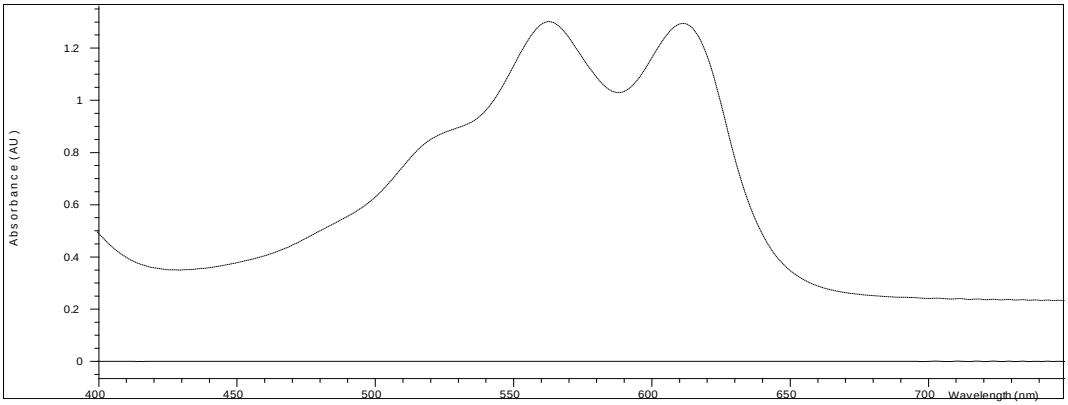


Figure 15: TVC spectra. The spectrum shows two absorption spikes due to the presence of the redox indicator TMPD

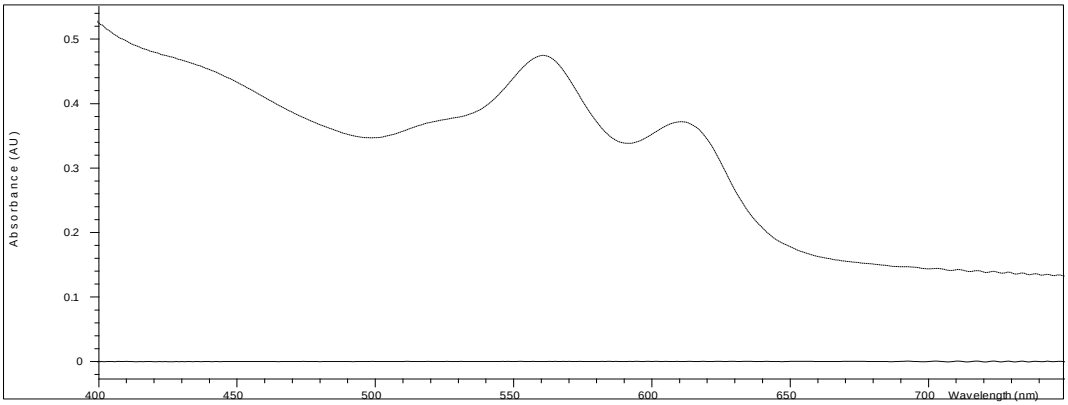


Figure 16: COLI spectra. The spectrum depends from the absorption of the redox indicator TMPD and the pH indicator Phenol red. The highest spike is the sum of the two indicators absorbance

3.2.2.2 Microbiological stability test

Microbiological tests were carried out using the MBS method together with the traditional plate count method, in order to ensure that the change of colour of vials could be exclusively attributable to microbial growth.

For these test we used culture broths obtained by a microbial strain that were cultivated at 37 ° C in the liquid medium BP (Bacteriological Peptone). For our experiments we used *E. coli*. Starting from the bacterial culture in stationary phase we prepared serial dilutions in sterile saline water starting from 10^{-1} up to 10^{-9}; these dilutions were used to contaminate vials and were plated on selective and enriched medium.

Plate count method: To perform microbiological analysis according to the traditional plate count method we used serial dilutions of a culture of *E. coli* in BP: 0.1 ml of each dilution of *E. coli* was inoculated, with the spread plating technique, on selective and enriched TSA medium; the same dilutions were used to contaminate MBS vials. The inoculated plates were incubated at 37°C in a thermostat to favour microbial growth and colonies were counted after about 24-48 hours.

MBS Method: To perform microbiological analysis according to the Micro Biological Survey we used the specific reagents for Total Viable Count (TVC) and for the determination of coliforms (COLI). The vials were filled with sterile water, shaken on a vortex, inoculated with 1 ml of each *E. coli* dilution and incubated at 37°C in a thermostat to favour the bacterial growth. For each reagent and for each packaging condition of in accelerated aging analyses, four vials were necessary: an uncontaminated vial, used as control, and three other vials contaminated with different dilutions, 10^{-3}, 10^{-6} and 10^{-9}. Once incubated, vials were checked at regular time intervals: the time taken for the vials to change colour, index of microbial growth, was annotated and compared with the expected ones (Table 5).

Expected time for colour change			
Reagente	**Dilution 10^{-3}**	**Dilution 10^{-6}**	**Dilution 10^{-9}**
TVC	6 h	12 h	18 h
COLI	4-6 h	10-12 h	16-18 h

Table 5: Time expected for each reagent to change colour for different dilutions

3.3 Results

3.3.1 Stability tests

Natural aging tests were performed analysing the stability of the reagents kept at room temperature and as mentioned above physical-chemical and microbiological tests were performed for each reagent after 3, 6 and 9 months.

The parameters investigated in chemical-physical tests were the time of dissolution, the colours of reagents, the pH values and the spectra; in microbiological test were analysed the colours of the uncontaminated vials as a control, and the time taken to change colour for the inoculated ones. Table 6 shows the results obtained from these experiments. The test is intended successful (+ in the table) when all parameters were in line with expectations and negative (- in the table) where all or most of the parameters, did not reflect expected values.

Stability test at room temperature			
Reagent	**3 months**	**6 months**	**9 months**
COLI	**+**	**-**	Not performed
TVC	**+**	**+**	**-**

Table 6: Results of stability tests performed at room temperature; each result (positive, +, or negative, -) includes chemical-physical and microbiological tests

Results in Table 6 are successful for TVC reagent, at both 3 and 6 months, for COLI reagent only after 3 months, it is achieved a good result. In fact for both reagents the time taken for vials to change colour was as on schedule, and controls showed the expected colour (Figure 17). Also physical-chemical test (i.e. both the measurements of pH that the spectra) were successful.

TVC (6 months) **COLI (3 months)**

Figure 17: Uncontaminated (left) and contaminated (right) vial for each reagent, after 6 months for TVC and after 3 months for COLI

From the results obtained in these tests it was possible to develop a summary table (Table 7) that shows the shelf life of each reagent at room temperature, 20°C, and at a 5°C; shelf life at 5°C is an estimate and it was calculated doubling the results obtained for 20°C.

Reagent	20°C *(experimental)*	5°C *(estimate)*
COLI	> 90 days	> 180 days
TVC	> 180 days	> 360 days

Table 7: Validity/effectiveness period for each reagent expressed in days

To improve the performance of both reagents the original composition of the reagents was changed and accelerated aging tests were conducted .

3.3.2 Reagents modifications

3.3.2.1 Modification of TVC reagent

The MBS TVC reagent was tested with two different formulas; Table 8 shows the original recipe.

Type of component	Component
Source of amino acids	Bacteriological Peptone (BP)
Source of carbohydrates	Yeast extract
Salt	Sodium chloride (NaCl)
Buffer solution	KH_2PO_4
Buffer solution	K_2HPO_4
Indicator	N,N,N',N'-tetramethyl-p-pnenyl-enendiaminedihydrochloride (TMPD)

Table 8: Components of the original recipe of the TVC reagent

In order to optimize performances, in particular to improve the optical detection of the colour change, we carried out a series of experiments varying the initial composition of the recipe.

Colour tests were carried out on TVC vials with different formulations and in different conditions. In these tests we tried two new sources of amino acids: component T and component P in different combinations with BP:

1) BP 5 g/l + T 5 g/l
2) BP 2 g/l + T 8 g/l
3) BP 1 g/l + T 9 g/l
4) T 10g/l
5) BP 5 g/l + P 5 g/l
6) BP 2 g/l + P 8 g/l
7) BP 1 g/l + P 9 g/l
8) P 10g/l

In the original recipe the concentration of BP was 10 g/l.

We examined the colours of the uncontaminated and inoculated vials, and also the change of colour of the latter. Results obtained in these experiments showed that the colour of vials containing component T was better than those containing component P and that the optimal combination was BP 5g/l and T 5g/l.

The new recipe was modified adding the component T (in concentration of 5 g/l) and changing the concentration of BP (from 10 g/l 5 g/l).

Accelerated aging tests at 60 ° C were performed without packing after 7, 14, 21 days for both the original and the new recipe. The latter gave better results, fore as the other, although stable at room temperature for 6 months, lost its functionality in 21 days at 60°C.

On the new TVC formulation we performed further accelerated aging tests at 60°C.

Table 9 shows the positive or negative outcomes of the physical-chemical and microbiological tests. The test was considered successful (+) only when all the examined parameters corresponded to the ones obtained for the control kept at room temperature, and negative (-) when the parameters differed from this.

Accelerated TVC tests without packaging				
	Old TVC		**New TVC**	
Time	**60°C**	**Room temperature**	**60°C**	**Room temperature**
1 week	+	+	+	+
2 weeks	+	+	+	+
3 weeks	_	+	+	+
1 month	Not performed	Not performed	+	+
2 months	Not performed	Not performed	_	+
3 months	Not performed	Not performed	_	+

Table 9: Accelerated aging tests results for TVC reagent

Results in the table show that the new TVC reagent recipe, although more stable, lost its stability after 2 months. Physical-chemical test show a slow dissolution time (10 minutes) and a dark grey final colour (expected colour was dark blue). In microbiological tests, all contaminated vials changed colour but in more time compared to the expected. The tests after 3 months confirmed the loss of stability. Also in this case, the physical-chemical showed a slow dissolution time (9 minutes) and a dark grey final colour. In microbiological tests only the vial inoculated with the 10^{-3} dilution changed colour while the others remained red (Figure 18**).**

It should be noted that the control vials kept at room temperature always performed positive results so negative results were due to accelerated aging, and not to other reasons as malfunctions or problems in the reagent preparation.

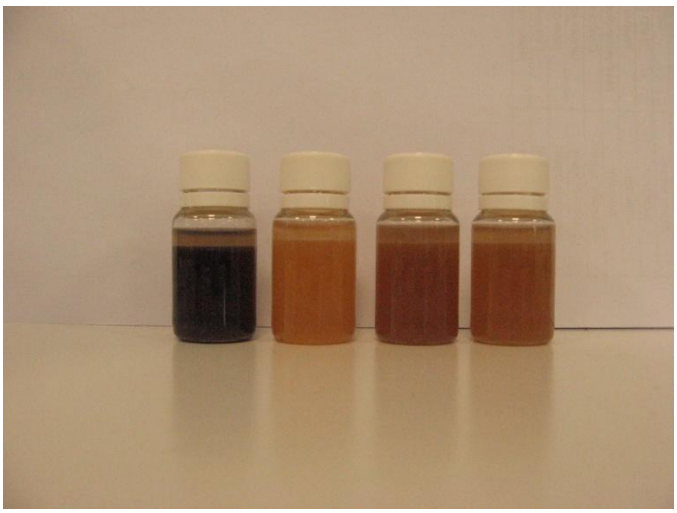


Figure 18: TVC; vials incubated in the thermostat without packaging after 3 months. From left to right: control, 10^{-3}, 10^{-6}, and 10^{-9}

3.3.2.2 Modification of COLI reagent

The first accelerated aging tests on COLI reagent were carried out storing vials at 60°C, in a plastic bag. These initial tests were unsuccessful: after a few days of storage reagents turned brown, as if they were burned, also when water was added (instead they should become red). When vials were inoculated with dilutions of *E. coli* and incubated at 37°C they remained of a red-brown colour, rather than turning yellow.

Thus, COLI reagent cannot be maintained at 60°C, because at that temperature it loses its stability and functionality. This is due to a reaction of "browning", the Maillard reaction, characterized by a complex series of phenomena that take place after the heat treatment of sugars and proteins (Munanairi *et al.*, 2007). Especially this reaction involves the carbonyl groups of reducing sugars and the free amino groups of proteins. The compounds that arise from these transformations are responsible for the formation of the brown colour.

For this reason, the further accelerated aging tests on COLI were performed at 45°C. These tests were initially carried out on the original reagent, which has then been modified and re-tested.

Table 10 shows the old formula; in the new formula the concentration of TRIS was changed from 2.5 g/l to 5 g/l, the one of BP from 20 g/l to 10 g/l and the component T was added at a concentration of 10 g/l.

Type of component	Component
Source of amino acids	Bacteriological Peptone (BP)
Source of carbohydrates	Lactose
Source of carbohydrates	Dextrose
Selective agent	Bile salts n.3
Selective agent	L-tryptophan
Selective agent	Cicloeximide
Buffer solution	4-(2-idrossietil)-1-piperazinetasulfonic acid (HEPES)
Buffer solution	TRIS
Indicator	Phenol red (PR)
Indicator	N,N,N',N'-tetramethyl-p-pnenyl-enendiaminedihydrochloride (TMPD)

Table 10: Components of the original recipe of the CO reagent

Vials were kept in oven without a humidity protective packaging and the physical-chemical and microbiological tests were performed at different times. Table 11 shows the results for physical-chemical and microbiological tests. The test was considered successful (+) when all the examined parameters corresponded to the ones obtained for the control kept at room temperature, negative (-) when the parameters differed from this and with an intermediate outcome (+ / -) when only a minority of parameters did not reflect the expected values.

Accelerated TVC tests without packaging				
	Old COLI		New COLI	
Time	**45°C**	**Room temperature**	**45°C**	**Room temperature**
1 week	+/_	+	Not performed	Not performed
3 weeks	_	+	+	+
5 weeks	Not performed	Not performed	_	+
8 weeks	Not performed	Not performed	_	+

Table 11: Accelerated aging tests results for the original and the new COLI reagent

This table shows that the new COLI reagent displays a greater stability. In the original COLI reagent microbiological tests already begun to show some issues from the first week: all the inoculated vials changed colour but some were too slow. Physical-chemical test instead gave

good results. Tests performed after 3 week were completely negative, because all the vials inoculated, either with or without the plastic bag envelope, didn't change colour and remained orange.

The new COLI reagent, instead, displayed positive results both in accelerated aging test after 3weeks, and in physical-chemical and microbiological tests. After 5 weeks the results were negative: in chemical-physical tests the final colour was brown (the expected colour was red) and pH was 6.86; in microbiological test vials didn't change colour and remained orange (Figure 19). The tests after 8 weeks confirmed that the reagent had lost its stability: in chemical-physical tests the final colour was brown (the expected colour was red) and pH was 6.75; n microbiological test vials didn't change colour and remained red (Figure 20). As seen for TVC, also in this case, the control vials kept at room temperature always performed positive results so negative results were only due to accelerated aging.

Figure 19: New COLI reagent; vials incubated in the thermostat without packaging after 5 weeks. From left to right: control, 10^{-3}, 10^{-6}, and 10^{-9}

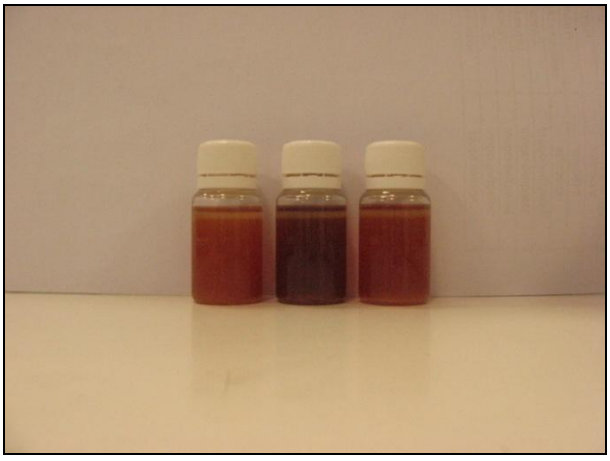


Figure 20: New COLI reagent; vials incubated in the thermostat without packaging after 8 weeks. From left to right: 10^{-3}, 10^{-6}, and 10^{-9}

3.3.3 Packaging

3.3.3.1 The use of Vaseline in MBS reagents

To increase stability of the reagents it has been very important to protect them from humidity; between all those factors capable of meeting this need Vaseline displayed a key role.
Vaseline is a hydrophobic substance, consisting of hydrocarbons, characterized by low reactivity against chemical agents, it acts as a lubricant and settling on the surface it creates a thin layer that covers and protects the reagent, from humidity and oxygen.
The choice of using vaseline in the reagents formulations originates by the need to avoid that atmospheric oxygen could enter the vials, in order to allow reduction of TMPD, thus the change of colour. In fact, as described above, in the MBS method the redox indicator TMPD plays an important role enabling the detection of bacterial enzymatic activity in the reagent, since it is capable to change colour passing from its oxidized blue form to a reduced yellow form. Initially oxygen is present in the medium so the indicator remains in its oxidized state. The activity of respiratory chain enzymes leads to the reduction of oxygen that acts as the final electron acceptor. Once oxygen is completely consumed electrons are transferred to TMPD that will be reduced turning yellow. For this reason it's clear that a further input of oxygen in the vial would maintain TMPD in its oxidized state. To use Vaseline several colour tests were carried out on all reagents. Initially vials were tested without Vaseline, since given its oily nature it was seen to increase dissolution time. Precisely vials were prepared without vaseline and then sterilized with γ-rays. When vials were ready for use, Vaseline was added together with water. Although vials prepared this way displayed very fast dissolution times, they lost their stability for humidity.
Thus Vaseline was added to vials before sterilization. These reagents were much more stable.
For this reason vials were always prepared with Vaseline ensuring a good protection against humidity.

3.3.3.2 Humidity protective packaging

Accelerated aging tests at 45 and 60°C were performed in saturated humid air: vials were incubated in stoves containing a beaker filled with water.

To ensure effective protection from humidity it was necessary to package products making them water-proof; various types of protective packaging have been tested. Among these "barrier envelopes" resulted particularly effective and also suitable for commercialization. These are shielding envelopes, made by a metallic multilayer film, which maintain vacuum, and act as a protective barrier against humidity and water vapour. Their composition is as follows:

- Outer layer: dissipative polyester static
- Middle layer: aluminium shielding sheet EMI / RFI
- Inner layer: antistatic polyethylene

They are used for vacuum packaging and heat sealing, usually together with desiccants to remove the remaining internal humidity. The desiccants in silica gel are able to absorb and neutralize humidity within a packaging or a container. Silica gel is a polymer of silicon dioxide (SiO2) n, formed by a colloidal suspension (gel); it displays a considerable porosity, and this ensures a high total surface area available for the absorption of water. The dehydrated salts are packaged in the form of granules in paper bags impermeable to dust and resistant to tearing. They maintain the quality of the product over time, preserving it from oxidation and corrosion.
The accelerated aging tests with protective packaging have been conducted on the new TVC formulation using glass containers and metal containers and on the new COLI formulation using metal barrier envelopes. Dehydrated salts in silica gel were put in all containers and envelopes.
Results are shown in Table 12 and Table 13; these tables also show results of tests carried out without packaging, previously discussed, in order to make a comparison.

TVC at 60 °C			
Time	**Without packaging**	**With packaging**	**Room temperature**
1 week	+	+	+
2 weeks	+	+	+
1 month	+	+	+
2 months	_	+	+
3 months	_	+	+

Table 12: Accelerated aging tests results for TVC

As shown in the table, both physical chemical and microbiological tests for TVC were successfull, performing excellent results in pH values, spectra, dissolution time, colour of the

uncontaminated vials used as control (blue-purple) and time taken to change for inoculated vials (Figure 21). We can definitely state that reagent is maintained better when stored in humidity protective packaging, compared to vials kept in plastic bags that lost their stability after 2 months.

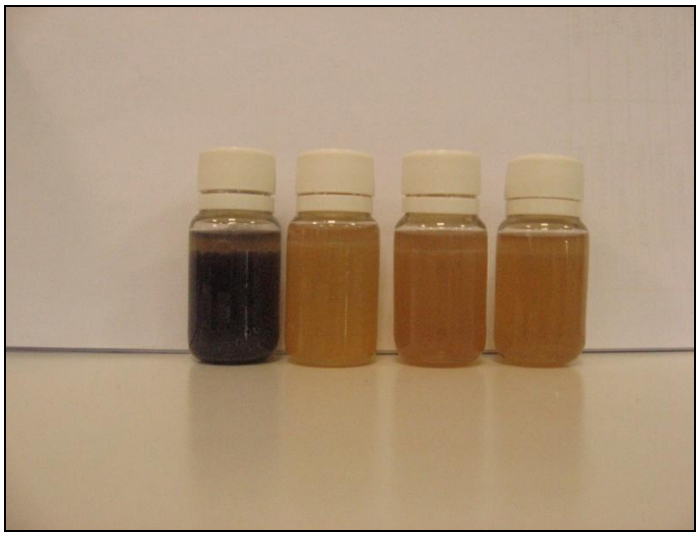


Figure 21: TVC reagent after 3 months; vials incubated in the thermostat in a glass container. From left to right: control 10^{-3}, 10^{-6}, and 10^{-9}

NEW COLI at 45 °C			
Time	**Without packaging**	**With packaging**	**Room temperature**
3 weeks	+	+	+
5 weeks	_	+	+
8 weeks	_	+	+

Table 13: Accelerated aging tests results for the new COLI reagent

The results for the COLI reagent in Table 13 show that also in this case the reagent was maintained better in the metal envelope rather than in humidity unprotected storage conditions (Figure 22).

Figure 22: New COLI reagent after 8 weeks; vials incubated in the thermostat in a metal envelope. From left to right: control 10^{-3}, 10^{-6}, 10^{-9}

3.3.4 Accelerated aging tests summarizing tables

All accelerated aging experiments performed on COLI and TVC reagents allowed to develop summarizing tables that highlight the storage time of reagents in various conditions (Table 14 and Table 15). It is usually estimated that stability increases two times for every decrease of temperature of 10°C. In these tables the estimates were calculated evaluating as a precautionary measure a doubled storage time for each decrease of temperature: from 60°C to 20°C, from 45°C to 20°C, from 20°C to 5°C.

	60°C ***(experimental)***	**20°C** ***(estimate)***	**5°C** ***(estimate)***
TVC O.R.	20 days	80 days.	160 days.
TVC N.R.	60 days	240 days.	480 days.
TVC N.R. + packaging	> 90 days	> 360 days	> 720 days

Table 14: Validity/effectiveness period (expressed in days) of TVC reagent for all storage condition. (-O.R.: original recipe, -N.R.: new recipe)

	45°C ***(experimental)***	**20°C** ***(estimate)***	**5°C** ***(estimate)***
COLI O.R.	20 days	40 days	80 days
COLI N.R.	35 days	70 days	140 days
COLI N.R. + packaging	> 60 days	> 120 days	> 240 days

Table 15: Validity/effectiveness period (expressed in days) of CO reagent for all storage condition. (-O.R.: original recipe, -N.R.: new recipe)

In order to obtain a more general view these tables also show the results of tests carried out without packaging at room temperature.

CHAPTER IV

Multi reader Set-up

4.1 Introduction

The Multi reader has been made possible thanks to a close collaboration with the Department of Physics, Tor Vergata University. My role was essentially to verify the compliance of the adopted solutions from the biological point of view, to indicate the operating parameters and to verify the functionality of the system.

4.1.1 Device description

The optical device called "multi reader" (MR) is required to control multiple samples, without having to visually inspect at regular intervals the vials under examination. The MR is a modular optical device furnished of specific software which can incubate the vials to analyze, detect automatically the change of colour of the solution and calculate the microorganisms concentration in the sample. The MR is connected to a computer and the results of the analysis are made available as a report in digital format ready to be printed.

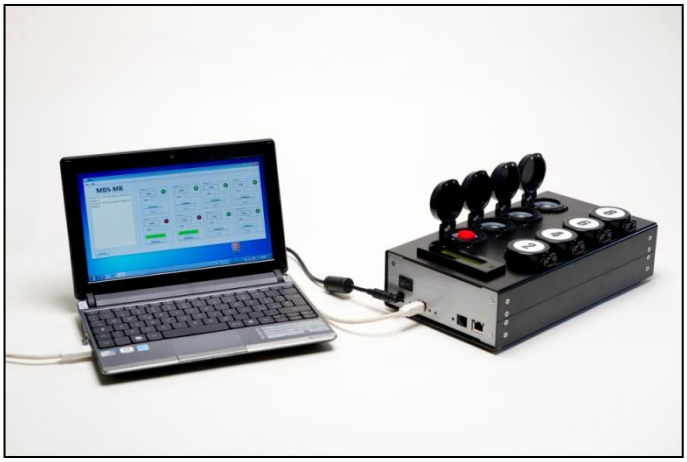


Figure 23: MR device connected to computer

The MR was conceived in successive steps dividing the apparatus into different sections. In particular, these sections can be identified with the following subsystems:

- **Heating system and temperature control of the reaction cell**

The reaction cell that lodges the disposable vial is constituted by an aluminium pipe of opportune diameter that must be maintained at a constant temperature. The incubation temperature is variable since this can vary according to the typology of analysis to perform. The temperature range selected is from 30°C to 44°C. The heating circuit was originally constituted of a coil of heating wire thread in tungsten with a resistivity of about 50 ohm/m. Such system, even though very efficient and inexpensive, was too difficult to place around the tube housing (in order to ensure the appropriate heat transmission it must be wound with attention around the aluminium pipe to maintain a tight contact between the wire and the tube pipe) and it was difficult to weld (tungsten is not wieldable with the pond from electrical circuits). Such system is present in the prototypes 1 and 2. In the following prototypes (3, 4 and 5) the tungsten wire was replaced by a circuit shaped ad hoc (considering the hole for the LEDs, for the photo diode and for the temperature sensor). The circuit is constituted by a sheet in kapton on which thin copper tracks of development are placed (the resistivity of about 20 ohms/m is provided by the small size of the deposit of copper).

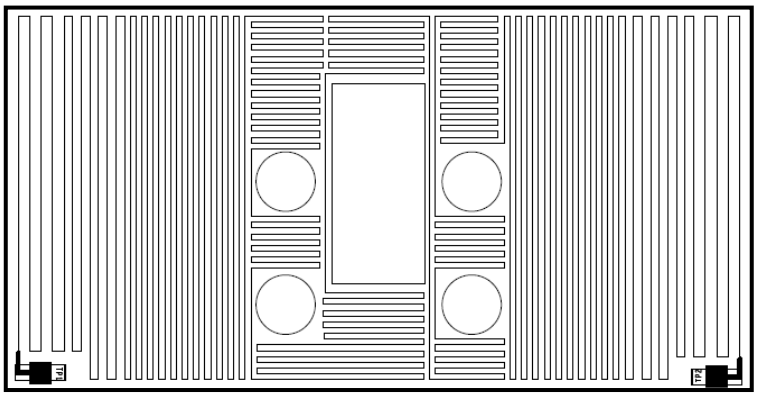


Figure 24: Heating circuit in kapton

The temperature control system is provided by a chip (U3), assembled in contact and parallel to the aluminium pipe, which can measures the surface temperature. The chip, programmable through serial doors, checks directly the heating circuit furnishing or removing power when needed. Another safety chip that controls the programmed temperature is placed in contact with the cylinder base (also in aluminium). If the temperature exceeds 55°C, for a malfunction of the first chip, the second chip intervenes stopping the heating circuit.

The different chips were set using a thermocouple in order to ensure a very precise and accurate measurement of the temperature inside of the reaction vial. With these devices, the planned temperature is guaranteed with a discard of ± 0.5°C.

- **Detection optical system of the reaction cell**

The optical system is constituted by a part assigned to light emission and another one assigned to receive the return signal.

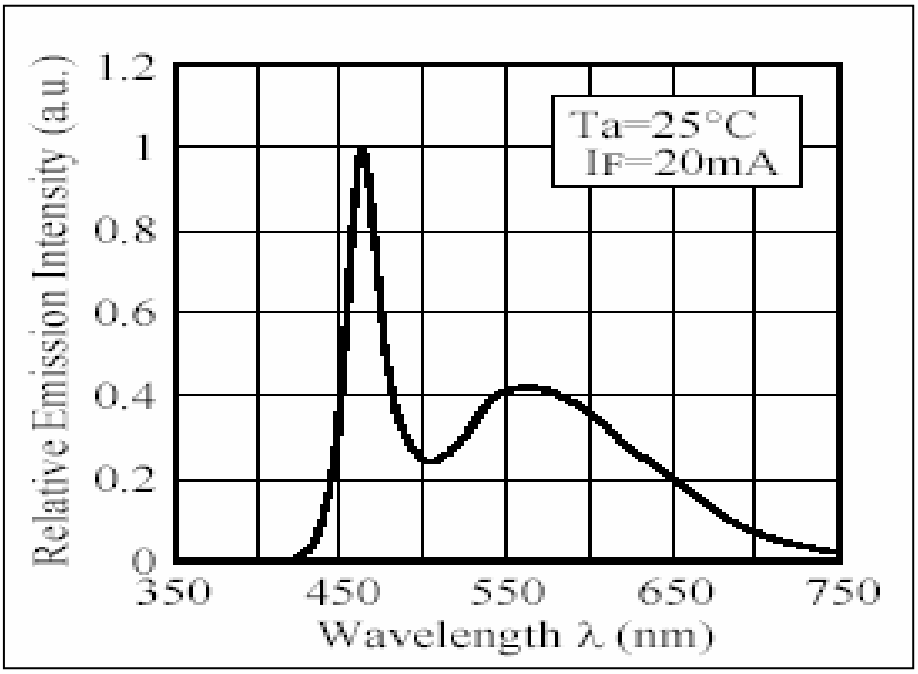


Figure 25: Emission spectrum of LED

The light emission device consists of 3 LEDs (Light Emitting Diode) symmetrically arranged around the receiving photodiode. Such configuration allows performing grants the measurement exclusively of the light scattered by the solution (excluding the light transmitted and reflected), in order to avoid the receiver saturation due to the clearness of the initial solution (especially for liquids analysis) and the subsequent turbidity due to bacterial growth. The LED (LD1, LD2, LD3) are centred on the RGB bands. They are a very high efficiency and brightness (from 15000 → 22000 mcd). Brightness is checked by a current amplifier able to control brightness according to the light scattered by the solution in the vial.

The signal detection device is a colour sensor (U1), consisting in a matrix of 3 photodiodes placed on a chip that is generally used for quality control, to monitor colour production and detection. The photodiodes are screened with spectral filters in the 3 primary colours: red, green and blue (RGB). Externally to the case of the whole photodiode, is placed an infrared spectral

filter (IR) that cuts the lower frequency of infrared (NIR). The particular constitution of the sensor allows it to have a very good response in the visible spectrum (400 - 700 nm). At the photodiode exit there are the 3 signals (RGB); these signals (very week) are sent to a transimpedance amplifier (U2) that amplifies them to fit to the entry of ADC (Analog to Digital Converter).

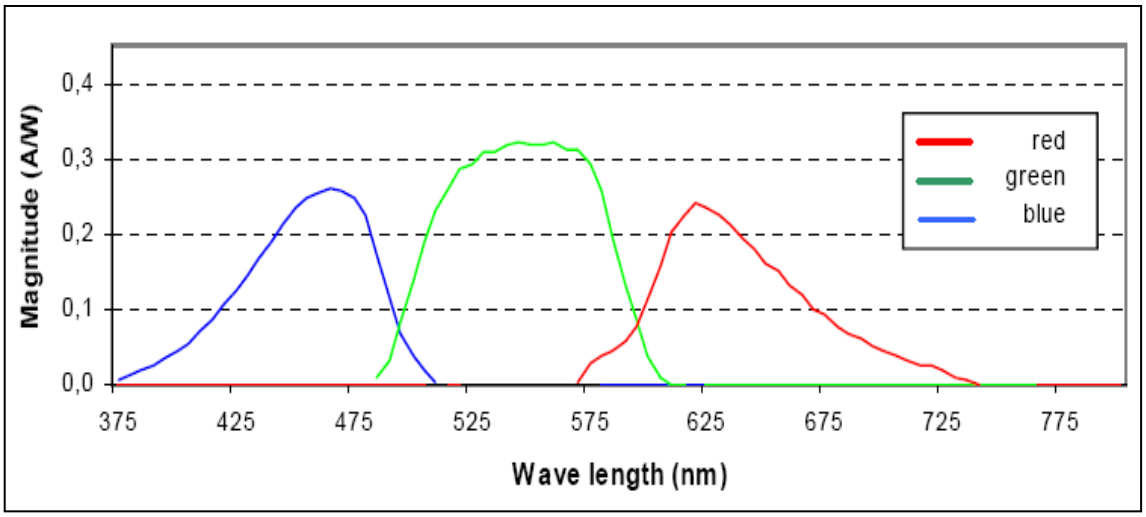


Figure 26: Spectral response of the photodiode

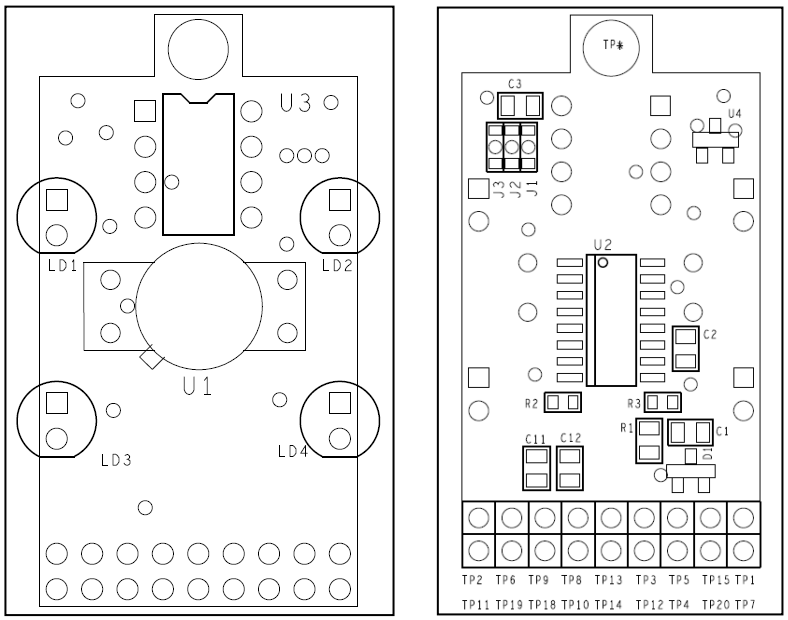


Figure 27: Optical system and temperature controller (front sx and rear dx)

- **Motherboard (PCB)**

All of the different MR systems are integrated in the motherboard of processing and complete control of the different analysis cells. The heart of the PCB is the PIC18F97J60 10-bit microcontroller (Microchip Semiconductor), which ADC can receive up to 16 analogue channels. The microcontroller is assigned to control all the functions of the MR, to receive all the details set by the user through the computer and to send the measurements at the control program (installed on the computer). Through a series of planned and specific controls, it determines if the analysis result is negative (no significant colour changes) or positive (colour changes due to bacterial growth), calculating directly the final result. The PCB is interfaced with the computer through 2 alternative serial ports, an USB and an Ethernet. This type of connection, a part from using the common standards of connection, grants the simultaneous use of different MR and the simultaneous analysis of several independent samples. The PCB is powered with 12 V by an external power feeder in order to operate only at low voltage, avoiding problems related to the isolation and therefore ensuring a simple procedure for the CE signature of the device.

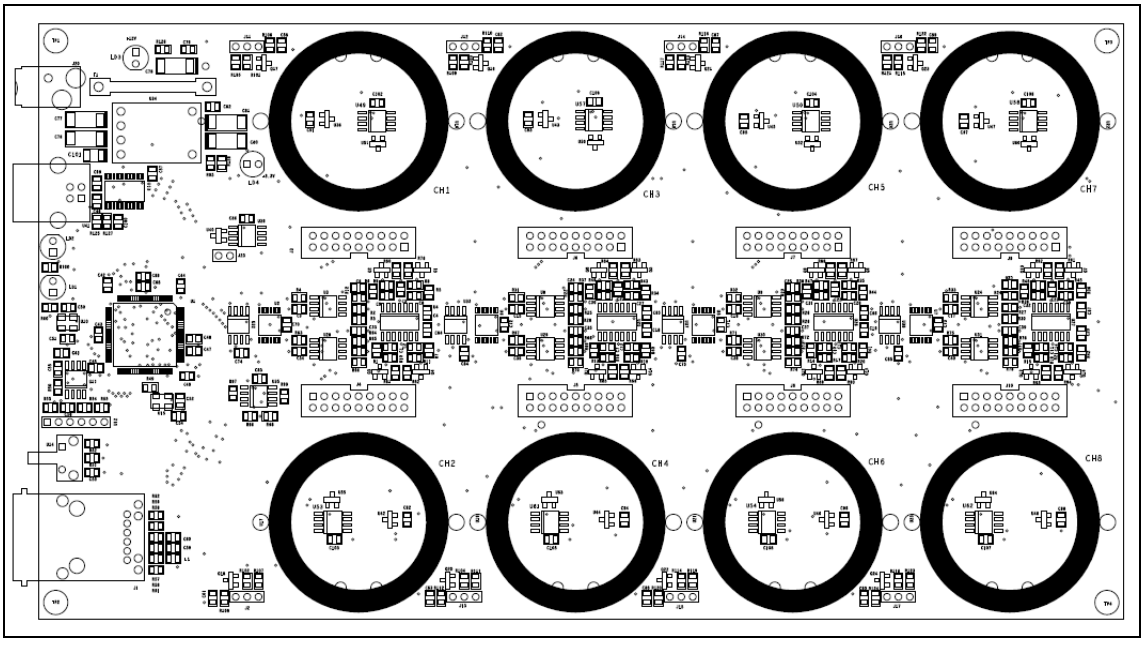


Figure 28: Motherboard of the MR

The device is connected to the computer through an USB port and powered by external power supply.

4.1.2 Software design

The control software was very delicate to design because the device must monitor and control all the various settings and functions. The software is made more complex also for the possibility to use simultaneously and independently different MR. The software must perform a great amount of controls and operations with particular attention to the measures detected and to the combinations of these. In fact it is absolutely crucial to reduce the probability to get "false negatives".

We can divide the software in 2 different parts:

- ROM code sw in the motherboard microcontroller;
- User interface sw on the computer.

For the definition of the first one, we used the PCW compiler included in the MPAB IDE version 6 package. The functions used are all available in the library of the compiler. The microcontroller has been placed on the PICDEM 2 PLUS demo board to which the system has been connected. In the realized version it is possible to change the set point value, the constants value of the algorithm and the type of algorithm used for the control. Basically we were able to have a "total" control of the functions of the microcontroller (PIC).

For the second we chose to use the Java language that is very common and easily integrative in the actual HTML browsers. Moreover, the java is free and therefore it does not impose licensing fees or royalties.

In the user interface software are also displayed the following information (Figure 29):

- "real time" colour of the analyzing vial;
- green or red light indicating the availability or unavailability of the station;
- CFU box indicating the "real time" value of the result, (red border means positive result, green border means negative result, gray border means analysis is still running).

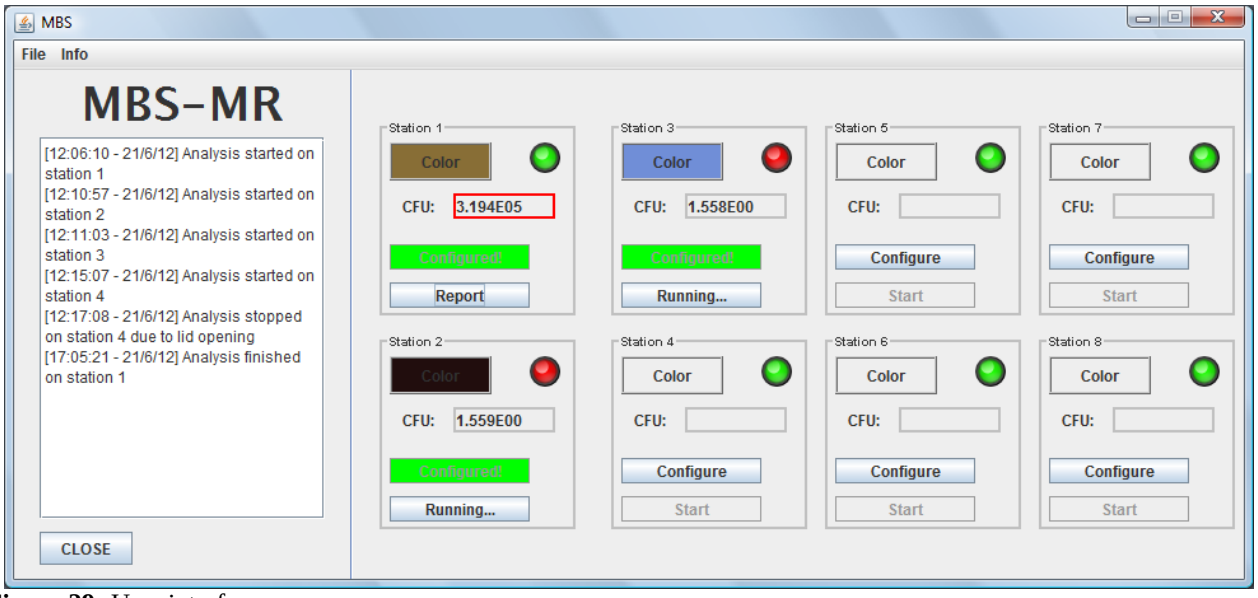


Figure 29: User interface sw

4.2 Operating principle

During the execution of a test the device sends periodically (every 10 seconds) the data to the computer that saves it and analyzes it in order to provide the results.

These are different and multiple data that are : RGB signal, RGB LED streams, state of the stations (in use, free, open) etc. The Java software installed on the computer, apart from memorizing and analyzing all the data received from the device during the analysis, sets also all the parameters required for the start of the analysis in a specific station. During the set-up of the testing station, apart from inserting the sample details the operator can in fact choose from a menu the microorganism to search for (and consequently the vial in use) and the macro category to which the sample belongs (water, meat, dairy products, vegetables etc.). The choice of these two elements refers automatically and univocally to a specific set of parameters that is sent to the device for the preliminary set-up. The possible choices in the menu correspond to all the possible combinations of the items of the following figures:

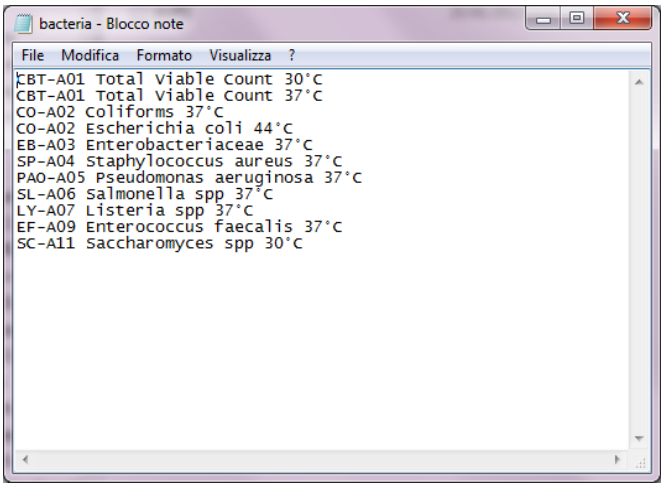


Figure 30: Settings file for bacteria

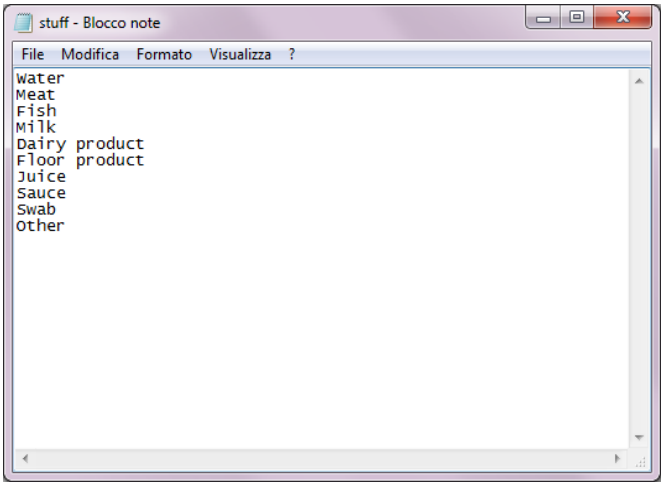


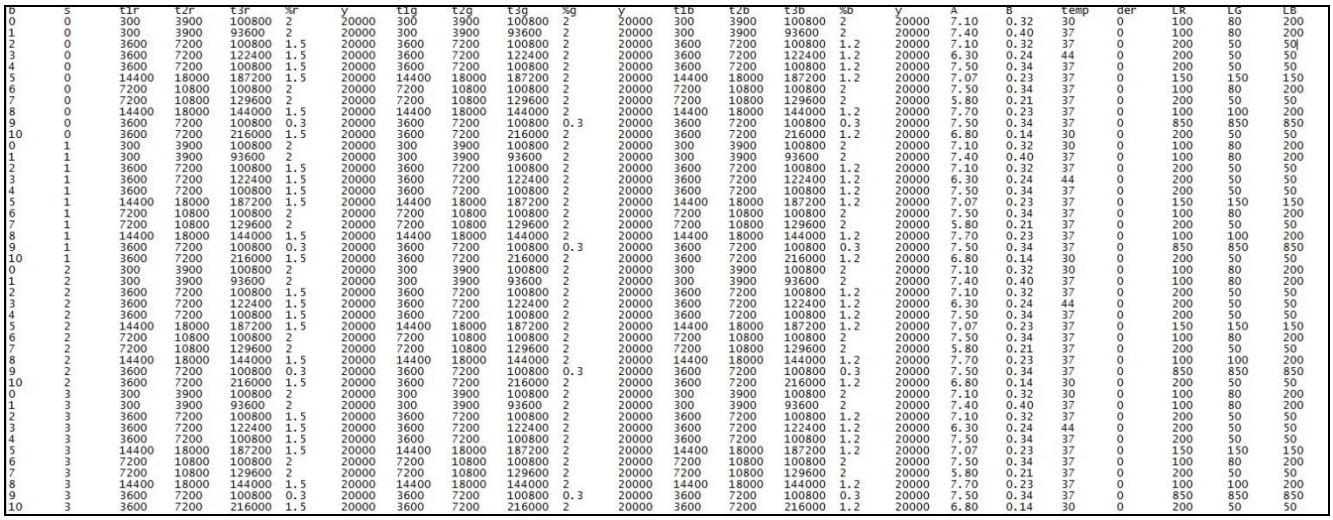
Figure 31: Settings file for stuff

As mentioned before, choosing from the menu the microorganism to search for and the macro category of interest determines univocally the setting of the parameters of the device.

The parameters for each colour are:

- Time of offset of the colours (about 10 minutes from the start) - t1
- Minimum time required for the system to analyze the data and give the results - t2
- Maximum time required for the analysis - t3
- Minimum percentage of increase of the signal (after the offset at t2) after which the test can be positive - % (RGB)
- Minimum value of the derivative of the RGB signal - d(RGB)
- Value that the signal must reach after the offset at t2 - y
- Temperature of execution of the test - T

- Expected sign of the derivative of the RGB signal - d
- Calculation parameters for the value of contamination - A, B (parameters of the calibration curve)



b	s	t1r	t2r	t3r	%r	y	t1g	t2g	t3g	%g	y	t1b	t2b	t3b	%b	y	A	B	temp	der	LR	LG	LB
0	0	300	3900	100800	2	20000	300	3900	100800	2	20000	300	3900	100800	2	20000	7.10	0.32	30	0	100	80	200
1	0	300	3900	93600	2	20000	300	3900	93600	2	20000	300	3900	93600	2	20000	7.40	0.40	37	0	100	80	200
2	0	3600	7200	100800	1.5	20000	3600	7200	100800	2	20000	3600	7200	100800	1.2	20000	7.10	0.32	37	0	200	50	50
3	0	3600	7200	122400	1.5	20000	3600	7200	122400	2	20000	3600	7200	122400	1.2	20000	6.30	0.24	44	0	200	50	50
4	0	3600	7200	100800	1.5	20000	3600	7200	100800	2	20000	3600	7200	100800	1.2	20000	7.50	0.34	37	0	200	50	50
5	0	14400	18000	187200	1.5	20000	14400	18000	187200	2	20000	14400	18000	187200	1.2	20000	7.07	0.23	37	0	150	150	150
6	0	7200	10800	100800	2	20000	7200	10800	100800	2	20000	7200	10800	100800	2	20000	7.50	0.34	37	0	100	80	200
7	0	7200	10800	129600	2	20000	7200	10800	129600	2	20000	7200	10800	129600	2	20000	5.80	0.21	37	0	200	50	50
8	0	14400	18000	144000	1.5	20000	14400	18000	144000	2	20000	14400	18000	144000	1.2	20000	7.70	0.23	37	0	100	100	200
9	0	3600	7200	100800	0.3	20000	3600	7200	100800	0.3	20000	3600	7200	100800	0.3	20000	7.50	0.34	37	0	850	850	850
10	0	3600	7200	216000	1.5	20000	3600	7200	216000	2	20000	3600	7200	216000	1.2	20000	6.80	0.14	30	0	200	50	50
0	1	300	3900	100800	2	20000	300	3900	100800	2	20000	300	3900	100800	2	20000	7.10	0.32	30	0	100	80	200
1	1	300	3900	93600	2	20000	300	3900	93600	2	20000	300	3900	93600	2	20000	7.40	0.40	37	0	100	80	200
2	1	3600	7200	100800	1.5	20000	3600	7200	100800	2	20000	3600	7200	100800	1.2	20000	7.10	0.32	37	0	200	50	50
3	1	3600	7200	122400	1.5	20000	3600	7200	122400	2	20000	3600	7200	122400	1.2	20000	6.30	0.24	44	0	200	50	50
4	1	3600	7200	100800	1.5	20000	3600	7200	100800	2	20000	3600	7200	100800	1.2	20000	7.50	0.34	37	0	200	50	50
5	1	14400	18000	187200	1.5	20000	14400	18000	187200	2	20000	14400	18000	187200	1.2	20000	7.07	0.23	37	0	150	150	150
6	1	7200	10800	100800	2	20000	7200	10800	100800	2	20000	7200	10800	100800	2	20000	7.50	0.34	37	0	100	80	200
7	1	7200	10800	129600	2	20000	7200	10800	129600	2	20000	7200	10800	129600	2	20000	5.80	0.21	37	0	200	50	50
8	1	14400	18000	144000	1.5	20000	14400	18000	144000	2	20000	14400	18000	144000	1.2	20000	7.70	0.23	37	0	100	100	200
9	1	3600	7200	100800	0.3	20000	3600	7200	100800	0.3	20000	3600	7200	100800	0.3	20000	7.50	0.34	37	0	850	850	850
10	1	3600	7200	216000	1.5	20000	3600	7200	216000	2	20000	3600	7200	216000	1.2	20000	6.80	0.14	30	0	200	50	50
0	2	300	3900	100800	2	20000	300	3900	100800	2	20000	300	3900	100800	2	20000	7.10	0.32	30	0	100	80	200
1	2	300	3900	93600	2	20000	300	3900	93600	2	20000	300	3900	93600	2	20000	7.40	0.40	37	0	100	80	200
2	2	3600	7200	100800	1.5	20000	3600	7200	100800	2	20000	3600	7200	100800	1.2	20000	7.10	0.32	37	0	200	50	50
3	2	3600	7200	122400	1.5	20000	3600	7200	122400	2	20000	3600	7200	122400	1.2	20000	6.30	0.24	44	0	200	50	50
4	2	3600	7200	100800	1.5	20000	3600	7200	100800	2	20000	3600	7200	100800	1.2	20000	7.50	0.34	37	0	200	50	50
5	2	14400	18000	187200	1.5	20000	14400	18000	187200	2	20000	14400	18000	187200	1.2	20000	7.07	0.23	37	0	150	150	150
6	2	7200	10800	100800	2	20000	7200	10800	100800	2	20000	7200	10800	100800	2	20000	7.50	0.34	37	0	100	80	200
7	2	7200	10800	129600	2	20000	7200	10800	129600	2	20000	7200	10800	129600	2	20000	5.80	0.21	37	0	200	50	50
8	2	14400	18000	144000	1.5	20000	14400	18000	144000	2	20000	14400	18000	144000	1.2	20000	7.70	0.23	37	0	100	100	200
9	2	3600	7200	100800	0.3	20000	3600	7200	100800	0.3	20000	3600	7200	100800	0.3	20000	7.50	0.34	37	0	850	850	850
10	2	3600	7200	216000	1.5	20000	3600	7200	216000	2	20000	3600	7200	216000	1.2	20000	6.80	0.14	30	0	200	50	50
0	3	300	3900	100800	2	20000	300	3900	100800	2	20000	300	3900	100800	2	20000	7.10	0.32	30	0	100	80	200
1	3	300	3900	93600	2	20000	300	3900	93600	2	20000	300	3900	93600	2	20000	7.40	0.40	37	0	100	80	200
2	3	3600	7200	100800	1.5	20000	3600	7200	100800	2	20000	3600	7200	100800	1.2	20000	7.10	0.32	37	0	200	50	50
3	3	3600	7200	122400	1.5	20000	3600	7200	122400	2	20000	3600	7200	122400	1.2	20000	6.30	0.24	44	0	200	50	50
4	3	3600	7200	100800	1.5	20000	3600	7200	100800	2	20000	3600	7200	100800	1.2	20000	7.50	0.34	37	0	200	50	50
5	3	14400	18000	187200	1.5	20000	14400	18000	187200	2	20000	14400	18000	187200	1.2	20000	7.07	0.23	37	0	150	150	150
6	3	7200	10800	100800	2	20000	7200	10800	100800	2	20000	7200	10800	100800	2	20000	7.50	0.34	37	0	100	80	200
7	3	7200	10800	129600	2	20000	7200	10800	129600	2	20000	7200	10800	129600	2	20000	5.80	0.21	37	0	200	50	50
8	3	14400	18000	144000	1.5	20000	14400	18000	144000	2	20000	14400	18000	144000	1.2	20000	7.70	0.23	37	0	100	100	200
9	3	3600	7200	100800	0.3	20000	3600	7200	100800	0.3	20000	3600	7200	100800	0.3	20000	7.50	0.34	37	0	850	850	850
10	3	3600	7200	216000	1.5	20000	3600	7200	216000	2	20000	3600	7200	216000	1.2	20000	6.80	0.14	30	0	200	50	50

Figure 32: Matrix of setting parameters

4.2.1 Algorithm

During the test execution the software uses a routine calculation that examines every minute the result of the analysis. Basically, it examines whether the variations of the RGB signals detected and memorized are really due to a change of colour of the vial content.

For this reason an algorithm was implemented which is able to search for the maximum values of the derivative of the RGB signal. The algorithm calculates whether the maximum value of the derivative is an absolute value or not.

The program memorizes the maximum stream of the derivative functions of the RGB signals (real time) and considers it maximum only if apart from being higher than the minimum value set at the start of the analysis – d(RGB) – it isn't surpassed by another maximum for at least 1,1 hours. This value has been determined experimentally from the values obtained after thousands of tests.

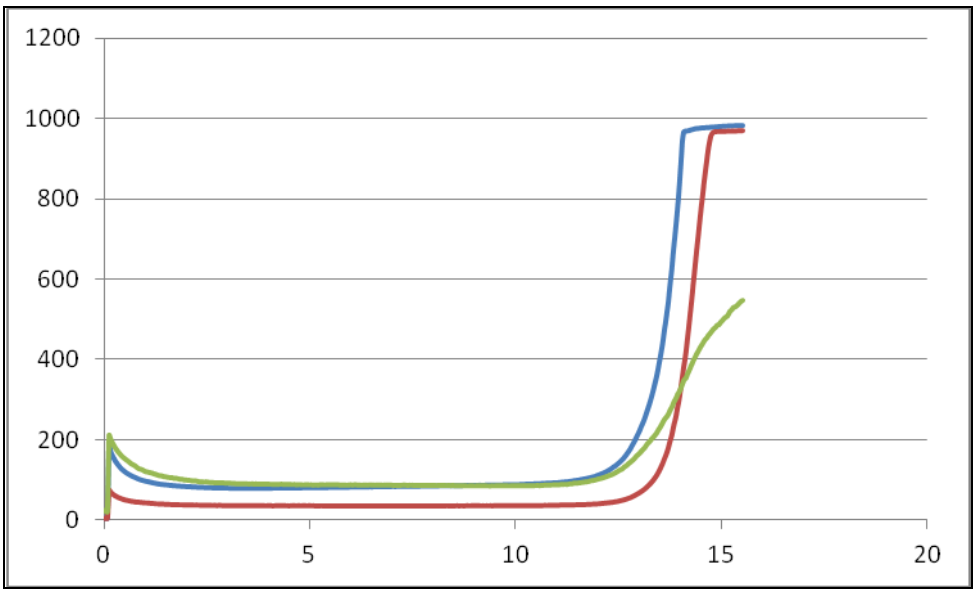


Figure 33: RGB Signal

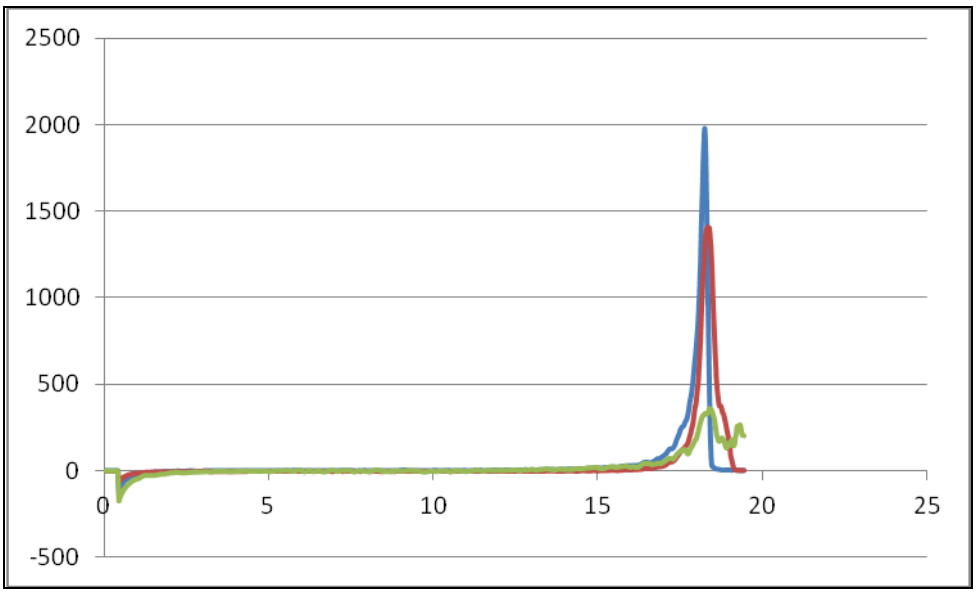


Figure 34: 1st derivative of RGB signals d(RGB)

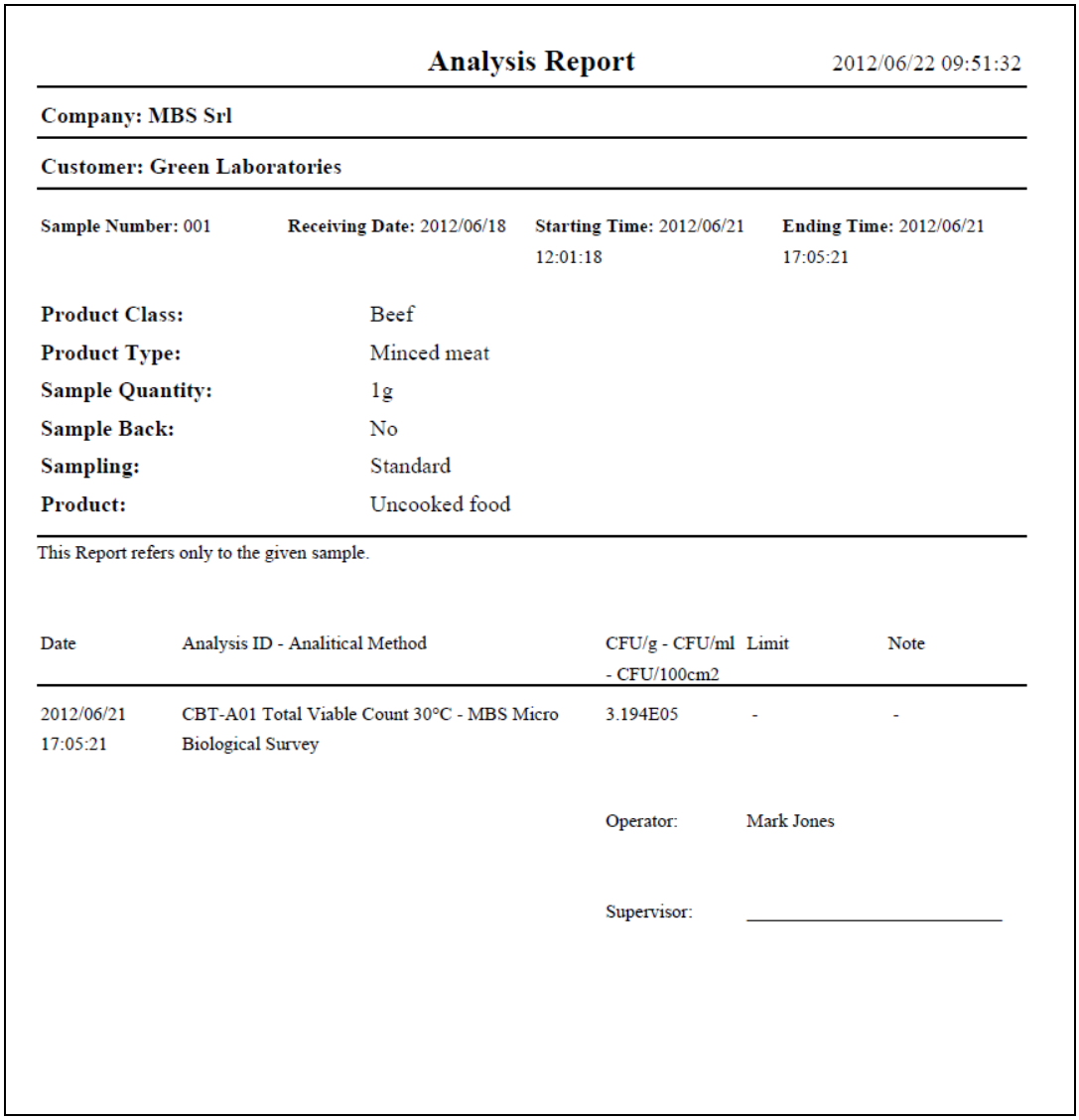
When the program finds an absolute value it evaluates the variations in the correspondent RGB signal. If the signal has undergone a variation at least equal to the one specified from the %(RGB) input parameter then there is an “OK” for one colour.

The final stop condition, that is the one that determines the end of the analysis, is constituted by the occurrence of 2 “OK” of at least 2 colours (RG – GB – RB).

When these two conditions occur the test is considered concluded and the value of contamination is calculated based on the parameters (A, B) and the time passed until the stop according to the following equation:

$$CFU = 10^{(A - Bt)}$$

The results obtained are printed, together with the data inserted at the beginning of the analysis, in a pdf report.



Analysis Report 2012/06/22 09:51:32

Company: MBS Srl

Customer: Green Laboratories

Sample Number: 001 **Receiving Date:** 2012/06/18 **Starting Time:** 2012/06/21 12:01:18 **Ending Time:** 2012/06/21 17:05:21

Product Class:	Beef
Product Type:	Minced meat
Sample Quantity:	1g
Sample Back:	No
Sampling:	Standard
Product:	Uncooked food

This Report refers only to the given sample.

Date	Analysis ID - Analitical Method	CFU/g - CFU/ml - CFU/100cm2	Limit	Note
2012/06/21 17:05:21	CBT-A01 Total Viable Count 30°C - MBS Micro Biological Survey	3.194E05	-	-

Operator: Mark Jones

Supervisor: _____

Figure 35: Report of the MR

CHAPTER V

Validation of the MBS method for Total Viable Count and *E. coli* in food samples

5.1 Introduction

5.1.1 The importance of validation

Microbiological food safety and food-borne infections are important public health concerns worldwide. Contaminated food consumption often results in illnesses called food borne infections or food borne intoxications (Saikia & Joshi, 2010; Arzina *et al*., 2011).

Microbiological analysis of food is an important part for managing microbiological safety within the entire food chain. The hygienic criteria of the process for many foodstuffs now include a test for Total Viable Count (TVC) and *Escherichia coli* (*E. coli*). TVC gives a quantitative idea of the presence of mesophilic aerobic microorganisms of animal origin (Greenwood *et al.*, 1984). It is an important criterion for evaluating the microbial quality of various food products and can also be used to evaluate their degree of freshness (Nanu *et al*., 2007). *E. coli* is part of the normal microflora of the gastrointestinal tract of mammals and birds. Already in the 19th century, *E. coli* was recognized as a good indicator of faecal contamination. It was identified as the only species in the coliform group found exclusively in the intestinal tract of humans and other warm-blooded animals and subsequently excreted in large numbers in faeces, approximately 10^9 per gram (Cabral, 2010). Some strains have developed the ability to cause disease in the gastrointestinal and urinary tract or in the central nervous system even in the most resistant human hosts. The majority of cases worldwide are caused by strains of the serotype O157:H7, an enterohemorrhagic strain of *E. coli* (EHEC). It can colonize the intestines of humans and cause diarrhea, hemorrhagic colitis and a series of symptoms that are recognized under the name of haemolytic-uremic syndrome (Nataro & Kaper, 1998; Yin *et al*., 2011).

Both control authorities and individual food business operators use microbiological analysis to monitor the microbiological quality of raw materials and finished products and the microbiological status of manufacturing procedures. Microbiological analysis is also a valuable

tool for compliance testing of alternative methods or for the assessment of the efficacy of control strategies based upon Hazard Analysis Critical Control Points (HACCP) (Jasson *et al*., 2010). Standardized methods (e.g. ISO methods) are acknowledged as reference analytical methods for official controls. These standardized methods are based on traditional microbiological culture methods that are widely used in food analysis laboratories. These techniques present several issues, such as subjectivity in the interpretation of some biochemical or morphological tests, possible interference of matrices, especially when they present high levels of contamination, high cost of the method, both in terms of labour and supplies, and above all, the long time (from 3 to 7 days) needed to obtain definitive results, depending on the need of biochemical and serological confirmation (Tomas *et al.*, 2009).

During the last decades, the requirement for food industry to rapidly assess food safety, has led to the development and refinement of alternative microbiological methods of analysis that are quicker and easier to perform than the corresponding reference method; nowadays there is also a growing attention to the possibility of automated systems (Feng, 1996). Therefore, the prerequisite for sale and use of any alternative method is to provide evidence that this could yield equivalent results to those provided by the corresponding reference method. For this reason, ISO 16140 (Anonymous, 2003a) for the validation of alternative methods, represents a key issue for the suppliers of alternative methods, the food and drink industries and for public health (Feinberg *et al*., 2009).

5.1.2 Reference methods

The two types of analysis we have considered in our study, Total Viable Count (TVC) and *E. coli,* belong to the category of quantitative methods and share the same protocol of validation. The only differences, as we shall see, are related to the requirements of selectivity.

Indeed, for the enumeration of *E. coli* it is required that, within a certain limit, the method detects only this species of microorganisms; in addition different microorganisms should not give false positive results, and their presence should not affect the count of the target microorganism. This watchfulness is not necessary for the TVC exam. Indeed, in such case, the analysis aims to count all the bacteria that grow at 30°C without precise nutritional requirements and without any kind of selectivity restraints.

The reference standard methods describe the laboratory protocols to follow and represent the international uniformly accepted standard for this test. The reference documents are:

- EN ISO 4833:2004 "Horizontal method for enumeration of microorganisms – techniquecount at 30°C", the technique used is the count on plates of the non-selective mediumPCA.
- EN ISO 16649-2:2001 "Horizontal method for enumeration of *Escherichia coli* β-glucuronidase positive - colony count technique at 44°C using 5-bromo-4-chloro-3-indolyl β-D-glucuronide "; the standard provides, as in 4833, the plate counton plates of s selective and highly specific medium called TBX.

5.2 Materials and methods

5.2.1 Statistical analysis on artificially contaminated water samples

A comparison between the colorimetric MBS method and the plate count method for the detection and differential count of TVC and *E. coli*, in artificially contaminated samples, was performed according to ISO 17025 (Anonymous, 2005), following the International Standards ISO/TR13843 (Anonymous, 2000), using those described in ISO 9998 as reference methods (Anonymous, 1991).

5.2.1.1 Bacterial strains

All the strains used in these statistical analyses were available from ATCC (American Type Culture Collection). The strains used in these statistical analyses were *Salmonella typhimuriu m*(ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853), *Listeria innocua* (ATCC 33090), *Escherichia coli*(ATCC 25992), *Escherichia coli* O157:H7 (ATCC 35150), *Staphylococcus aureus*(ATCC 12600) and *Enterococcus faecalis* (ATCC 29212).

5.2.1.2 Preparation of artificially contaminated samples

Sterile water samples were contaminated with the above indicated microorganisms coming from overnight cultures with serial dilutions in sterile saline solution up to 10^{-8}.

5.2.1.3 Colorimetric MBS method procedure

As reported in the introduction, the analytical procedure for the quantitative colorimetric MBS method is based on a colorimetric survey, using a redox indicator that detects changes of the oxidoreductive state in the reaction medium. For the analysis performed with the MBS method we used, ready to use, disposable, sterile MBS vials, containing the specific reagent for the analysis. The vials used were the ones for the analysis of *E.coli* and TVC (Table 16 and Table 17).

Type of component	Component
Source of amino acids	Bacteriological Peptone (BP)
Source of amino acids	Tryptone
Source of carbohydrates	Lactose
Source of carbohydrates	Dextrose
Selective agent	Bile salts n.3
Selective agent	L-tryptophan
Selective agent	Cicloeximide
Buffer solution	4-(2-idrossietil)-1-piperazinetasulfonic acid (HEPES)
Indicator	Phenol red (PR)
Indicator	N,N,N',N'-tetramethyl-p-pnenyl-enendiaminedihydrochloride (TMPD)

Table 16: Reagent for *E.coli*

Type of component	Component
Source of amino acids	Bacteriological Peptone (BP)
Source of carbohydrates	Yeast extract
Selective of amino acids	Tryptone
Salt	Sodium chloride (NaCl)
Buffer solution	KH_2PO_4
Buffer solution	K_2HPO_4
Indicator	N,N,N',N'-tetramethyl-p-pnenyl-enendiaminedihydrochloride (TMPD)

Table 17: Reagent for Total Viable Count

To carry out the analysis, 10 ml of sterile distilled water and 1 ml of the samples were added to a vial of one or another type, depending on the type of analysis to carry out. The vial was shaken until all the reagent was dissolved. Later on, the vial was incubated at 30°C for TVC or at 44°C for *E. coli*. The TVC detected and counted by MBS is defined as the quantity of aerobic microorganisms able to grow in a complete medium. The *E. coli* are defined, according to European Directive 91/492/CEE, as thermophilic coliforms that produce indole from tryptophan after incubation at 44°C±2°C for 24 hours. The starting colours are blue for TVC and reddish for *E. coli* and, in the presence of microorganisms, the colours change to yellow indicating a positive result. The time for the yellow colour development is inversely related to the bacterial content of the analyzed sample. The persistence of the starting colour after 24 hours indicates a negative result, i.e. absence of microorganisms. The *E.coli* confirmation test was carried out by adding a few drops of the Kovac's reagent (isoamyl alcohol, p-Dimethylaminobenzaldehyde, concentrated hydrochloric acid) after colour change occurred in *E. coli* MBS vials. The development of a red ring reveals the production of indole (MacFaddin, 1980).

5.2.1.4 Reference method ISO 9998

100µl of sample were plated on Tryptic Soy Agar (TSA; Sigma Chemical CO., St. Louis) for TVC and on Tryptone Bile X-Glucuronide Agar (TBX; Cultimed, Barcelona, Spagna) for *E. coli* (MacFaddin, 1980; Anonymous, 2001).

The TSA medium (Trypticase Soy Agar) is used for the growth of several species of microorganisms and usually used for the analysis of water, foods and surfaces, and was used instead of PCA because it is non-selective and of fast reading. This medium was used in the validation experiments relating to the TVC for presumptive counts, in order to have an indication on the dilutions to use. The medium in powder is dissolved (40 g/l) in deionised water, brought to a boil to complete dissolution and sterilized by autoclave for 20 minutes at 121°C. After it is cooled to a temperature of 44-47°C it can be distributed sterile Petri dishes in reason of 15-20 ml per plate. 1 ml of food homogenate is pour plated and incubated at the specific temperature required for the analysis, and results are obtained after 24 h.

Component	**Quantity (pH 7,3)**
Caasein Peptone	15 g/l
Soia Peptone	5 g/l
Sodium Chloride (NaCl)	5 g/l
Agar	15 g/l

Table 18: TSA medium composition

The TBX medium (Tryptone Bile Agar X-Gluc) is indicated by the reference standard ISO 16649-2 for determination and enumeration of *Escherichia coli*, especially in products for human consumption (foods and, in some cases, water) or feed. The TBX Agar is a selective and chromogenic agar; the selective action is due to the presence of bile salts, inhibitors of Gram positive bacteria, while the differential action is performed by the chromogenic substrate 5-bromo-4-chloro-3 indolyl-β -D-glucuronide (trade name X-Gluc) whose hydrolysis, due to the intracellular enzyme β-glucuronidase, brings to the formation of a blue-green pigment.

This compound is released by bacteria, and because insoluble in the agar, it accumulates near the colonies identifying them; the 4-methylumbelliferyl-β-Dglucuronide (or MUG), the differential agent previously used for TBX, tended instead to diffuse into the agar affecting the accuracy of results. *E. coli*, is one of the few species β-glucuronidase positive among Enterobacteriaceae, together with a few strains of Salmonella and Shigella (<1% of total), forming blue or blue-green colonies, while the β-glucuronidase negative enterobacteria form colourless colonies. There are however strains of *E. coli* that do not possess this enzyme, but they represent only the 3-3.5%;

unfortunately among these there is also the important pathogenic strain O157: H7, but its identification requires serological analysis or the toxin research.

Incubation occurs at 44°C after spread plating or pour plating, and colonies are counted after 18-24h. In case of a suspect presence of stressed microorganisms there is a need for pre-incubation at 37°C for 4 hours. The medium is suspended (36.6 g/l) in deionised water, brought to boiling to complete dissolution and sterilized in an autoclave for about 15 minutes at 121°C. After bringing the medium at a temperature of 44-47°C, it can be distributed in sterile Petri dishes in reason of 15-20 ml per plate. Results are obtained after 24 h and only blue colonies were considered positive. The TBX Agar is particularly "sensitive"; it should be stored at 2-8°C and it should not be exposed to direct sunlight. It is quite sensitive to high temperatures, which can deteriorate the chromogenic selective agent. During the preparation the medium should never be boiled more than once and after sterilization it has to be cooled in a water bath as quickly as possible. During incubation the temperature must be kept strictly at less than 46°C.

Component	**Quantity (pH 7,2)**
Casein peptone	20 g/l
Bile Salt	1.5 g/l
5-Bromo-4-chloro-3-indolyl-β-D-glucuronicacid (BCIG)	0,075 g/l
DymethilSuulfoxide (DSMO)	3 ml
Agar	15 g/l

Table 19: TBX medium composition

5.2.1.5 Statistical analysis

Statistical analysis was carried out according to ISO/TR 13843 (Anonymous, 2000). One-way and two-ways analysis of variance were performed to determine the general estimate of precision in the colorimetric method compared to the reference method of plate counting. The reliability was calculated using statistical analysis of Coefficient of Variation (CV). Uncertainty was calculated using the statistical analysis of chi-square test (χ^2).

5.2.2 Primary validation

The primary validation of MBS method for TVC and for *E. coli* on food samples was made according to ISO 16140 (Anonymous, 2003a).

5.2.2.1 Bacterial strains

All the strains used in this validation were available from ATCC (American Type Culture Collection). The strains used in this validation were *E. coli* (ATCC 25992), *E. coli* O157: H7 (ATCC 35150), *C. Freundii* (ATCC 43864), *K. Pneumoniae* (ATCC 13883), *E. cloacae* (ATCC 13047), *E. sakazaki* (ATCC 51329), *S. enteritidis* (ATCC 13076), and *S. typhimurium* (ATCC 14028), *Y. enterocolitica* (ATCC 19543), *B. cereus* (ATCC 11778), *B. stearothermophylus* (ATCC 24567),*B. subtilis* (ATCC 6633), *L. innocua* (ATCC 33090), *L. ivanoii* (ATCC 19119), *L. monocytogenes* (ATCC 7644), *S. aureus* (ATCC 12600), *S. epidermidis* (ATCC 12228), *S. lentus* (ATCC 29070), *P. aeruginosa* (ATCC 27853), *R. equi* (ATCC 31543),*E. faecalis* (ATCC 29212), *L. delbrueckii subsp. Lactis* (ATCC 12315), *C. perfringens* (ATCC 13124), *A. niger* (ATCC 9642) and *S. cerevisiae* (ATCC 9763).

5.2.2.2 Preparation of artificially contaminated samples

Sterile water samples were contaminated with a mixture of the above indicated microorganisms coming from overnight cultures with serial dilutions in sterile saline solution up to 10^{-8}.
Artificial contamination, executable according to the protocol described in Annex C of the standard, is usually used in validation protocols allowing a more precise control and a more rapid study, since the level of contamination is certain. It is still preferable to use naturally contaminated samples, since in this way, results will reflect more realistically the aim of the method; in this way it is also possible to assess the eventual interference of the food matrix on results.

5.2.2.3 Preparation of different levels of contamination of naturally contaminated food samples

Five different food matrices were selected for validation. They were: cheese, white meat, red meat, vegetables and fruit. These were naturally contaminated. Homogenized "baby food'' of the same typologies were used as a negative control, because surely not contaminated.

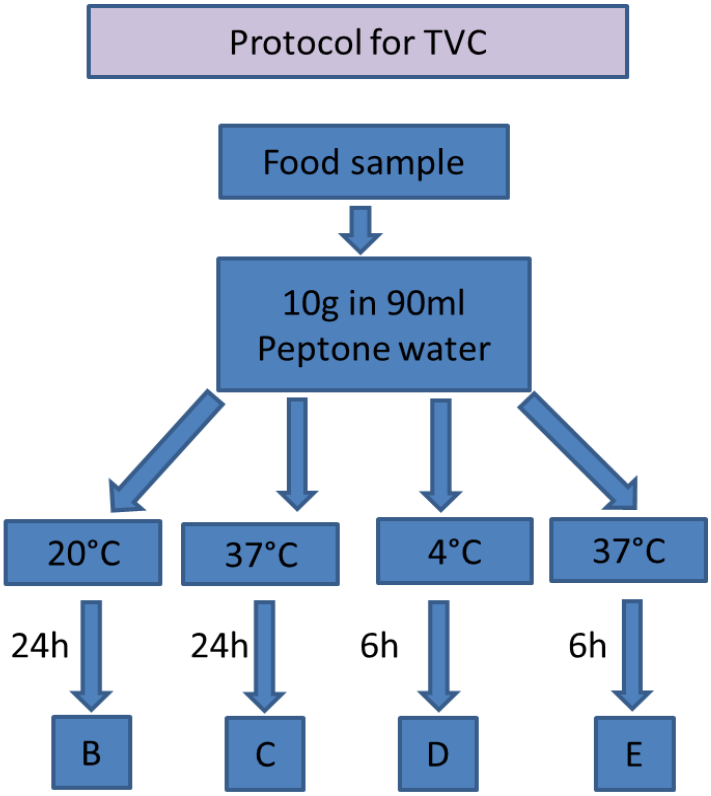
To use naturally contaminated samples we had to develop a specific procedure in order to achieve different levels of contamination of the same food matrices. Both for TVC and *E. coli* the lowest level of contamination, level of zero contamination, was obtained using samples of homogenized baby food"; the accurate sterilization procedures of this category of products were found to be ideal to verify the efficacy of the method in detecting the presence or absence of the analyte. Homogenate was first heated in a water bath at 50-60°C to make it more fluid and then, as required by the reference method and diluted 1:10 in peptone water. The sample made of 10g of homogenized dissolved in 90ml of peptone water, is indicated with the letter A and we will refer to it from now on with this abbreviation.

The food products chosen, collected in supermarkets and grocery shops, all showed the "basic" level of contamination expected, given the type of microorganisms sought and that all products were raw and unwashed. This was estimated by a presumptive count. Starting from this level four different levels of contamination were obtained by incubating the samples, after homogenization and dilution in peptone water, at different temperatures and for different periods of time (Table 20). The original sample was therefore divided into 4 samples and incubated at the conditions indicated in the table below: each level of contamination is identified with a letter and the different conditions of incubation are divided depending on the type of analysis (TVC or COLI):

Sample	**TVC**	***E.coli***
A	homogenate	homogenate
B	24h / 20°C	6h / 20°C
C	24h / 37°C	6h / 37°C
D	6h / 4°C	3h / 4°C
E	6h / 37°C	3h/37°C

Table 20: Temperatures and periods of incubation

For *E. coli* the time of incubation is shorter because previous experiments demonstrated that this microorganism reached stationary phase in less than 24 hours giving unsuitable results (Figure 36).



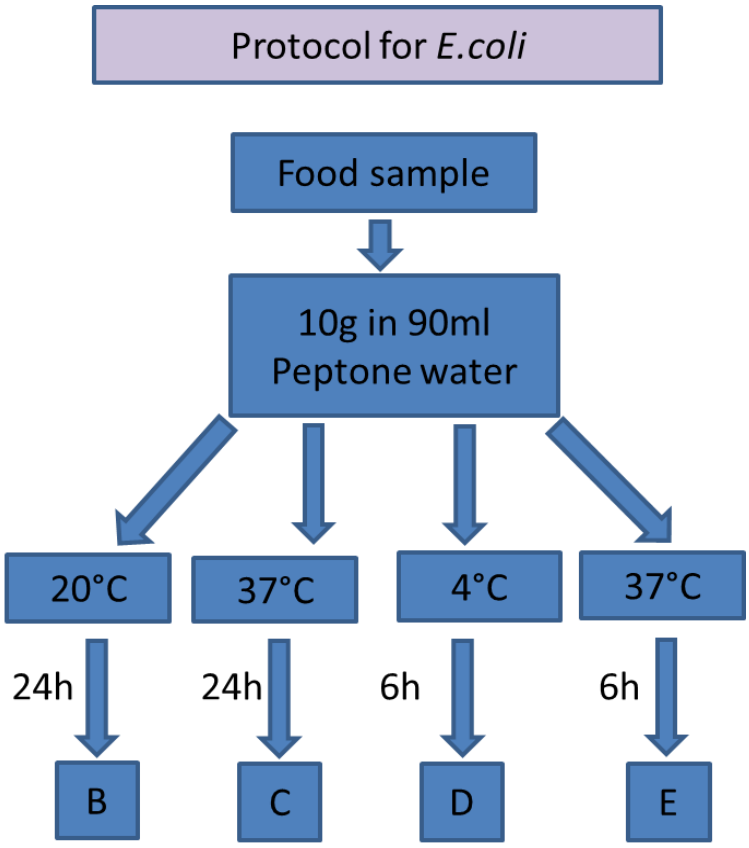


Figure 36: Protocols for the generation of different levels of contamination of naturally contaminated food

5.2.2.4 Analysis of 1 gram of non-homogenized food

The above-mentioned contamination levels were appropriately diluted, in order to obtain suitable results (i.e. from 15 and 300 CFU per plate). The different levels of contamination of naturally contaminated samples were obtained as follows: 10±0,5g of naturally contaminated samples were homogenized in 90ml of peptone water in a stomacher according to ISO 16140 (Anonymous, 2003a). To verify the equivalence between the MBS method and by the reference method, the samples were simultaneously tested.

For conditions D and E we also introduced to a variant, in order to verify another property of the MBS method: the ability to accurately detect bacterial concentration analyzing a whole piece of food, without previous treatment. Of 5 replicates scheduled, 3 were prepared as described above (homogenization in peptone water and further dilution) and 2 inserting directly into the MBS reaction vial 1 g of the sample. Both were incubated in the same conditions.

The diagram shows that the initial quantity of the sample was of 10 g. This is because during incubation, particularly in E2 at 37°C, most of the liquids contained in the sample evaporate causing a significant loss of mass; since some foods are very rich in liquids (e.g. mozzarella, pear), the incubation of 10g assures to have after incubation at least 1g of sample requested for the analysis according to the MBS method. As the piece of food could not be plated bacterial concentration was evaluated with the reference method, plating serial dilution of the sample. To obtain comparable results between the 5 samples and the two methods (reference method and MBS method) we proceeded this way: conditions D and E include 3 homogenized samples, indicated as D1 and E1 and 2 samples made of whole pieces, indicated as D2 and E2; the final protocol is shown in Figure 37.

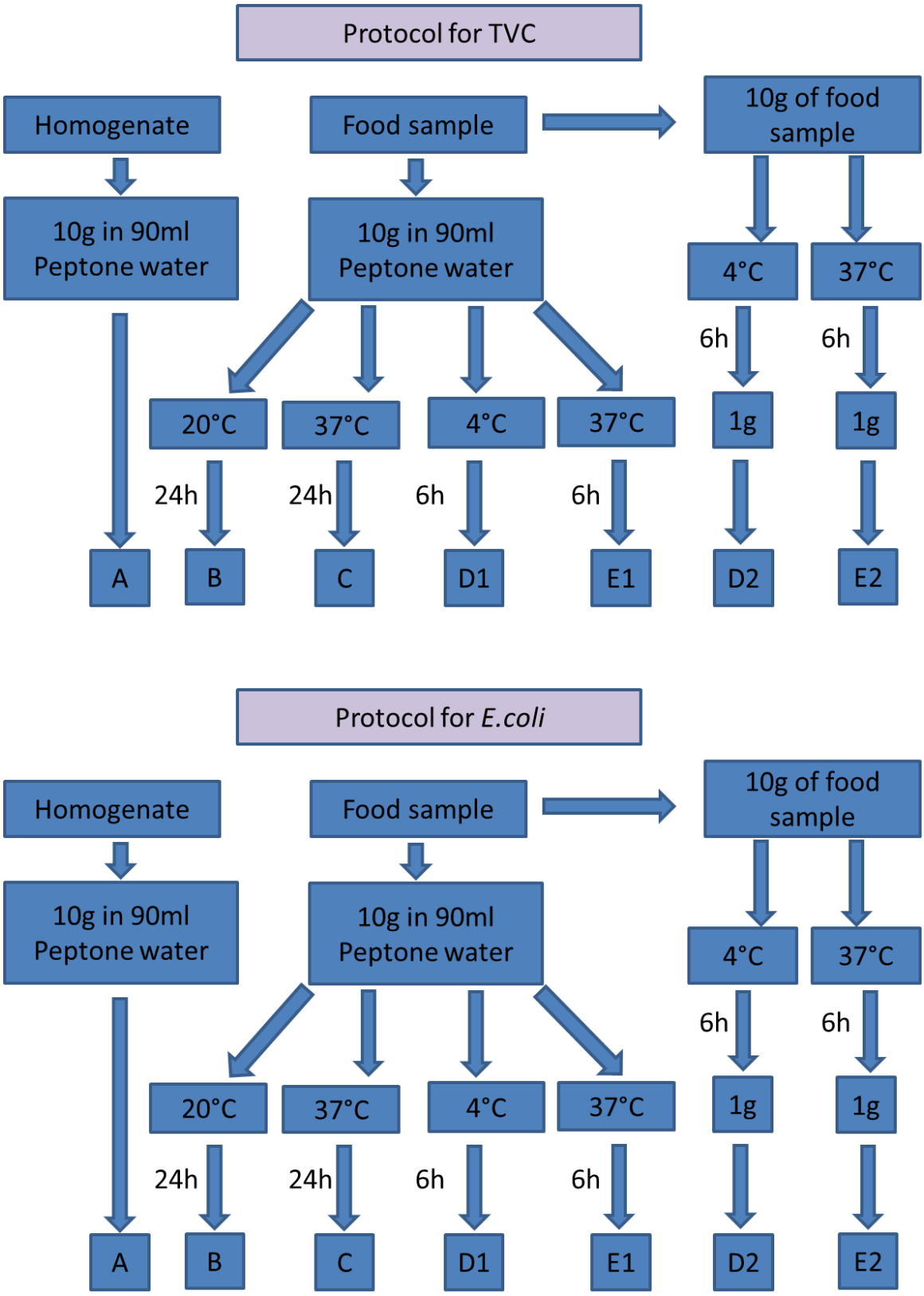


Figure 37: Protocols for the generation of different levels of contamination of naturally contaminated food, including whole pieces and negative control (level of zero contamination, sample A)

5.2.2.5 Colorimetric MBS method procedure

Analysis with the MBS method were carried out as previously described.

5.2.2.6 Reference method

For TVC the reference method was plate count on Plate Count Agar (PCA; Liofilchem, Roseto degli Abbruzzi, Italy) after 72 hours according to ISO 4833 (Anonymous, 2003b). For *E. coli* it was plate count on Tryptone Bile X-GLUC Agar (TBX; Cultimed, Barcelona, Spagna) after 24 hours according to ISO 16649-2 (Anonymous, 2001).

The PCA medium (Plate Count Agar) is indicated by the reference standard ISO 4833:2004 for the count of total viable cells growing aerobically at 30°C in food matrices intended for human consumption or in animals feed. PCA does not contain selective agents, so any mesophilic organism without special nutritional needs will grow. This type of analysis is in fact called TVC (Total Viable Count). In the analysis of dairy products it is optional (but recommended) to add 1.0 g/l of skimmed milk powder, that doesn't contain inhibitory substances. The medium powder is suspended (23.5 g/l) in deionised water, brought to boiling to complete dissolution and sterilized in an autoclave for 15-20 minutes at 121°C. It is then cooled to a temperature of 44-47°C and distributed in reason of 15-20ml in sterile Petri dishes. The samples are plated pour plate and colonies are counted after 48-72h at 30°C, paying particular attention to the little pieces of food that can be easily mistaken for colonies.

Component	**Quantitaty (pH 7,0)**
Casein peptone	5 g/l
Yeast extract	2,5 g/l
Glucose ($C_6H_{12}O_6$)	1,0 g/l
Agar	15 g/l

Table 21: PCA medium composition

5.2.2.7 Data analysis

Data analysis was carried out according to ISO 16140 (Anonymous, 2003). For the validation of MBS method 3 parameters were analyzed: linearity, accuracy and selectivity. The linearity of the method was assessed by analysing the correlation between bacterial concentrations (expressed as CFU/ml)and the time taken for the MBS vials to change colour. The accuracy was assessed by analysing the correlation between bacterial concentrations (expressed as log CFU/ml) obtained with the reference method and the alternative method MBS. Selectivity of the MBS method for *E. coli* was observed by comparing MBS method with the reference method on both artificially contaminated samples and naturally contaminated samples.

5.3 Results

5.3.1 Statistical analysis on artificially contaminated water samples

Sterile water was initially used to avoid any chemical interference due to organic matrices. In MBS colorimetric method the change of colour of the vials from blue, for TVC, and reddish, for *E. coli*, to yellow indicates a positive result that is the presence of microorganisms. The time occurred for MBS vials to change colour is inversely related to the bacterial concentration in the analysed samples. The water samples were artificially contaminated (see Materials and Methods). The statistical analysis for the MBS method on TVC and on *E. coli* vials was carried out according to ISO/TR 13843 (2000) using as reference method the plate counting method ISO 9998 (1991) on ten different dilutions of ten different samples. MBS reliable operating limits were comparable to the reference methods for plate counts at concentrations between $1x10^7$ and <10 CFU/ml. The results of the statistical analysis are shown on Table 1 and are expressed in terms of:

- Estimate of Precision;
- Coefficient of Variance
- Uncertainty

General estimate of precision was made according to ISO/TR 13843 (2000) using Analysis of Variance (ANOVA) tests. Results obtained by both one-way and two-way analysis of variance have shown that there were no statistical differences on bacterial count between the results obtained with MBS method and the results obtained with the reference method. Reliability of the bacterial count using MBS method was also assessed by statistical analysis using Coefficient of

Variation (CV) analysis according to ISO/TR 13843 (2000). It appeared that the MBS method was more reliable than the reference method. Likewise, the uncertainty of the bacterial count using MBS method was less than that of the reference method as determinated by χ^2 statistical test according to ISO/TR 13843 (2000) (Table 22).

	TVC	***E. coli***
ESTIMATE OF PRECISION		
One-way analysis of variance DF 1,30 (limit1% = 4.17)	F = 1,13	F = 0,2615
Two-way analysis of variance DF 7,28 (limit1% = 4.17)	F = 1,8576	F = 1,16
COEFFICIENT OF VARIANCE (CV)		
Plate count	0,1815	0,4533
MBS	0,0295	0,0628
UNCERTAINTY (χ^2)		
Plate count DF 9 (limit 0,5% = 4,17)	$\chi^2 < 0,25$	$\chi^2 < 1,2$
MBS DF 9 (limit 0,5% = 4,17)	$\chi^2 < 0,22$	$\chi^2 < 0,30$

F = ANOVA F-test, CV = Coefficient of variation, DF = Degrees of freedom, χ^2 = Chi-square test

Table 22: Statistical analysis carried out on the MBS method on TVC and *E. coli* according to the ISO/TR 13843:2000

5.3.2 Primary validation

The primary validation of the MBS method for TVC and for *E. coli* was made according to ISO 16140 (2003). The following tables are showed the results obtained from the different experiments.

The main performance parameters that the alternative method must demonstrate are: linearity, accuracy and selectivity.

The tables shown the results obtained for the different food matrices at different conditions (see table 20)

The results obtained, indicated the concordance between results obtained with MBS method and reference method to detect TVC and *E. coli*.

Results for Total Viable Cells

Red Meat

Reference Method (Plate Count on PCA, after 72 h, expressed as CFU/ml)

	A	B	C	D1	E1
1	0	$3{,}0 \times 10^3$	$7{,}1 \times 10^3$	$1{,}01 \times 10^2$	$4{,}5 \times 10^2$
2	0	$6{,}0 \times 10^3$	$7{,}84 \times 10^3$	$8{,}9 \times 10^2$	$3{,}9 \times 10^2$
3	0	$5{,}0 \times 10^3$	$8{,}01 \times 10^3$	$1{,}06 \times 10^2$	$4{,}2 \times 10^2$
4	0	$6{,}0 \times 10^3$	$7{,}39 \times 10^3$	$1{,}01 \times 10^2$	$4{,}0 \times 10^2$
5	0	$2{,}0 \times 10^3$	$7{,}51 \times 10^3$	$1{,}12 \times 10^2$	$3{,}6 \times 10^2$
MEAN	0	$4{,}4 \times 10^3$	$7{,}57 \times 10^3$	$1{,}02 \times 10^2$	$4{,}04 \times 10^2$

Calculated values of 1 gram of non-homogenized food (expressed as CFU/g): D2=$1{,}02 \times 10^4$ E2=$4{,}04 \times 10^6$

Alternative Method (MBS vial at 30°C)

	A	B	C	D1	D2	E1	E2
1	no color change after 24 h	11 h	10 h	16 h	not performed	15,5 h	not performed
2	no color change after 24 h	11 h	9,5-13,5 h	16 h	not performed	15,5 h	not performed
3	no color change after 24 h	11 h	9,5-13,5 h	16 h	not performed	15,5 h	not performed
4	no color change after 24 h	11 h	9,5-13,5 h	not performed	4,5 h	not performed	7 h
5	no color change after 24 h	11 h	9,5-13,5 h	not performed	4,5 h	not performed	7 h

Cheese

Reference Method (Plate Count on PCA, after 72 h, expressed as CFU/ml)

	A	B	C	D1	E1
1	0	$7,2x10^3$	$2,15\ x10^4$	1,55 x10	$2,3\ x10^3$
2	0	$7,79\ x10^3$	$2,29\ x10^4$	1,59 x10	$2,29\ x10^3$
3	0	$8,02\ x10^3$	$1,98\ x10^4$	1,72 x10	$2,66\ x10^3$
4	0	$7,19\ x10^3$	$2,1\ x10^4$	1,5 x10	$2,55\ x10^3$
5	0	$7,6\ x10^3$	$2,2\ x10^4$	1,66 x10	$2,12\ x10^3$
MEAN	0	$7,56\ x10^3$	$2,14\ x10^4$	1,6 x10	$2,38\ x10^3$

Calculated values of 1 gram of non-homogenized food (expressed as CFU/g): D2=$1,6x10^4$ E2=$2,38x10^6$

Alternative Method (MBS vial at 30°C)

	A	B	C	D1	D2	E1	E2
1	no color change after 24 h	9,5 h	8 h	16 h	not performed	10,5 h	not performed
2	no color change after 24 h	9,5 h	8 h	16 h	not performed	9,5–13,5 h	not performed
3	no color change after 24 h	9,5 h	8 h	16 h	not performed	9,5–13,5 h	not performed
4	no color change after 24 h	9,5 h	8 h	not performed	7,5 h	not performed	3 h
5	no color change after 24 h	9,5 h	8 h	not performed	7,5 h	not performed	3 h

Fruit

Reference Method (Plate Count on PCA, after 72 h, expressed as CFU/ml)

	A	B	C	D1	E1
1	0	$8,84x10^3$	$4,56x10^4$	$9,0x10^2$	$1,48x10^4$
2	0	$8,51x10^3$	$4,86x10^4$	$1,12x10^3$	$1,35x10^4$
3	0	$8,6x10^3$	$4,75x10^4$	$1,03x10^3$	$1,42x10^4$
4	0	$9,23x10^3$	$4,88x10^4$	$9,6x10^2$	$1,35x10^4$
5	0	$9,4x10^3$	$4,61x10^4$	$9,2x10^2$	$1,5x10^4$
MEAN	0	$8,92x10^3$	$4,73x10^4$	$9,9x10^2$	$1,42x10^4$

Calculated values of 1 gram of non-homogenized food (expressed as CFU/g): D2=$9,9x10^5$ E2=$1,42x10^7$

Alternative Method (MBS vial at 30°C)

	A	B	C	D1	D2	E1	E2
1	no color change after 24 h	10 h	7 h	14 h	not performed	9 h	not performed
2	no color change after 24 h	10 h	7 h	14 h	not performed	9 h	not performed
3	no color change after 24 h	10 h	7 h	14 h	not performed	9 h	not performed
4	no color change after 24 h	10 h	7 h	not performed	6 h	not performed	3 h
5	no color change after 24 h	10 h	7 h	not performed	6 h	not performed	3 h

White meat

Reference Method (Plate Count on PCA, after 72 h, expressed as CFU/ml)

	A	B	C	D1	E1
1	0	$2,1x10^3$	$7,55\ x10^3$	5,0 x10	$1,3\ x10^2$
2	0	$2,19\ x10^3$	$7,09\ x10^3$	2,0 x10	$1,29\ x10^2$
3	0	$1,99\ x10^3$	$7,32\ x10^3$	2,0 x10	$1,46\ x10^2$
4	0	$2,1\ x10^3$	$6,9\ x10^3$	3,0 x10	$1,55\ x10^2$
5	0	$2,05\ x10^3$	$6,96\ x10^3$	2,0 x10	$1,12\ x10^2$
MEAN	0	$2,09\ x10^3$	$7,16\ x10^3$	2,8 x10	$1,34\ x10^2$

Calculated values of 1 gram of non-homogenized food (expressed as CFU/g): D2=$2,8x10^4$ E2=$1,34x10^6$

Alternative Method (MBS vial at 30°C)

	A	B	C	D1	D2	E1	E2
1	no color change after 24 h	13 h	10,5 h	18 h	not performed	15,5 h	not performed
2	no color change after 24 h	13 h	9,5-13,5 h	18 h	not performed	15,5 h	not performed
3	no color change after 24 h	13 h	9,5-13,5 h	18 h	not performed	15,5 h	not performed
4	no color change after 24 h	13 h	10,5 h	not performed	6 h	not performed	3 h
5	no color change after 24 h	13 h	9,5-13,5 h	not performed	6 h	not performed	3 h

Vegetable

Reference Method (Plate Count on PCA, after 72 h, expressed as CFU/ml)

	A	B	C	D1	E1
1	0	$3,3x10^3$	$4,25\ x10^4$	5,5 x10	$1,65\ x10^2$
2	0	$3,71\ x10^3$	$4,32\ x10^4$	4,6 x10	$1,36\ x10^2$
3	0	$3,51\ x10^3$	$4,45\ x10^4$	6,0 x10	$1,79\ x10^2$
4	0	$3,66\ x10^3$	$4,29\ x10^4$	5,2 x10	$1,64\ x10^2$
5	0	$3,39\ x10^3$	$4,44\ x10^4$	4,0 x10	$1,75\ x10^2$
MEAN	0	$3,51\ x10^3$	$4,35\ x10^4$	5,1 x10	$1,64\ x10^2$

Calculated values of 1 gram of non-homogenized food (expressed as CFU/g): D2=$5,1x10^2$ E2=$1,64x10^5$

Alternative Method (MBS vial at 30°C)

	A	B	C	D1	D2	E1	E2
1	no color change after 24 h	11 h	7 h	16,5 h	not performed	14 h	not performed
2	no color change after 24 h	11 h	7 h	16,5 h	not performed	14 h	not performed
3	no color change after 24 h	11 h	7 h	16,5 h	not performed	14 h	not performed
4	no color change after 24 h	11 h	7 h	not performed	13 h	not performed	5 h
5	no color change after 24 h	11 h	7 h	not performed	13 h	not performed	5 h

RESULTS FOR *E.coli*

Red Meat

Reference Method (Plate Count on TBX, after 48 h, expressed as CFU/ml)

	A	B	C	D1	E1
1	0	$8,83 \times 10^3$	$2,35 \times 10^4$	2,00 x10	$2,11 \times 10^3$
2	0	$8,97 \times 10^3$	$2,26 \times 10^4$	3,00 x10	$2,29 \times 10^3$
3	0	$9,25 \times 10^3$	$2,15 \times 10^4$	3,00 x10	$1,99 \times 10^3$
4	0	$9,01 \times 10^3$	$2,21 \times 10^4$	2,10 x10	$2,06 \times 10^3$
5	0	$9,00 \times 10^3$	$2,10 \times 10^4$	2,70 x10	$2,10 \times 10^3$
MEAN	0	$9,01 \times 10^3$	$2,21 \times 10^4$	2,70 x10	$2,11 \times 10^3$

The D2 and F2 pieces are not present in this test because the change of colour occurred only around the pieces enabling the right interpretation of the results.

Alternative Method (MBS vial at 44°C)

	A	B	C	D	E
1	no colour change after 30h	9,5 h	7 h	20,5 h	13,5 h
2	no colour change after 30h	9,5 h	7 h	20,5 h	13,5 h
3	no colour change after 30h	9,5 h	7 h	20,5 h	13,5 h
4	no colour change after 30h	8,5 h	6,5 h	9,5-21,5 h	9,5-13,5 h
5	no colour change after 30h	9,5-21,5 h	7,5 h	9,5-21,5 h	9,5-21,5 h

Cheese

Reference Method (Plate Count on TBX, after 48 h, expressed as CFU/ml)

	A	B	C	D1	E1
1	0	$8{,}93 \times 10^3$	$1{,}65 \times 10^4$	$3{,}80 \times 10^3$	$1{,}02 \times 10^4$
2	0	$9{,}32 \times 10^3$	$1{,}56 \times 10^4$	$3{,}95 \times 10^3$	$1{,}03 \times 10^4$
3	0	$9{,}15 \times 10^3$	$1{,}61 \times 10^4$	$4{,}32 \times 10^3$	$9{,}26 \times 10^3$
4	0	$8{,}46 \times 10^3$	$1{,}60 \times 10^4$	$4{,}29 \times 10^3$	$8{,}95 \times 10^3$
5	0	$7{,}99 \times 10^3$	$1{,}42 \times 10^4$	$4{,}81 \times 10^3$	$1{,}02 \times 10^4$
MEAN	0	$8{,}79 \times 10^3$	$1{,}57 \times 10^4$	$4{,}23 \times 10^3$	$9{,}81 \times 10^3$

Calculated values of 1 gram of non-homogenized food (expressed as CFU/g): D2=$4{,}23 \times 10^4$ E2=$9{,}81 \times 10^4$

Alternative Method (MBS vial at 44°C)

	A	B	C	D1	D2	E1	E2
1	no colour change after 30 h	9,5 h	8,5 h	10-12 h	not performed	9 h	not performed
2	no colour change after 30 h	9,5 h	8,5 h	10-20 h	not performed	9 h	not performed
3	no colour change after 30 h	9,5 h	8,5 h	10-20 h	not performed	9 h	not performed
4	no colour change after 30 h	8,5 h	8,5 h	not performed	6,5 h	not performed	4,5 h
5	no colour change after 30 h	9,5 h	8,5 h	not performed	6,5 h	not performed	4,5 h

Fruit

Reference Method (Plate Count on TBX, after 48 h, expressed as CFU/ml)

	A	B	C	D1	E1
1	0	$1{,}08 \times 10^4$	$2{,}25 \times 10^4$	$2{,}90 \times 10^2$	$3{,}30 \times 10^2$
2	0	$1{,}13 \times 10^4$	$2{,}27 \times 10^4$	$3{,}00 \times 10^2$	$2{,}50 \times 10^2$
3	0	$9{,}95 \times 10^3$	$2{,}61 \times 10^4$	$1{,}90 \times 10^2$	$3{,}20 \times 10^2$
4	0	$1{,}04 \times 10^4$	$2{,}50 \times 10^4$	$1{,}80 \times 10^2$	$2{,}90 \times 10^2$
5	0	$1{,}04 \times 10^4$	$2{,}40 \times 10^4$	$2{,}40 \times 10^2$	$3{,}10 \times 10^2$
MEAN	0	$1{,}06 \times 10^4$	$2{,}41 \times 10^4$	$2{,}39 \times 10^2$	$3{,}00 \times 10^2$

Calculated values of 1 gram of non-homogenized food (expressed as CFU/g): D2=$2{,}39 \times 10^4$ E2=$3{,}0 \times 10^6$

Alternative Method (MBS vial at 44°C)

	A	B	C	D1	D2	E1	E2
1	no colour change after 30 h	9 h	7,5 h	16,5 h	not performed	16 h	not performed
2	no colour change after 30 h	9 h	7,5 h	9,5-22 h	not performed	9,5-22 h	not performed
3	no colour change after 30 h	9 h	7,5 h	9,5-22 h	not performed	9,5-22 h	not performed
4	no colour change after 30 h	9 h	7,5 h	not performed	7 h	not performed	3 h
5	no colour change after 30 h	9,5-21,5 h	7,5 h	not performed	7 h	not performed	3 h

White meat

Reference Method (Plate Count on TBX, after 48 h, expressed as CFU/ml)

	A	B	C	D1	E1
1	0	$5,77x10^3$	$9,6\ x10^3$	$3,3\ x10^2$	$1,22\ x10^3$
2	0	$6,01x10^3$	$1,12\ x10^4$	$3,9\ x10^2$	$9,6\ x10^2$
3	0	$5,63x10^3$	$1,03\ x10^4$	$4,2\ x10^2$	$1,11\ x10^3$
4	0	$6,29\ x10^3$	$9,7\ x10^3$	$3,8\ x10^2$	$1,06\ x10^3$
5	0	$6,02\ x10^3$	$1,29\ x10^4$	$3,0\ x10^2$	$9,2\ x10^2$
MEAN	0	$5,94\ x10^3$	$1,07\ x10^4$	$3,6\ x10^2$	$1,05\ x10^3$

Calculated values of 1 gram of non-homogenized food (expressed as CFU/g): D2=$3,6x10^3$ E2=$1,05x10^4$

Alternative Method (MBS vial at 44°C)

	A	B	C	D1	D2	E1	E2
1	no colour change after 30 h	10 h	9 h	16 h	not performed	13,5 h	not performed
2	no colour change after 30 h	10 h	9 h	10-20 h	not performed	10-20 h	not performed
3	no colour change after 30 h	10 h	9 h	10-20 h	not performed	10-20 h	not performed
4	no colour change after 30 h	10 h	9 h	not performed	9 h	not performed	7 h
5	no colour change after 30 h	10 h	9 h	not performed	9 h	not performed	7 h

Vegetable

Reference Method (Plate Count on TBX, after 48 h, expressed as CFU/ml)

	A	B	C	D1	E1
1	0	$2,89 \times 10^4$	$6,65 \times 10^4$	$9,0 \times 10^2$	$4,52 \times 10^3$
2	0	$3,12 \times 10^4$	$6,78 \times 10^4$	$9,1 \times 10^2$	$4,7 \times 10^3$
3	0	$3,09 \times 10^4$	$6,95 \times 10^4$	$8,4 \times 10^2$	$5,01 \times 10^3$
4	0	$3,0 \times 10^4$	$6,82 \times 10^4$	$8,0 \times 10^2$	$4,5 \times 10^3$
5	0	$2,86 \times 10^4$	$6,78 \times 10^4$	$8,5 \times 10^2$	$4,77 \times 10^3$
MEAN	0	$2,99 \times 10^4$	$6,80 \times 10^4$	$8,6 \times 10^2$	$4,7 \times 10^3$

Calculated values of 1 gram of non-homogenized food (expressed as CFU/g): D2=$8,6 \times 10^3$ E2=$4,7 \times 10^4$

Alternative Method (MBS vial at 44°C)

	A	B	C	D1	D2	E1	E2
1	no colour change after 30 h	7 h	5,5 h	14 h	not performed	9,5-13 h	not performed
2	no colour change after 30 h	7 h	5,5 h	9,5-22 h	not performed	9,5-13 h	not performed
3	no colour change after 30 h	7 h	5,5 h	9,5-22 h	not performed	9,5-13 h	not performed
4	no colour change after 30 h	7 h	5,5 h	not performed	6,5 h	not performed	5 h
5	no colour change after 30 h	7 h	5,5 h	not performed	6,5 h	not performed	5 h

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. An increase in analyte should correspond to a linear or proportional increase in results (ISO 16140 2003).

This was achieved graphically as illustrated in Figure 38 (for Total Viable Count) and in Figure 39 (for *E. coli*), by plotting bacterial concentrations (expressed as the log of CFU/ml) obtained with the reference method with the time occurred for taken to change colour with the identical samples analyzed with MBS method.

A linear inverse relationship between the MBS method and the bacteria concentration, with a correlation factor (R^2) close to 1.00 confirming the linearity of the data can be observed.

Using naturally and artificially contaminated food samples, bacterial concentrations (expressed as the log of CFU/ml) obtained with the reference method are plotted against the time taken to change colour for the identical samples analyzed with MBS method with Total Viable Count vials and *E. coli* vials on five different food matrices: red meat, cheese, vegetable, fruit and white meat.

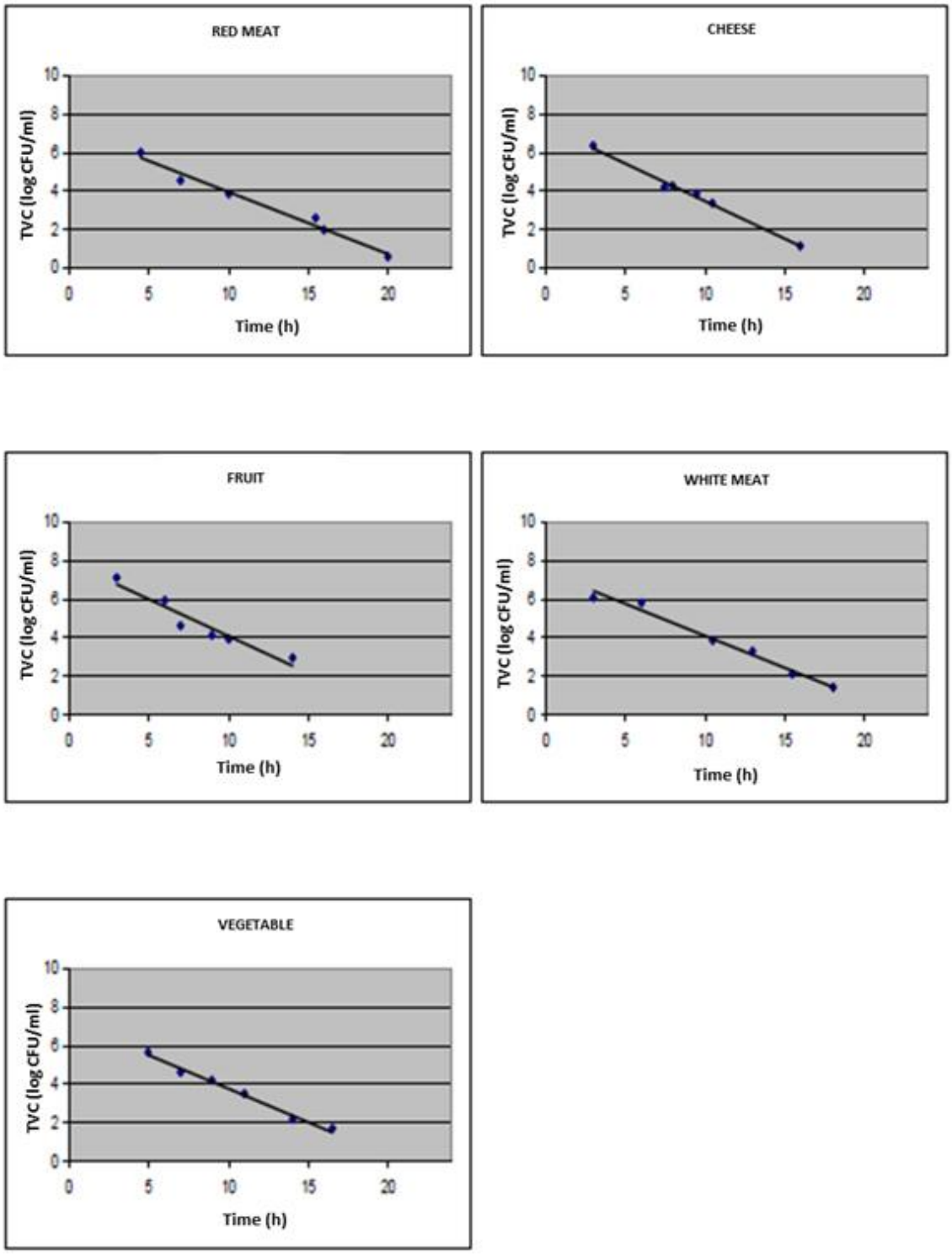


Figure 38: Correlation curve between the MBS method and the reference method for TVC in all 5 food matrices at 37°C

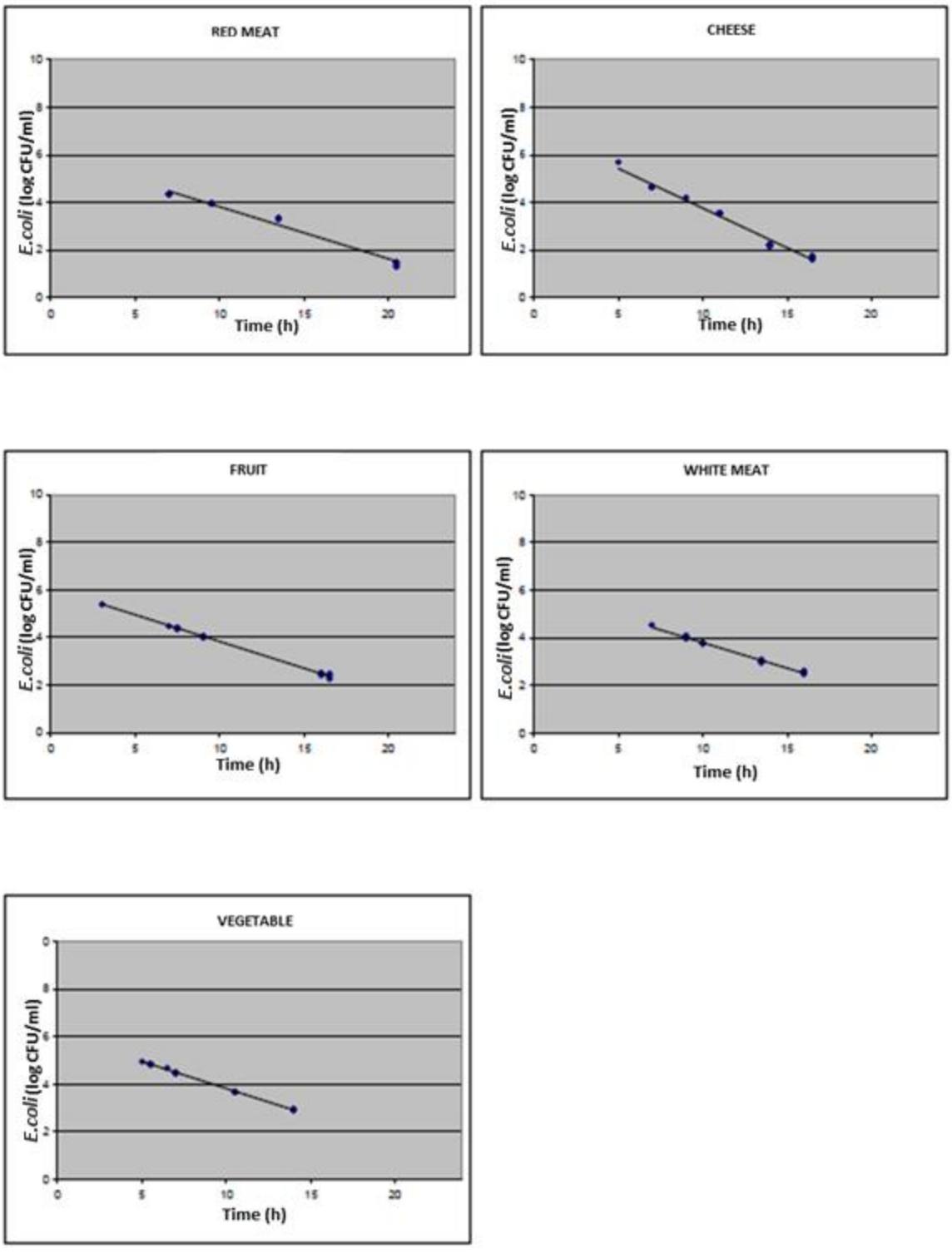


Figure 39: Correlation curve between the MBS method and the reference method for *E. coli* in all 5 food matrices at 44°C

The linearity is confirmed calculating the equations of the curves, the relative coefficients and its correlation factor (R2). The R2 is close to 1.00 for all different food matrices.

We can identify two different global curves of correlation (TVC and *E. coli*).The tables here reported (23 A and B) display these equations and correlation factors that take account of all data obtained for the different food samples.

This values are used to study the accuracy of MBS method compared to the reference method.

A)

Food Sample - TVC		**R^2**
Red Meat	**y = -0,3205x +7,192**	**0,9804**
Cheese	**y = -0,3904x +7,192**	**0,9907**
Fruit	**y = -0,3844x +7,959**	**0,9199**
White meat	**y = -0,3315x +7,435**	**0,9201**
Vegetable	**y = -0,3471x +7,286**	**0,9199**
Global line TVC	**y = -0,3181x +7,027**	**0,9526**

B)

Food Sample – *E.coli*		**R^2**
Red Meat	**y = -0,2193x +6,025**	**0,9783**
Cheese	**y = -0,3323x +7,074**	**0,9839**
Fruit	**y =-0,2228x +6,042**	**0,9973**
White meat	**y = -0,2135x +5,939**	**0,9923**
Vegetable	**y = -0,2244x +6,058**	**0,9986**
Global line *E.coli*	**y = -0,2254x +6,072**	**0,9923**

Table 23: Equations and correlation factors for different food samples and global equations for TVC (A) and *E. coli* (B)

The figures below show the correlation between the time taken to change colour for the MBS vials and the bacterial concentrations kin the samples used to obtain the global line for TVC (Figure 40 A) and *E. coli* (Figure 40 B).

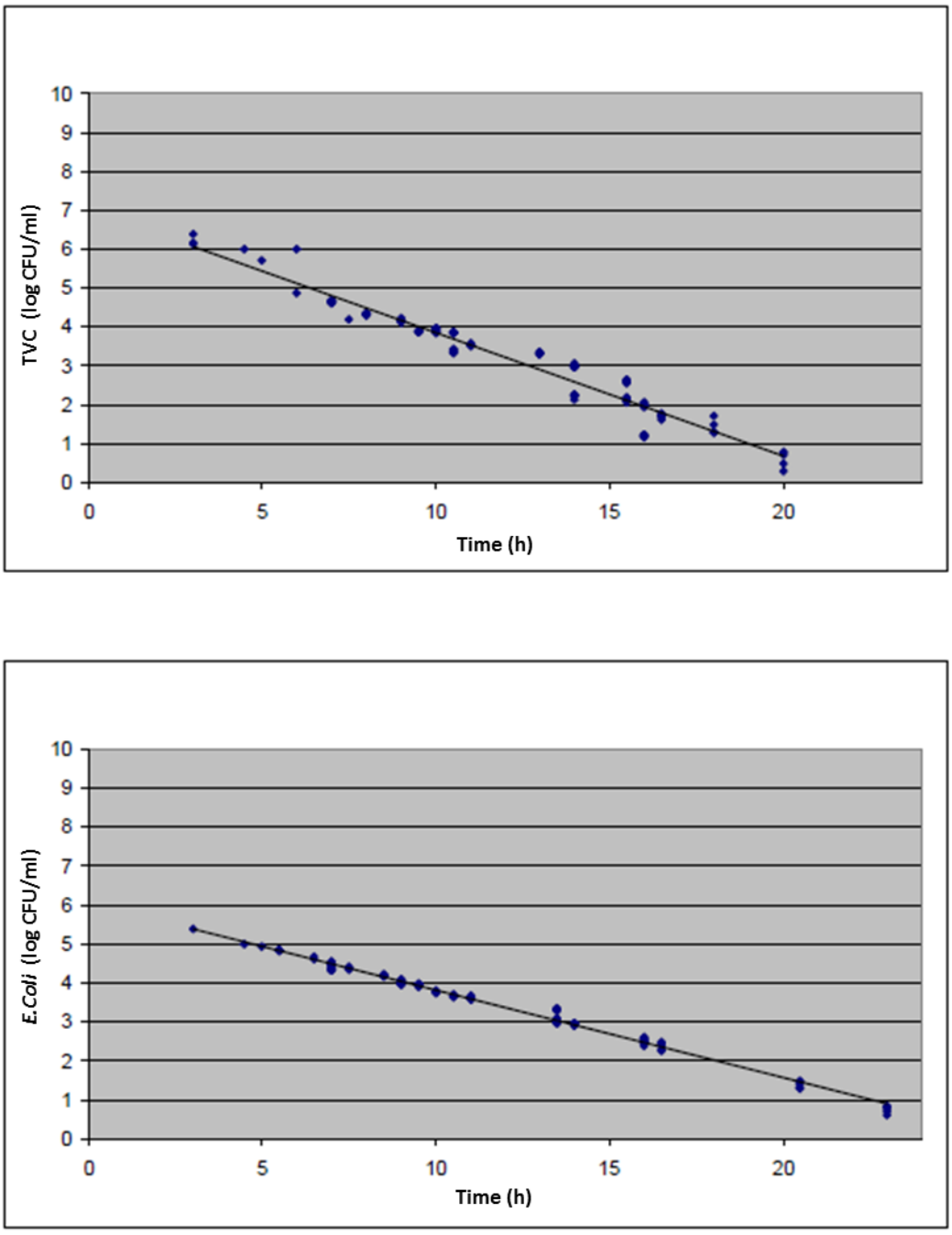
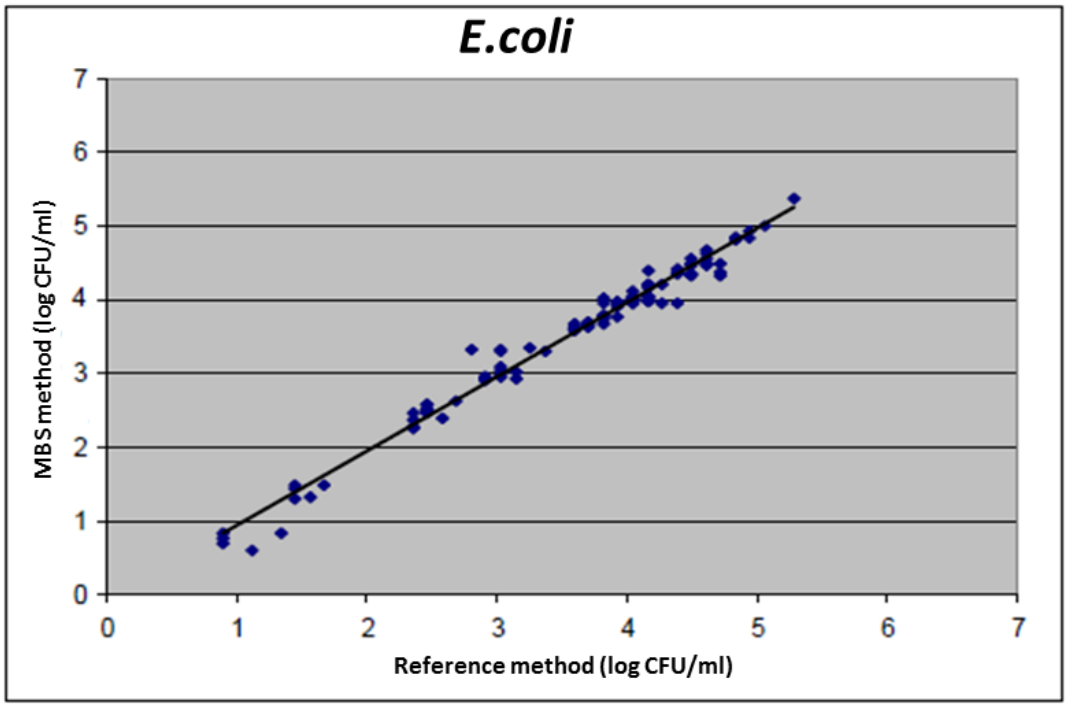


Figure 40: Global correlation lines between analytes (TVC and *E.coli*) and time taken for MBS vials

As showed in figure there is a linear inverse relationship between the time taken for MBS vials to changeand bacterial concentration calculated with the reference method, with a correlation factor (R^2) close to 1.00 (i.e. 0.95 for TVC and 0.99 for *E. coli*).

Accuracy is the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples. The term “relative accuracy” used here is complementary to the “accuracy” and “trueness” as defined in ISO 5725-1:1994/COR 1 (1998). This states that accuracy is “the closeness of agreement between a test result and the accepted reference value”, and that trueness is "the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value". For the purpose of this standard, the accepted reference values are chosen as the values obtained by the reference method. Thus, the term "relative" implies that the reference method does not automatically provide the accepted reference value as indicated by ISO 16140 (2003). In Figure 41 the bacteria concentrations (expressed as log CFU/ml) obtained with the reference counting methods for TVC (Figure 41 A) and *E. coli* (Figure 41 B) are plotted against the bacteria numbers (expressed as log CFU/ml) obtained with the alternative MBS method for TVC and *E. coli*. The straight lines in both graphs show a perfect correlation between the reference methods and the MBS methods. In fact the slopes are close to theoretical value of 1.00 (i.e. 1.00 for TVC and 0.99 for *E. coli*).



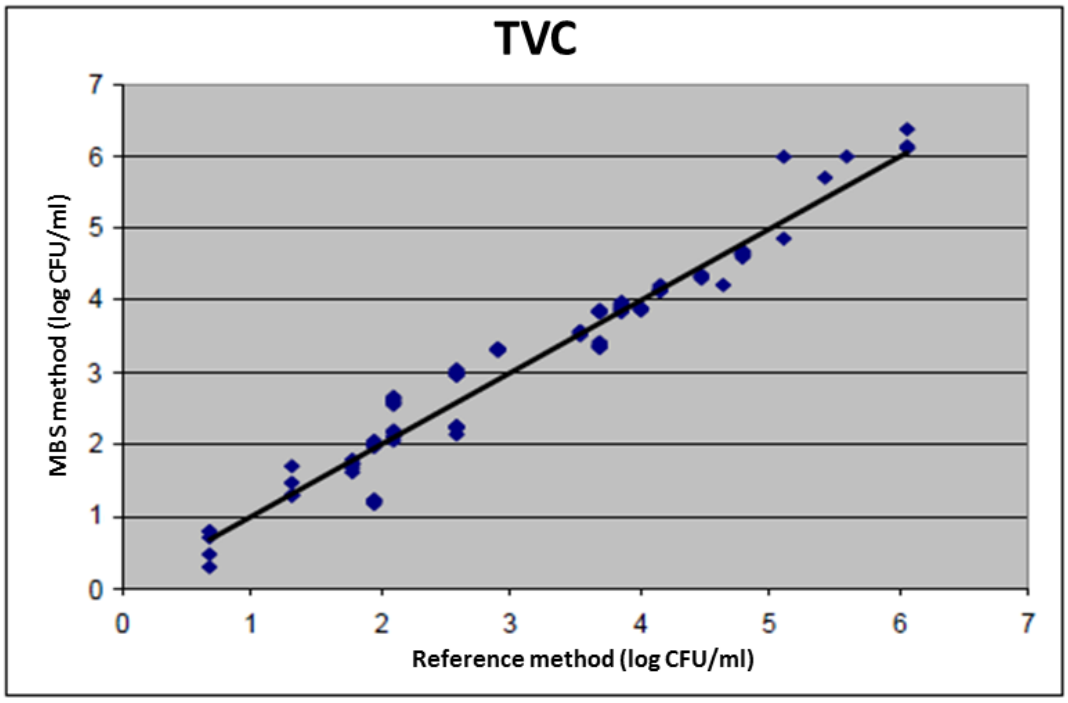


Figure 41: Correlation curve between the MBS method and the reference method for TVC and *E.coli*

A good correlation between bacterial concentrations (expressed as log CFU/ml) obtained with the traditional counting method and with the alternative MBS method. The correlation factor (R^2) is close to 1.00 (i.e. 0.95 for TVC and 0.99 for *E. coli*).

Selectivity is the ability of an alternative method to detect the target analyte from a wide range of strains and is the lack of interference from a relevant range of non-target strains of the alternative method as indicated by ISO 16140 (2003). Figure 42 illustrates selectivity of the MBS method for *E. coli*. The figure shows the vials for *E. coli* inoculated with five different bacteria strains 24 hours after inoculation. Only the vial inoculated with *E. coli* shows a change of the colour from red to yellow.

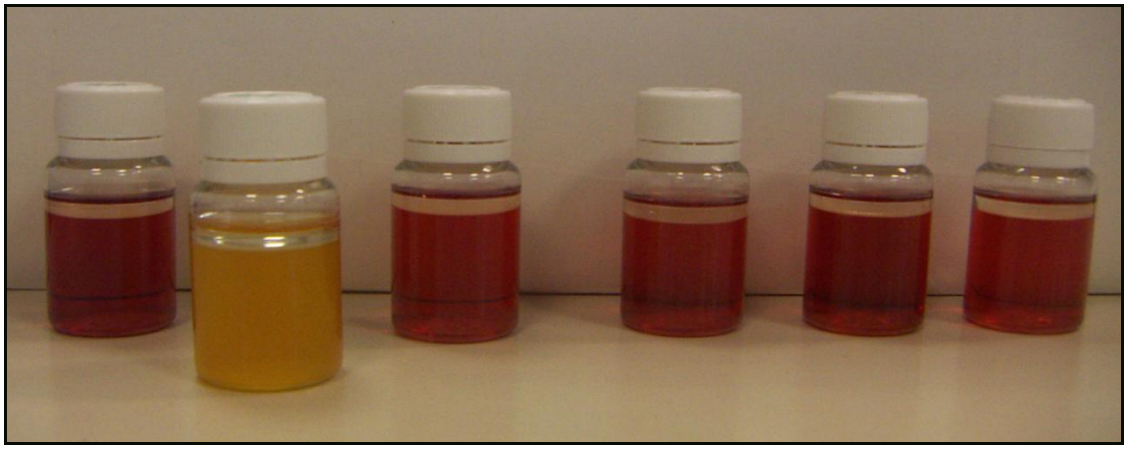


Figure 42: Selectivity of the MBS method for *E. coli*

The selectivity has been observed by testing samples artificially contaminated with different bacterial strains. The figure shows the vials for *E. coli* inoculated with five different bacteria after 24 hours after inoculation. Only the vial inoculated with *E. coli* shows a change of colour from red to yellow.

Table 24 shows the lowest detection limit (expressed as CFU/ml) of MBS vials for TVC and for *E. coli* towards different bacterial strains in artificially contaminated samples. The lowest detection limit represents the minimal bacterial concentration required to induce the change of colour in either MBS vials for TVC or for *E. coli*. The lowest detection limit is utilized to assess the selectivity of the MBS method.

Very high concentrations of all bacteria other than *E. coli* were necessary to induce the change of colour (i.e. a positive result of the test) of *E. coli* vials, instead only one *E. coli* cell (on average) was sufficient. These results indicate that *E. coli* vials are selective for *E. coli*, although they showed a lower level of selectivity towards other coliforms strains, a medium level of selectivity towards Enterobacteriaceae and a higher level of selectivity towards Gram-positive bacteria.

For TVC vials, instead, only one cell (on average) of all aerobic bacteria strains tested was sufficient to induce change of colour of TVC vials, indicating a very low selectivity.

Bacteria strains	Reference Id.	Lowest detection limits for *E. coli* reagent (cfu/ml)	Lowest detection limits for TVC reagent (cfu/ml)
Enterobacter cloacae	ATCC 13047	$> 10^5$	1
Enterobactersakazakii	ATCC 31329	$> 10^5$	1
Pseudomonas aeruginosa	ATCC 27853	$> 10^6$	1
Salmonella enteritidis	ATCC 13076	$> 10^6$	1
*Salmonella .enterica*serovar*typhimurium*	ATCC 14028	$> 10^4$	1
Yersinia enterocolitica	ATCC 19543	$> 10^6$	1
Citrobacterfreundii	ATCC 43864	$> 10^3$	1
Klebsiellapneumoniae	ATCC13883	$> 10^3$	1
Eschierica coli	ATCC 25922	1	1
Eschierica coli O157:H7	ATCC 35150	1	1
Enterococcus. faecalis	ATCC 29212	$> 10^6$	1
Bacillus cereus	ATCC 11778	$> 10^6$	1
Bacillus. stearothermophylus	ATCC 24567	$> 10^6$	1
Bacillus subtilis	ATCC 6633	$> 10^6$	1
Listeria innocua	ATCC 33090	$> 10^6$	1
Listeria ivanovii	ATCC 19119	$> 10^6$	1
Listeria monocytogenes	ATCC 7644	$> 10^6$	1
Rhodococcusequi	ATCC 31543	$> 10^6$	1
Sthaphylococcusaureus	ATCC 12600	$> 10^6$	1
Sthaphylococcusepidermidis	ATCC 12228	$> 10^6$	1
Staphylococcus lentus	ATCC 29070	$> 10^6$	1
Lactobaccilusdelbrueckii subsp. lactis	ATCC 12315	$> 10^6$	$>10^3$
Clostridium perfringens	ATCC 13124	$> 10^6$	$> 10^6$

Table 24: Results of selectivity

Table 25 shows the results obtained with the MBS method and the reference method for the detection of *E. coli* in naturally contaminated samples of 5 different food matrices. Both methods have identified 100 on 100 target strains as positives, with total absence of false negatives; moreover both have identified 25 on 25 non target strains with a total absence of false positives.

		REFERENCE METHOD		
		Present	**Absent**	**Total**
MBS METHOD	**Positive**	100	0	100
	Negative	0	25	25
	Total	100	25	125

Table 25: Results for the selectivity test on *E. coli* reagent using naturally contaminated samples of 5 different food matrices

CHAPTER VI

Conclusions and future prospective

Food-borne diseases represent a serious issue for human health in underdeveloped but also in highly industrialized countries, despite all the prevention strategies adopted.

The introduction of HACCP system represented a big step forward, increasing responsibility of food operators and extending controls to all levels of the food chain.

This new approach required the development of new analytical methods; the traditional methods in fact, although sensitive and effective, are often of complicated execution (needing skilled personnel) and do not give results in time. These features are incompatible with the frequent and multi-level plan of controls expected by the HACCP system.

In recent years, the need for food industries to rapidly assess microbiological quality of raw materials and finished products, led to the development and refinement of alternative microbiological methods of analysis. Such alternative methods are quicker and easier to perform than the corresponding reference methods (Feinberg et al., 2009). In this context, the goal of the present study was the primary validation of the Micro Biological Survey (MBS) method for both TVC and *E. coli*, defined, according to European Directive 91/492/CEE, as thermophilic coliforms that produce indole from tryptophan after incubation at 44°C±2°C for 24 hours.

The MBS method is a fast colorimetric system for the detection and selective counting of bacteria in food products, in water and in environmental samples. The MBS method measures the catalytic activity of redox enzymes of the main metabolic pathways of bacteria (Antonini *et al.*, 2007; Schultz & Chan, 2001; Slater, 2003), allowing an unequivocal correlation between the observed enzymatic activity and the number of viable cells present in the samples. The time taken to change colour is inversely related with the log of bacterial concentration; like an enzymatic reaction, the greater the number of bacteria, the faster the colour change (Berlutti *et al.*, 2003). The results reported in this paper further support the previous findings concerning the existence of a stringent correlation between metabolic activity of bacteria and the number of viable cells.

The stability of MBS reagent was analysed. This is a very important issue for the proper use of the method, and also for the possibility to market it successfully.

Stability studies represent a critical point to assess the quality of a substance. The correct storage is very important to maintain the products characteristics for the entire period of validity. This can only be achieved thanks to a complete and correct stability program.

Accelerated and natural aging tests were performed at various times on the different MBS reagents. Microbiological and physical-chemical parameters were then evaluated. Accelerated aging tests have been carried out maintaining the reagents in an oven in humidity saturated atmosphere and comparing different storage conditions.

Thanks to these studies we obtain information on the best storage conditions of reagents, their shelf life, and on how to improve stability: compositions of the reagents was slightly modified in order to accomplish these results. Another crucial information furnished by these test was the importance of providing protection from humidity, which has resulted in improved stability. In this regard it has been observed that both the presence of vaseline and the use of a humidity protective packaging were essential to ensure as long as possible the functionality of the reagents.

All tests were carried out on several food samples using a Multi Reader (MR), a modular optical device made by Physics Department of the Tor Vergata University. It has been verified the compliance of the adopted solutions from the biological point of view, setting the optimal operating parameters of the MR and it has been confirmed the functionality of the system.

The last step was the validation of the MBS method. Validation aims to compare the results obtained with an alternative method, in this case the MBS method, with the results obtained with the reference method verifying the equivalence between the two methods by looking at linearity, accuracy and selectivity. The results were statistically analysed and compared according to the norm ISO/IEC 17025 (2005) and ISO 16140 (2003) verifying the equivalence between the two methods. All the performance parameters indicated a total equivalence between the reference method and the MBS method for detection and counting of TVC and *E. coli* in artificially contaminated water samples and in naturally contaminated food samples. When a method is validated for environmental sample analysis, it is important to include naturally contaminated samples. In this study, we have selected five different food matrices: cheese, vegetables, white meat, red meat and fruit.

The validation of the MBS method strongly supports its use as an alternative method for food analysis. The linearity over a range of bacterial concentrations was excellent. The selectivity was more than satisfactory with the absence of false negatives and false positives. The accuracy, evaluated on 125 naturally contaminated samples, showed a high correlation between the MBS method and the reference methods.

Comparing the MBS method to other analytical methods currently in use the following considerations come to light. With traditional count plate methods bacteria replication can be observed with the naked eye, but greater expertise in the operators and operational complexity are required. On the other hand, alternative methods often turn out to be very expensive also requiring highly equipped laboratories. The use of immunological or genetic probes (with the assistance of PCR to increase sensitiveness) had a great impact in microbial analysis (AL Haj *et al*., 2008; Cook *et al*., 2011; Loongyai *et al*., 2011; Parekh & Subhash, 2008; Settanni & Corsetti, 2007; Sherfi *et al*., 2006; Thacker *et al*., 1996). Indeed they are very quick and sensitiveness can be improved by using automated o semi-automated systems. The disadvantages are not only related to the need for specialized personnel and equipment, but also for a high limit of sensitiveness (immunological methods) and/or complexity and high costs of analysis (genetic methods). In addition the exact quantification of the number of bacteria over a large range of concentrations is not always possible. Colorimetric methods currently available are mainly based upon microorganisms secondary metabolism measuring. One of these methods detects the presence of *E. coli* on the basis of the activity of the enzyme β-glucuronidase (Al-Turki & El-Ziney, 2009). However, it should be mentioned that using this method it is not possible to detect the pathogenic, although relatively uncommon forms *E. coli* O157: H7 verocytotoxin producers (Ling *et al*., 2000; Donkor *et al*., 2008), which do not exhibit β-glucuronidase activity (Karmali *et al.*, 2010; Thompson *et al.*, 1990). Instead, *E. coli* O157: H7 is detected by the MBS method on the basis of its indole production from tryptophan.

For the above reported reasons, the MBS method can represent a worthy aid in food screening without replacing the analysis carried out with traditional methods which are very precise though often long and complex.

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ATTACHMENTS

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Validation of the Micro Biological Survey Method for Total Viable Count and *E. coli* in Food Samples

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ABSTRACT

The aim of this study was the validation of the Micro Biological Survey (MBS) method for microbiological analysis of food for Total Viable Count (TVC) and *Escherichia coli* (*E. coli*). The MBS method is a rapid quantitative alternative method for the detection and selective counting of bacteria in agro-food, in water and in environmental samples. It is based on colorimetric survey in mono-use disposable reaction vials that must be filled with the samples without any preliminary treatment (e.g., homogenization, dilution, etc.); the greater the number of bacteria presents into the sample, the faster the color change. However, an independent evaluation of the analytical results obtained with MBS method would be required before commercialization. Therefore, this alternative method was validated in comparison to the reference method. The general estimate of precision, reliability, uncertainty, linearity, accuracy and selectivity were determined. All the performance parameters have demonstrated total correlation between the alternative method and the reference method for the detection and counting of TVC and *E. coli* both in artificially contaminated and in naturally contaminated samples. MBS assay can be used as rapid and user friendly screening method for detection of TVC and *E. coli* in food industry.

Key words: Food microbiological analysis, food safety, alternative microbiological method, total viable count, *E. coli*

INTRODUCTION

Microbial analysis of food is an integrated part of the management of microbial safety in the food chain. The hygiene process criteria for many foodstuffs now include a test for Total Viable Count (TVC) and *Escherichia coli*. TVC gives a quantitative idea of the presence of mesophilic aerobic microorganisms of animal origin (ISO 4833, 2003). It serves as important criteria for evaluating the microbial quality of various foods and also degree of freshness of food (Nanu *et al*., 2007). *E. coli* is part of the normal microflora of the gastrointestinal tract of mammals and birds. As early as the 19th century, *E. coli* was recognized as a good indicator of faecal contamination. It is the only species in the coliform group found exclusively in the intestinal tract of humans and other warm-blooded animals, excreted in large numbers (ca., 10^9 CFU g^{-1}) in faeces (Cabral, 2010). Some strains have developed an ability to cause disease in the gastrointestinal,

urinary or central nervous system in even the most robust human hosts. The worldwide majority of the cases of the disease are caused by strains of serotype O157:H7, an enterohemorrhagic *E. coli* (EHEC). It can colonize the intestine of humans and cause diarrhea, hemorrhagic colitis and hemolytic-uremic syndrome (Yin *et al*., 2011; Nataro and Ketalaper, 1998).

Both control authorities and individual food business operators use microbial analysis for the purpose of monitoring the microbiological quality of raw materials and finished products and the microbiological status of manufacturing procedure. Even for compliance testing or assessment of Hazard Analysis Critical Control Points (HACCP) management strategies, microbial analysis is a valuable tool (Nicolas *et al*., 2007; Jasson *et al*., 2010). Standardized methods (e.g. ISO methods) are acknowledged as the reference analytical methods for official control. These standardized methods are based on traditional microbiological culture standard methods that are widely used in food analysis laboratories. These techniques present several difficulties, such as the subjectivity in the interpretation of some biochemical or morphological tests and the possible interference of matrices, specially when they present high levels of contamination. In addition, they are characterized by the high cost of the method, both in terms of labor and supplies and above all, by the long time needed to obtain definitive results (from 3 to 7 days) (Thomas *et al*., 2009). These reasons have led to the development and refinement of alternative microbiological methods of analysis (Mandal *et al*., 2011). Such alternative methods are quicker and easier to perform than the corresponding reference method and some can also be automated (Feng, 1996). Therefore, the prerequisite for the sale and use of any alternative method is to provide evidence that this could yield results which are equivalent to those provided by the corresponding reference method. The suppliers of the alternative methods, the food and drink industry, the public health services and other authorities need a reliable common protocol for the validation of such alternative methods. As a result, ISO 16140 (2003) represents a key issue (Feinberg *et al*., 2009).

In this context, MBS srl (a spin-off of Roma Tre University, Rome, Italy) has developed an alternative method, called Micro Biological Survey (MBS) method. It is a colorimetric fast system for the detection and the selective counting of bacteria present in agro-food, in water and in environmental samples. The MBS method consist of an analytical kit utilizing disposable, ready-to-use reaction vials for fast microbiological analyses. The analysis is based on the color change of the vial content which is induced by the presence of bacteria. The analyses can be carried out by untrained personnel and anywhere where they are necessary, without the need for any other instrumentation than a thermostat provided on request. The MBS method measures the catalytic activity of the redox enzymes in the main metabolic pathways of bacteria (Shultz and Chan, 2001; Slater, 2003; Antonini *et al*., 2007) which allows an unequivocal correlation between the observed enzymatic activity and the number of viable cells present in the samples. The time required for a color change is inversely related to the log of bacterial concentration; like an enzymatic reaction, the greater the number of bacteria, the faster the color change (Berlutti *et al*., 2003). The goal of the present study was the primary validation of the MBS method in accord with ISO 4833 (2003) for both TVC and *E. coli* assays.

MATERIALS AND METHODS

This study was initiated in 2009, lasting about two years for conclusion.

Statistical analysis on artificially contaminated water samples: A comparison between the colorimetric MBS method and the plate count method for the detection and differential

count of TVC and *E. coli* in artificially contaminated samples was performed according to ISO/IEC 170 25 (2005) following the International Standards ISO/TR 13843 (2000), using as reference methods those described by ISO 9998 (1991).

Bacterial strains: All the strains used in this statistical analysis were available at ATCC (American Type Culture Collection). The strains used in this statistical analysis were *Salmonella typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853), *Listeria innocua* (ATCC 33090), *Escherichia coli* (ATCC 25992), *Escherichia coli* O157:H7 (ATCC 35150), *Staphylococcus aureus* (ATCC 12600) and *Enterococcus faecalis* (ATCC 29212).

Preparation of artificially contaminated samples: Sterile water samples were contaminated with a mixture of the above indicated microorganisms coming from overnight cultures with serial dilutions in sterile saline solution up to 10^{-8}. Ten different dilutions of ten different samples were analyzed for both TVC and *E. coli*. Each dilution was tested in duplicate with both the MBS method and the plate counting reference method.

Colorimetric MBS method procedure: The analytical procedure for the quantitative colorimetric MBS method is based on colorimetric survey, using a redox indicator of the change of the oxidoreductive state in the reaction medium. For the analysis by the MBS method, ready-to-use MBS vials, sterilised and containing the reagent for the analysis were used. Two different kinds of vials were used: the vials for TVC analysis and the vials for *E. coli* analysis. To carry out the analysis, 10 mL of sterile distilled water and 1 mL of the samples were added to a vial of one or another type, depending on the type of analysis to be carried out. The vial was shaken until all the reagent was dissolved. Later on, the vial was incubated at 30°C for TVC or at 44°C for *E. coli*. The TVC which were detected and counted by MBS are defined as aerobic microorganisms able to grow in a complete medium. The *E. coli* are defined, according to European Directive 91/492/CEE, as thermophilic coliforms that produce indole from tryptophan after incubation at 44±2°C for 24 h. The starting colors are blue for TVC vials and reddish for *E. coli* vials and in the presence of microorganisms, the colors of the vials changed to yellow color indicating a positive result. The time for the yellow color development is inversely related to the bacterial content of the analysed sample. The persistence of the starting color after 24 h indicates a negative result, i.e., absence of microorganisms. The *E. coli* confirmation test was carried out by adding few drops of the Kovac's reagent (isoamyl alcohol, p-Dimethylaminobenzaldehyde, concentrated hydrochloric acid) after color change from reddish to yellow occurred in *E. coli* MBS vials. The development of a red ring revealed the production of indole (MacFaddin, 1980).

Reference method ISO 9998 (1991): One hundred microliter of the different samples were plated on Tryptic Soy Agar (TSA; Sigma Chemical CO., St. Louis) for TVC and on Tryptone Bile X-Glucuronide Agar (TBX; Cultimed, Barcelona, Spagna) for *E. coli* (MacFaddin, 1980). Then the plates were incubated at 30°C for TVC and at 44°C for *E. coli*. During TVC plate count all colonies visible after 24 and 36 h on TSA were considered positive while during *E. coli* plate count only blue colonies on TBX were considered positive. For the statistical analysis, only the positive plates containing less than 300 colonies were utilized.

Statistical analysis: Statistical analysis was carried out according to ISO/TR 13843 (2000). One-way analysis of variance and two-way analysis of variance were performed to determine the

general estimate of precision in the colorimetric method when using it as compared to the reference method of plate counting. The reliability was calculated using statistical analysis of Coefficient of Variation (CV). Uncertainty was calculated using the statistical analysis of chi-square test (χ^2).

Primary validation: The primary validations of MBS method for TVC and for *E. coli* on food samples were made according to ISO 16140 (2003).

Bacterial strains: All the strains used in this validation were available at ATCC (American Type Culture Collection). The strains used in this validation were *E. coli* (ATCC 25992), *E. coli* O157: H7 (ATCC 35150), *C. freundii* (ATCC 43864), *K. pneumoniae* (ATCC 13883), *E. cloacae* (ATCC 13047), *E. sakazakii* (ATCC 51329), *S. enteritidis* (ATCC 13076) and *S. typhimurium* (ATCC 14028), *Y. enterocolitica* (ATCC 19543), *B. cereus* (ATCC 11778), *B. stearothermophilus* (ATCC 24567), *B. subtilis* (ATCC 6633), *L. innocua* (ATCC 33090), *L. ivanovii* (ATCC 19119), *L. monocytogenes* (ATCC 7644), *S. aureus* (ATCC 12600), *S. epidermidis* (ATCC 12228), *S. lentus* (ATCC 29070), *P. aeruginosa* (ATCC 27853), *R. equi* (ATCC 31543), *E. faecalis* (ATCC 29212), *L. delbrueckii* subsp. *lactis* (ATCC 12315), *C. perfringens* (ATCC 13124), *A. niger* (ATCC 9642) and *S. cerevisiae* (ATCC 9763).

Preparation of artificially contaminated samples: Sterile water samples were contaminated with a mixture of the above indicated microorganisms coming from overnight cultures with serial dilutions in sterile saline solution up to 10^{-8}. To verify the equivalence between the MBS method and the reference method the samples were simultaneously tested.

Preparation of different levels of contamination of naturally contaminated food samples: Five different food matrices were selected for validation. They were cheese, white meat, red meat, vegetable and fruit. Only the samples naturally contaminated were selected for the experiments. Baby food of the same foodstuffs typologies were used as a negative control, because they were definitely not contaminated. Five different levels of contamination of naturally contaminated samples were obtained as follows: 10±0.5 g of naturally contaminated samples were homogenized in 90 mL of peptone water by a stomacher according to ISO 16140 (2003). Then homogenates were incubated for different times and different temperatures obtaining different levels of contamination. To verify the equivalence between the MBS method and the reference method, the samples were simultaneously tested.

Colorimetric MBS methods procedure: Analysis with the MBS method was carried out as previously described.

Reference method: For TVC the reference method was plate count on Plate Count Agar (PCA; Liofilchem, Roseto degli Abbruzzi, Italy) after 72 h according to ISO 4833 (2003). For *E. coli* it was plate count on Tryptone Bile X-GLUC Agar (TBX; Cultimed, Barcelona, Spagna) after 24 h according to ISO 16649-2 (2001).

Data analysis: Data analysis was carried out according to ISO 16140 (2003). For the validation of MBS method 3 parameters were analyzed: linearity, accuracy and selectivity. The linearity of the method was assessed by analyzing the correlation using a plot of bacteria concentrations

(expressed as CFU mL^{-1}) against the time occurred for color change. The accuracy was assessed by analyzing the correlation using a plot of bacteria concentrations (expressed as log CFU mL^{-1}) obtained with the reference method with the alternative method MBS. Selectivity of the MBS method for *E. coli* was observed by comparing MBS method with the reference method on both artificially contaminated samples and naturally contaminated samples.

RESULTS

Statistical analysis on artificially contaminated water samples: Sterile water was initially used to avoid any chemical interference due to organic matrices. In MBS colorimetric method the change of the starting color of the vials from blue for TVC and reddish for *E. coli* to yellow color indicates a positive result, presence of microorganisms. The time occurred for color change is inversely related to bacteria content of analysed samples (Fig. 1a, b). The water samples were artificially contaminated (see Materials and Methods). The statistical analysis for the MBS method on TVC and on *E. coli* vials was carried out according to ISO/TR 13843 (2000) using as reference method the plate counting method ISO 9998 (1991) on ten different dilutions of ten different samples. MBS reliable operating limits were comparable to the reference methods for plate counts at concentrations between 1×10^7 and <10 CFU mL^{-1}. The results of the statistical analysis are shown on Table 1 and are expressed in terms of (1) Estimate of Precision; (2) Coefficient of Variance and (3) Uncertainty. General estimate of precision was made according to ISO/TR 13843 (2000) using Analysis of Variance (ANOVA) tests. Results obtained by both one-way analysis of variance and two-way analysis of variance have shown that there were no statistical differences on bacterial

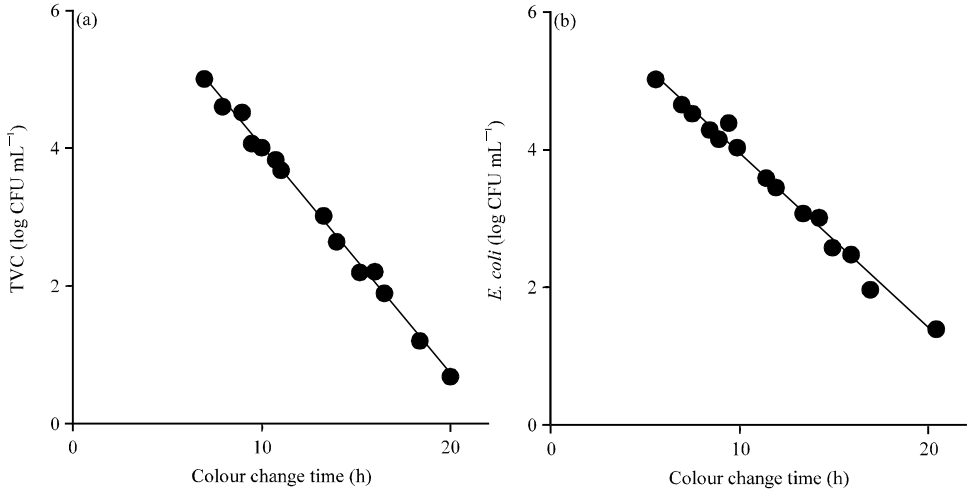

Fig. 1(a-b): Linearity: correlation line between analytes (TVC and *E. coli*) concentration with the time occurred for color change in the MBS vials. Using naturally contaminated food samples, bacteria numbers (expressed as the log of CFU mL^{-1}) obtained with the reference method are plotted against the time occurred for color change of the identical samples analyzed with MBS method. A linear inverse relationship between the time occurred for color change in the MBS vials and the bacteria concentration could be observed on five different food matrices with either TVC vials or *E. coli* vials. The correlation factor (R^2) is 0.95 for TVC and 0.98 for *E. coli*

Table 1: Statistical analysis carried out on the MBS method on TVC and *E. coli* according to the ISO/TR 13843 (2000)

Analysis	TVC	*E. coli*
Estimate of precision		
One-way analysis of variance		
DF 1.30 (limit 1% = 4.17)	F = 1.13	F = 0.2615
Two-way analysis of variance		
DF 7.28 (limit 1% = 4.17)	F = 1.8576	F = 1.16
Coefficient of Variance (CV)		
Plate count	0.1815	0.4533
MBS	0.0295	0.0628
Uncertainty (χ^2)		
Plate count		
DF 9 (limit 0.5% = 4.17)	χ^2<0.25	χ^2<1.2
MBS		
DF 9 (limit 0.5% = 4.17)	χ^2<0.22	χ^2<0.30

F: ANOVA F-test, CV: Coefficient of variation, DF: Degrees of freedom, χ^2: Chi-square test

count between the results obtained with MBS method and the results obtained with the reference method. The reliability of the bacterial count using MBS method was also assessed by statistical analysis using Coefficient of Variation (CV) analysis according to ISO/TR 13843 (2000). It appeared that the MBS method was more reliable than the reference method. Likewise, the uncertainty of the bacterial count using MBS method was less than that of the reference method as determinated by χ^2 statistical test according to ISO/TR 13843 (2000).

Primary validation: The primary validation of the MBS method for TVC and for *E. coli* was made according to ISO 16140 (2003). The main performance parameters which the alternative method must demonstrate are: linearity, accuracy and selectivity.

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. An increase in analyte should correspond to a linear or proportional increase in results (ISO 16140, 2003). This was achieved graphically as illustrated in Fig. 1 by plotting bacteria numbers (expressed as the log of CFU mL^{-1}) obtained with the reference methods for TVC and *E. coli* with the time occurred for color change of the identical samples analyzed with MBS methods for TVC and *E. coli*. A linear inverse relationship between the MBS methods and the bacteria concentration, with a correlation factor (R^2) close to 1.00, confirming the linearity of the data, can be observed.

Accuracy is the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples. The term relative accuracy used here is complementary to the “accuracy” and “trueness” as defined in ISO 5725-1:1994/COR 1 (1998). This states that accuracy is “the closeness of agreement between a test result and the accepted reference value” and that trueness is "the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value". For the purpose of this standard, the accepted reference values are chosen as the values obtained by the reference method. Thus, the term "relative" implies that the reference method does not automatically provide the accepted reference value as indicated by ISO 16140 (2003). In Fig. 2a and b the bacteria numbers (expressed as log CFU mL^{-1}) obtained with the reference counting methods for TVC and *E. coli* are plotted against the bacteria numbers (expressed as log

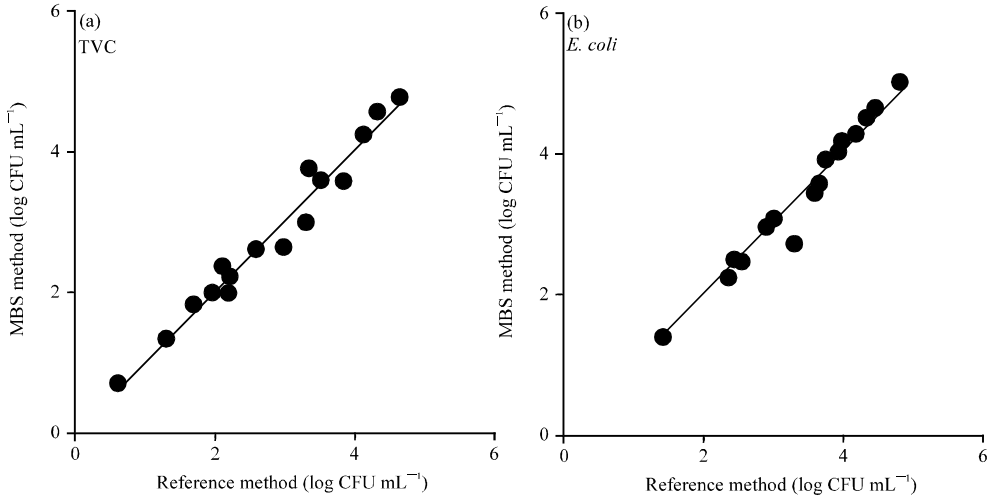

Fig. 2(a-b): Accuracy: correlation line between alternative MBS methods and reference methods (TVC and *E. coli*). Using naturally contaminated food samples, bacteria numbers (expressed as the log of CFU mL^{-1}) obtained with the reference method are plotted against bacteria numbers (expressed as the log of CFU mL^{-1}) obtained with MBS method on identical samples. A good correlation between the bacteria numbers (expressed as log CFU mL^{-1}) obtained with the traditional counting method and the alternative MBS method could be observed. In fact the slope is close to the theoretical value 1.00 (i.e. 1.00 for TVC and 0.99 for *E. coli*). The correlation factor (R^2) is 0.94 for TVC and 0.99 for *E. coli*

CFU mL^{-1}) obtained with the alternative MBS method for TVC and *E. coli*. The straight lines in both graphs show a perfect correlation between the reference methods and the MBS methods. In fact the slopes are close to theoretical value of 1.00.

Selectivity is the ability of an alternative method to detect the target analyte from a wide range of strains and is the lack of interference from a relevant range of non-target strains of the alternative method as indicated by ISO 16140 (2003). Among different inoculations only the vial inoculated with *E. coli* shows a change of the color from red to yellow. Table 2 shows the lowest detection limit (expressed as CFU mL^{-1}) of the MBS vials for TVC and for *E. coli* towards different bacterial strains in artificially contaminated samples. The lowest detection limit represents the minimal bacterial quantity required for inducing the color change in either MBS vials for TVC or for *E. coli*. The lowest detection limit is utilized to assess of the MBS vial selectivity. It could be noted that, using the *E. coli* vials, very high concentrations of all the bacteria other than *E. coli* were required for inducing the color change (i.e., a positive result of the test) while just one *E. coli* cell (on average) was sufficient to induce the color change of the same vials. These results indicate that the *E. coli* vials are selective for *E. coli*, although the *E. coli* vials showed a lower selectivity towards other coliforms strains, medium level selectivity towards *Enterobacteriaceae* and a higher selectivity towards Gram-positive bacteria. On the contrary, using TVC vials, just one cell of all the aerobic bacteria strains (on average) was sufficient to induce the color change, indicating the very low selectivity of the TVC vials.

Table 3 shows the results obtained with MBS method and reference method to detect *E. coli* in naturally contaminated samples of 5 different food matrices. Both methods have identified 100 target strains on 100 as positives, with total absence of false negatives; moreover both have identified 25 non target strains on 25 with a total absence of false positives.

Table 2: Results of selectivity tests

Bacteria strains		Lowest detection limits for *E. coli* vials (CFU mL^{-1})	Lowest detection limits for TVC vials (CFU mL^{-1})
Enterobacter cloacae	ATCC 13047	$>10^5$	1
Enterobacter sakazakii	ATCC 31329	$>10^5$	1
Pseudomonas aeruginosa	ATCC 27853	$>10^6$	1
Salmonella enteritidis	ATCC 13076	$>10^6$	1
Salmonella. enterica ser. *typhimurium*	ATCC 14028	$>10^4$	1
Yersinia enterocolitica	ATCC 19543	$>10^6$	1
Citrobacter freundii	ATCC 43864	$>10^3$	1
Klebsiella pneumoniae	ATCC13883	$>10^3$	1
Eschierica coli	ATCC 25922	1	1
Eschierica coli O157:H7	ATCC 35150	1	1
Enterococcus faecalis	ATCC 29212	$>10^6$	1
Bacillus cereus	ATCC 11778	$>10^6$	1
Bacillus stearothermophylus	ATCC 24567	$>10^6$	1
Bacillus subtilis	ATCC 6633	$>10^6$	1
Listeria innocua	ATCC 33090	$>10^6$	1
Listeria ivanovii	ATCC 19119	$>10^6$	1
Listeria monocytogenes	ATCC 7644	$>10^6$	1
Rhodococcus equi	ATCC 31543	$>10^6$	1
Sthaphylococcus aureus	ATCC 12600	$>10^6$	1
Sthaphylococcus epidermidis	ATCC 12228	$>10^6$	1
Staphylococcus lentus	ATCC 29070	$>10^6$	1
Lactobaccilus delbrueckii subsp. *lactis*	ATCC 12315	$>10^6$	$>10^3$
Clostridium perfringens	ATCC 13124	$>10^6$	$>10^6$

The lowest detection limit represents the minimal bacterial quantity of each bacterial strain required for inducing the color change in the corresponding MBS vials for TVC or *E. coli*

Table 3: Results for the selectivity test on *E. coli* vials using naturally contaminated samples of five different food matrices

	Reference method		
MBS method	Present	Absent	Total
Positive	100	0	100
Negative	0	25	25
Total	100	25	125

The food matrices utilized were cheese, white meat, red meat, vegetable, fruit. Both methods have been used to identify 100 target strains on 100 as positives, with total absence of false negatives; moreover both have identified 25 non target strains on 25 with total absence of false positives

DISCUSSION

In recent years, the need for the food industry to rapidly assess the microbiological quality of raw materials and finished products, has led to the development and refinement of alternative microbiological methods of analysis. Such alternative methods are quicker and easier to perform than the corresponding reference method (Feinberg *et al*., 2009). In this context, the goal of the present study was the primary validation of the Micro Biological Survey (MBS) method for both TVC and *E. coli*, defined, according to European Directive 91/492/CEE, as thermophilic coliforms that produce indole from tryptophan after incubation at 44±2°C for 24 h.

The MBS method is a colorimetric fast system for the detection and selective counting of bacteria in agro-food, in water and in environmental samples. The MBS method measures the catalytic activity of redox enzymes of the main metabolic pathways of bacteria (Shultz and Chan, 2001; Slater, 2003; Antonini *et al*., 2007), allowing an unequivocal correlation between the observed enzymatic activity and the number of viable cells present in the samples. The time required for color change is inversely related with the log of bacterial concentration; like an enzymatic reaction, the greater the number of bacteria, the faster the color change (Berlutti *et al*., 2003). The results reported in this study further support the previous findings concerning the existence of a stringent correlation between metabolic activity of bacteria and the number of viable cells.

Validation aims to compare the results obtained with an alternative method, in this case the MBS method, with the results obtained with the reference method verifying the equivalence between the two methods by looking at linearity, accuracy and selectivity. The results were statistically analyzed and compared according to the norm ISO/IEC 17025 (2005) and ISO 16140 (2003) verifying the equivalence between the two methods. All the performance parameters indicated a total equivalence between the reference method and the MBS method for detection and counting of TVC and *E. coli* in artificially contaminated water samples and in naturally contaminated food samples. When a method is validated for environmental sample analysis, it is important to include naturally contaminated samples. In this study, we have selected five different food matrices: cheese, vegetable, white meat, red meat and fruit.

The validation of the MBS method strongly supports its use as an alternative method for food analysis. The linearity over a range of bacterial concentrations was excellent. The selectivity was more than satisfactory with the absence of false negatives and false positives. The accuracy, evaluated on 125 naturally contaminated samples, showed a high correlation between the MBS method and the reference methods.

Comparing the MBS method to other analytical methods currently in use the following considerations come to light. With traditional count plate methods bacteria replication can be observed with the naked eye but greater expertise in the operators and operational complexity are required. On the other hand, alternative methods often turn out to be very expensive also requiring highly equipped laboratories. The use of immunological or genetic probes (with the assistance of PCR to increase sensitiveness) had a great impact in microbial analysis (Thacker *et al*., 1996; Sherfi *et al*., 2006; Settanni and Corsetti, 2007; AL-Haj *et al*., 2008; Parekh and Subhash, 2008; Cook *et al*., 2011; Loongyai *et al*., 2011). Indeed they are very quick and sensitiveness can be improved by using automated o semi-automated systems. The disadvantages are not only related to the need for specialized personnel and equipment but also for an high limit of sensitiveness (immunological methods) and/or complexity and high costs of analysis (genetic methods). In addition the exact quantification of the number of bacteria over a large range of concentrations is not always possible. Colorimetric methods currently available are mainly based upon microorganisms secondary metabolism measuring. One of these methods detects the presence of *E. coli* on the basis of the activity of the enzyme β-glucuronidase (Al-Turki and El-Ziney, 2009). However, it should be mentioned that using this method it is not possible to detect the pathogenic, although relatively uncommon forms *E. coli* O157: H7 verocytotoxin producers (Ling *et al*., 2000; Donkor *et al*., 2008) which do not exhibit β-glucuronidase activity (Thompson *et al*., 1990; Karmali *et al*., 2010). Instead, *E. coli* O157: H7 is detected by the MBS method on the basis of its indole production from tryptophan.

For the above reported reasons, the MBS method can represent a worthy aid in food screening without replacing the analysis carried out with traditional methods which are very precise though often long and complex.

CONCLUSIONS

The validation here reported provided evidence that the MBS method for TVC and *E. coli* gives similar results and is in agreement with the reference methods, also confirming the better reproducibility, specificity and selectivity of the MBS method. MBS method could therefore become a valid support for the control procedures for all the food farming companies willing to do a microbiological screening over their products to ensure utterly complete hygienic production.

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Qualitative and Quantitative Validation of the Micro Biological Survey Method for *Listeria* spp., *Salmonella* spp., Enterobacteriaceae and *Staphylococcus aureus* in Food Samples

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ABSTRACT

The objective of the present study was the preliminary validation of the qualitative MBS method for *Salmonella* spp. and *Listeria* spp. and the quantitative MBS method for Enterobacteriaceae and *Staphylococcus aureus*. The MBS method is a rapid alternative method for the detection and selective counting of bacteria in agro-food, in water and in environmental samples. It is based on a colorimetric survey in mono-use disposable reaction vials that can be filled with the samples without any preliminary treatment (e.g., homogenization, dilution, etc.); the greater the number of bacteria present in the sample, the faster the color changes. For the qualitative validation of the MBS method for *Salmonella* spp. and *Listeria* spp., selectivity, relative accuracy, relative specificity and relative sensitivity were determined. Selectivity, linearity and accuracy were analyzed for the quantitative validation of the MBS method for Enterobacteriaceae and *Staphylococcus aureus*. The validation here reported has shown that the MBS method for *Listeria* spp., *Salmonella* spp., Enterobacteriaceae and *S. aureus* give similar results and are in agreement with the reference method.

Key words: Food microbiological analysis, food safety, alternative microbiological method, *Listeria* spp., *Salmonella* spp., enterobacteriaceae, *S. aureus*

INTRODUCTION

Microbial food safety and food-borne infections are an important public health concern worldwide. Contaminated food consumption often results in an illness which is called food borne illness or food poisoning (Saikia and Joshi, 2010; Arzinz *et al*., 2011).

The hygiene process criteria for many foodstuff include tests for *Salmonella* spp., *Listeria* spp., Enterobacteriaceae and *Staphylococcus aureus* (*S. aureus*). Members of the genus *Salmonella* are Gram-negative and facultative anaerobic, rod-shaped bacteria (Malkawi and Gharaibeh, 2004). *Salmonella* is a major food and water borne pathogenic bacterium which causes an intestinal infection, accompanied by fever, abdominal cramps and diarrhea which is commonly known as salmonellosis (Rathnayaka, 2011). *Salmonella* is more often associated with any raw food of animal

origin which may be subject to fecal contamination, such as raw meat, poultry, fish/seafood, eggs and dairy product (Fadel and Ismail, 2009; McGuinness *et al*., 2009). *Listeria* spp. are ubiquitous bacteria widely distributed in the environment. Among the seven species of *Listeria*, only *Listeria monocytogenes* is commonly pathogenic for humans. *L. monocytogenes* is a Gram-positive rod that is catalase positive and shows a characteristic tumbling motility (Enan, 2006). It has been recognized as a veterinary pathogen and in humans it causes a disease known as listeriosis that could be very dangerous in older adults, persons with weakened immune systems, pregnant women and newborns. Food implicated in outbreaks of listeriosis have included various types of products such as dairy, meat, vegetable and sea food (Jalali *et al*., 2007; Adetunji and Arigbede, 2011).

Bacteria belonging to the Enterobacteriaceae family are the most common bacterial pathogens associated with gastrointestinal infections, particularly diarrhea. This bacterial family consists of a large heterogeneous group of facultative anaerobic, Gram-negative rods and includes species in the groups *Escherichia coli*, *Salmonella* and *Shigella*, the most common causative agents of intestinal infections (Rustam *et al*., 2006). Therefore, detection of Enterobacteriaceae, rather than for the traditional Coliform group, is advantageous because Enterobacteriaceae includes some potentially pathogenic species. Unlike the Coliform group, some Enterobacteriaceae species are often present in the processing environment. For this reason, enumeration of Enterobacteriaceae in foods now shows evidence of increasing interest (Rustam *et al*., 2006; Feinberg *et al*., 2009). *S. aureus* is a Gram-positive, catalase positive, coagulase positive non motile coccus bacterium (Ugbogu *et al*., 2007). *S. aureus* is a common cause of a bacterial food borne disease worldwide. However, quantitative evaluation of *S. aureus* contamination of food is not a simple task. Several studies have found *S. aureus* strains unable to produce black colonies with a clear halo on the Baird Parker Agar selective medium in dairy products and many *S. aureus* display peculiar biochemical properties (Da Silva *et al*., 2000). Therefore, the analysis of *S. aureus* eventually contaminated food stuff is made difficult by the risk of overlooking potentially pathogenic strains of *S. aureus* just because they have atypical morphological and/or biochemical characters. To avoid this danger, Harvey and Gilmour prescribe the coagulase test for all colonies with a diameter greater or less than 1 mm with or without halo or areas of clarification (Harvey and Gilmour, 1985).

Food borne pathogens are a growing concern for human illness and death. Therefore, there is an increasing demand to ensure a safe food supply and an urgent need to implement programmes such as Hazard Analysis Critical Control Points (HACCP) to monitor the quality of the products concerning produced for the presence of the pathogens (Nicolas *et al*., 2007). New method for the rapid and reliable detection of food borne pathogens are continuously proposed and improvements in the fields of immunology, molecular biology, automation and computer technology continue to have a positive effect on the development of faster, more sensitive and more convenient method in food microbiology (Biswas *et al*., 2011; Mandal *et al*., 2011).

Standardized method (e.g., ISO described method) are acknowledged as the reference analytical method for official control. They rely on traditional microbiological culture standard method that are widely used in food analysis laboratories. These traditional method involve the following basic steps: pre-enrichment, selective enrichment, selective plating, biochemical screening and serological confirmation (Mandal *et al*., 2011). These conventional method present several difficulties, such as subjectivity in the interpretation of some biochemical or morphological tests, the possible interference of matrices, especially when they present high levels of contamination, intense labor, high cost of supplies and above all, the prolonged time (from 3 to 7 days) needed to give definitive results, depending on the ability of the organisms to multiply into visible colonies (Thomas *et al*., 2009).

In this context, MBS srl (a spin-off of Roma Tre University, Rome, Italy) has developed an alternative rapid method, called Micro Biological Survey (MBS) method. It is a fast colorimetric fast system for the detection and the selective counting of bacteria present in agro-food, in water and in environmental samples. This method consists of an analytical kit containing disposable, ready-to-use reaction vials for fast microbiological analyses. The analysis is based on the change of color of the vial content which is induced by the presence of bacteria. The analyses can be carried out by untrained personnel and anywhere they are necessary, without the need for any instrumentation other than a thermostat which is provided on request. The MBS method measures the catalytic activity of the redox enzymes in the main metabolic pathways of bacteria (Shultz and Chan, 2001; Slater, 2003; Antonini *et al*., 2007) and allows an unequivocal correlation between the observed enzymatic activity and the number of viable cells present in the samples. The time required for a color change is inversely related to the log of bacterial concentration; like an enzymatic reaction, the greater the number of bacteria, the faster the color changes (Berlutti *et al*., 2003). In a previous study, we carried out the primary validation of the MBS method for Total Viable Count and for *Escherichia coli* with almost perfect agreement between reference method (Bottini *et al*., 2011). The objective of the present study was the primary validation of the qualitative MBS method for *Salmonella* spp. and *Listeria* spp. and the quantitative MBS method for Enterobacteriaceae and *S. aureus* in accord with ISO 16140 (2003). Qualitative method are method of analysis whose responses are either the presence or absence of the analyte detected either directly or indirectly in a certain amount of sample as indicated by ISO 16140 (2003). Quantitative method are method of analysis whose responses are the amount of the analyte measured either directly or indirectly in a certain amount of sample as indicated by ISO 16140 (2003). The validation here reported provide evidence that the new MBS method gave similar results and are in agreement with the reference method, confirming the reproducibility and specificity of MBS method.

MATERIALS AND METHODS

This study was conducted at the department of Biology, University Roma Tre during the period from 2009 to 2011.

Bacterial strains: All the strains used in these validations were available at ATCC (American Type Culture Collection): *E. coli* (ATCC 25992), *E. coli* O157:H7 (ATCC 35150), *C. freundii* (ATCC 43864), *K. pneumoniae* (ATCC 13883), *E. cloacae* (ATCC 13047), *E. sakazakii* (ATCC 51329), *S. enteritidis* (ATCC 13076), and *S. enterica* ser. Typhimurium (ATCC 14028), *Y. enterocolitica* (ATCC 19543), *B. cereus* (ATCC 11778), *B. stearothermophilus* (ATCC 24567), *B. subtilis* (ATCC 6633), *L. innocua* (ATCC 33090), *L. ivanovii* (ATCC 19119), *L. monocytogenes* (ATCC 7644), *S. aureus* (ATCC 12600), *S. epidermidis* (ATCC 12228), *S. lentus* (ATCC 29070), *P. aeruginosa* (ATCC 27853), *R. equi* (ATCC 31543), *E. faecalis* (ATCC 29212), *L. delbrueckii* subsp. *lactis* (ATCC 12315), *C. perfringens* (ATCC 13124), *A. niger* (ATCC 9642) and *S. cerevisiae* (ATCC 9763).

Preparation of naturally contaminated food samples with different levels of contamination: Naturally contaminated food samples were randomly selected among those found positive by reference method. Four different food matrices were selected for validation of qualitative method: Raw meat products, vegetables, pastry and dairy products. Three different food matrices

were selected for validation of quantitative method: Raw meat products, pastry and dairy products. Sterilized "Baby foods" of the same foodstuff typology were used as a negative control. Different levels of contamination of naturally contaminated samples were obtained as follows: 10±0.5 g of naturally contaminated samples were homogenized in 90 mL of peptone water by a stomacher according to ISO 16140 (2003). Then homogenates were incubated for different times at different temperatures obtaining different levels of contamination. To verify the equivalence between the MBS method and the reference method, these samples were simultaneously tested.

Artificially contaminated food samples: To achieve the number of samples required for statistical evaluation of the chosen parameters, artificially contaminated samples were also used. Food samples, found negative by reference method, were contaminated with a mixture of the above indicated microorganisms from overnight cultures with serial dilutions in sterile saline solution up to 10^{-8}. Ten different dilutions of ten different samples were analyzed for *Listeria* spp., *Salmonella* spp., Enterobacteriaceae and *S. aureus*. Each dilution was tested in duplicate with both the MBS method and the plate counting reference method.

Colorimetric MBS method procedure: The analytical procedure for qualitative and quantitative MBS method is based on colorimetric survey, using a redox indicator of the change of the oxidoreductive state in the reaction medium. For the analysis by the MBS method, ready-to-use MBS vials, sterilized and containing the reagent for the analysis were used. Four different kinds of vials were used: The vials for *Listeria* spp., *Salmonella* spp., Enterobacteriaceae and *S. aureus* analysis. To carry out the analysis, 10 mL of sterile distilled water and 1 mL of the samples were added to a vial of one or another type, depending on the type of analysis to be carried out. The vial was shaken until the entire reagent was dissolved. Later on, the vial was incubated at 37°C for all bacteria. The starting color is blue for vials for *Listeria* spp. and red for vials for *Salmonella* spp., Enterobacteriaceae and *S. aureus*. In the presence of the microorganisms of interest, the colors of vials changed to yellow indicating a positive result. The time taken to turn yellow is inversely related to the bacterial content of the analyzed sample. The persistence of the starting color after 36 h for *Listeria* spp. and *S. aureus*, 32 h for *Salmonella* spp. and 24 h for Enterobacteriaceae indicates a negative result, that is an absence of microorganisms.

Reference method: For *Listeria* spp. the reference method was plate count on Agar *Listeria* Ottaviani and Agosti (ALOA; Sigma, St. Louis, MO, USA) and *Listeria* Palcam Agar (PALCAM; Liofilchem, Roseto degli Abbruzzi, Italy) after 24 h of incubation at 37°C according to ISO 11290-1:1996/Adm 1 (2004). For *Salmonella* spp. it was plate count on Xylose Lysine Desoxycholate Agar (XLD, Sigma, St. Louis, MO, USA) and Brilliant Green Agar (BGA; Sigma, St. Louis, MO, USA) after 24-48 h of incubation at 37°C according to ISO 6579:2002/COR 1 (2004). For Enterobacteriaceae the reference method was plate count on Violet Red Bile Glucose Agar (VRBGA; Liofilchem, Roseto degli Abbruzzi, Italy) after 24 h of incubation at 37°C. For *S. aureus* it was plate Baird-Parker Agar (BPA; Sigma, St. Louis, MO, USA) after 46-48 h according to ISO 6888-1:1999/Adm 1 (2003). However, it should be kept in mind that, when a food sample is analysed with a reference method, a pretreatment is always required, according to the above reported ISO rules. Such pretreatment may vary from sample homogenization and dilution up to an additional enrichment, according to the different analysis to be carried out. The whole procedure for analysing a food sample may therefore last from a minimum of 36 h up to a maximum of 72 h.

Data analysis for qualitative validation: The primary validation of the qualitative MBS method for *Listeria* spp. and *Salmonella* spp. was made according to ISO 16140 (2003). The relative performance parameters indicated by the ISO 16140 (2003): accuracy, specificity and sensitivity were determined. They were calculated as follows: accuracy AC = ((PA+NA)/N) x 100%; specificity SP = (NA/N_) x 100%; sensitivity: SE = (PA/N_+) x 100%. Where, PA is the agreement for positive results; NA is the agreement for negative results; N is the total number of samples; N_ is the total number of negative results with the reference method (N_=NA+PD); N_+ is the total number of positive results with the reference method (N+ = PA+ND); PD is positive deviation (i.e., false positive result); ND is negative deviation (i.e., false negative result) (ISO 16140, 2003).

Data analysis for quantitative validation: Data analysis was carried out according to ISO 16140 (2003). Two parameters were analyzed: linearity and accuracy. The linearity of the method was assessed by analyzing the correlation using a plot of bacteria concentrations (expressed as CFU mL^{-1}) against the time taken to change color. The accuracy was assessed by analyzing the correlation using a plot of bacteria concentrations (expressed as log CFU mL^{-1}) obtained with the reference method and with the alternative MBS method (ISO 16140, 2003).

***S. aureus* confirmative coagulase test:** Preparation of samples for coagulase test was carried out as follows: selected colonies grown on BPA agar were transferred each with a sterile inoculation loop to different culture tubes containing Brain Heart Broth (Sigma, St. Louis, MO, USA) and were incubated at 37°C for 20-24 h; 10 mL of the supernatants of MBS method for *S. aureus,* that have changed color from red to yellow, were inoculated each within a vial containing 0.5 g of Amberlite MB-150 Mixed Bed Exchanger (Sigma, St. Louis, MO, USA), moderately shacked and left rest for 5-10 min. The confirmative coagulase test was carried out as follows: a vial with lyophilized rabbit plasma with EDTA (Sigma, St. Louis, MO, USA) was rehydrated with 3 mL of distilled water and 0.3 mL of the rehydrated rabbit plasma were pipetted into a sterile culture tube using a sterile pipette; 0.1 mL of the sample (either coming from culture tubes containing Brain Heart Broth or from MBS supernatants) was carefully mixed with the plasma in the sterile culture tube and then incubated at 37°C; the tubes were checked every hour for coagulation by gently tipping to the side; the coagulase test was positive if more than 75% of the tube contents had formed a coherent clot. If the test was negative after 4-6 h, the tube was left in the incubator and a final assessment was made after 24 h.

RESULTS

Selectivity of MBS method: Preliminary experiments were carried out to determine whether the food matrices may interfere with the MBS method. For this purpose tests on food samples artificially contaminated with target ATCC strains (*L. monocytogenes* ATCC 7644, *S. enterica* ser. Typhimurium ATCC 14028, *E. coli* ATCC 25922 and *S. aureus* ATCC 12600) were carried out (data not shown). A perfect agreement between reference method and MBS method was observed for all the strains, indicating that no interference came from any of the food matrices utilized (raw meat products, vegetables, pastry and dairy products).

Further preliminary tests were carried out to determine the selectivity of the different MBS method. The selectivity is defined as the ability of an alternative method to detect the target analyte from a wide range of strains and the lack of interference from a relevant range of non-target strains of the alternative method (ISO 16140, 2003). Table 1 reports the selectivity tests

Table 1: Selectivity tests

		Lowest detection limits for the different reagents (CFU mL^{-1})			
Bacteria strains		*Listeria* spp.	*Salmonella* spp.	*Enterobacter* spp.	*S. aureus*
Enterobacter cloacae	ATCC 13047	$>10^6$	$>10^5$	1	$>10^6$
Enterobacter sakazakii	ATCC 31329	$>10^6$	$>10^5$	1	$>10^6$
Pseudomonas aeruginosa	ATCC 27853	$>10^6$	$>10^6$	$> 10^6$	$>10^5$
Salmonella enteritidis	ATCC 13076	$>10^6$	1	1	$>10^6$
Salmonella enterica ser. Typhimurium	ATCC 14028	$>10^6$	1	1	$>10^6$
Yersinia enterocolitica	ATCC 19543	$>10^6$	$>10^6$	1	$>10^6$
Citrobacter freundii	ATCC 43864	$>10^6$	$>10^6$	1	$>10^6$
Klebsiella pneumoniae	ATCC13883	$>10^6$	$>10^6$	1	$>10^6$
Escherichia coli	ATCC 25922	$>10^6$	$>10^6$	1	$>10^6$
Escherichia coli O157:H7	ATCC 35150	$>10^6$	$>10^6$	1	$>10^6$
Enterococcus. faecalis	ATCC 29212	$>10^6$	$>10^6$	$>10^6$	$>10^5$
Bacillus cereus	ATCC 11778	$>10^6$	$>10^6$	$>10^6$	$>10^5$
Bacillus stearothermophilus	ATCC 24567	$>10^6$	$>10^6$	$>10^6$	$>10^5$
Bacillus subtilis	ATCC6633	$>10^6$	$>10^6$	$>10^6$	$>10^5$
Listeria innocua	ATCC 33090	1	$>10^6$	$>10^6$	$>10^5$
Listeria ivanovii	ATCC 19119	1	$>10^6$	$>10^6$	$>10^5$
Listeria monocytogenes	ATCC7644	1	$>10^6$	$>10^6$	$>10^5$
Rhodococcus equi	ATCC 31543	$>10^6$	$>10^6$	$>10^6$	$>10^6$
Staphylococcus aureus	ATCC 12600	$>10^6$	$>10^6$	$>10^6$	1
Staphylococcus epidermidis	ATCC 12228	$>10^6$	$>10^6$	$>10^6$	$>10^4$
Staphylococcus lentus	ATCC 29070	$>10^6$	$>10^6$	$>10^6$	$>10^4$
Lactobacillus delbrueckii subsp. *lactis*	ATCC 12315	$>10^6$	$>10^6$	$>10^6$	$>10^6$

indicating the minimum detection limit (expressed as CFU mL^{-1}) of the MBS method for *Listeria* spp., *Salmonella* spp., *Enterobacteriaceae* and for *S. aureus* towards different ATCC bacterial strains suspended in protonated water.

PRIMARY VALIDATION OF MBS METHOD FOR *Listeria* spp. AND *Salmonella* spp. IN QUALITATIVE ASSAYS

The primary validation of the qualitative MBS method for *Salmonella* spp. and *Listeria* spp. was performed according to ISO 16140 (2003).

Table 2 shows the results of analysis on both naturally and artificially contaminated food matrices (raw meat products, vegetable, pastry and dairy products) obtained with MBS method and reference method for *Listeria* spp. and *Salmonella* spp. These results indicate the concordance between results obtained with MBS method and reference method to detect *Listeria* spp. and *Salmonella* spp. Out of 71 positive samples obtained with the reference method for *Listeria* spp. 67 were found to be positive (N_+) and 4 were found to be negatives (ND) by the MBS method. Out of 17 negative samples with the reference method, 17 were found to be negative (N_-) by the MBS method and no positives were found (PD). For *Salmonella* spp. out of 81 positive samples obtained with the reference method, 79 were found to be positive (N_+) and 2 were found to be negatives (ND) by the MBS method. Out of 15 negative samples obtained with the reference method, 12 were found to be negative (N_-) and 2 were found to be positives (ND) by the MBS method.

The main performance parameters which the alternative method must demonstrate are the relative accuracy, specificity, sensitivity and selectivity.

Table 2: Results of analysis of food samples (raw meat products, vegetable, pastry and dairy products) either naturally or artificially contaminated obtained with MBS method and reference method for (a) *Listeria* spp. and (b) *Salmonella* spp.

	Reference method		
MBS method	Present	Absent	Total
***Listeria* spp.**			
Positive	67 (PA)	0 (PD)	67
Negative	4 (ND)	17 (NA)	21
Total	71 (N_+)	17 (N_-)	88 (N)
***Salmonella* spp.**			
Positive	79 (PA)	3 (PD)	82
Negative	2 (ND)	12 (NA)	14
Total	81 (N_+)	15 (N_-)	96 (N)

PA: Positive agreement, NA: Negative agreement, ND: Negative deviation (false negatives), PD: Positive deviation (false positives), N: Total number of samples (NA+PA+PD+ND), N_+: Total number of positive results obtained with reference method, N_-: Total number of negative results obtained with reference method

Table 3: Paired values of relative accuracy (AC), relative sensitivity (SE) and relative specificity (SP) for MBS method and reference method

	MBS method		Reference method	
	Listeria spp.	*Salmonella* spp.	*Listeria* spp. (ISO 11290-1:1996, 2004)	*Salmonella* spp. (ISO 6579:2002, 2004)
(%)	(%)		(%)	
AC = (PA+NA)/N	95.4	94.8	88.7	89.1
SE = PA/N+	100.0	80.0	85.2	94.4
SP = NA/N-	94.4	97.5	97.4	88.8

Relative accuracy is the degree of correspondence between the results obtained by the reference method and the results obtained by the alternative method on identical samples (ISO 16140, 2003). The term "relative accuracy" used here is complementary to the "accuracy" and "trueness" as defined in ISO 5725-1:1994/COR 1 (1998). This states that accuracy is "the closeness of agreement between a test result and the accepted reference value", and that trueness is "the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value".

Relative sensitivity is the ability of the alternative method to detect the analyte when it is detected by the reference method.

Relative specificity is the ability of the alternative method to not detect the analyte when it is not detected by the reference method.

Table 3 shows the values of the performance parameters for MBS method calculated using the positive and negative results shown in Table 2 and the same performance parameters for the reference method as reported in the literature (ISO 11290-1:1996/Adm 1, 2004 for *Listeria* and ISO 6579:2002/COR 1, 2004 for *Salmonella* spp.).

PRIMARY VALIDATION OF QUANTITATIVE MBS METHOD FOR ENTEROBACTERIACEAE AND *S. aureus*

The primary validations of the quantitative MBS method for *S. aureus* and Enterobacteriaceae were performed according to ISO 16140 (2003). The main performance parameters which the alternative method must demonstrate are: linearity and accuracy.

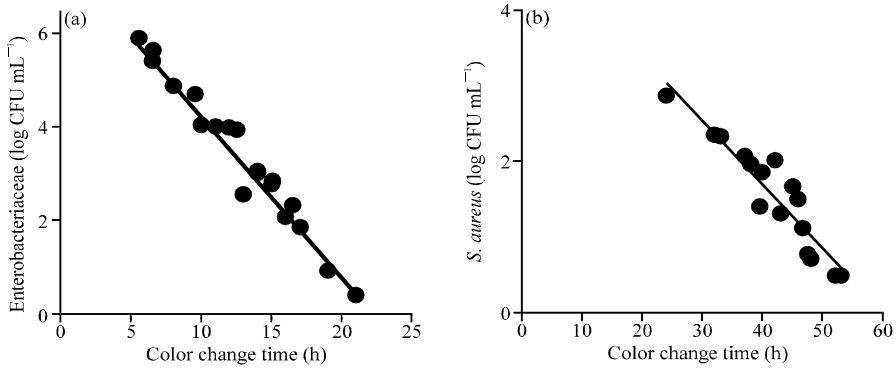

Fig. 1 (a-b): Linearity: Correlation line between analyte (a) Enterobacteriaceae and (b) *S. aureus* concentrations with the time taken to change color with in the MBS method

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 as indicated by ISO 16140 (2003). This was achieved graphically as illustrated in Fig.1 by plotting bacteria concentrations (expressed as the log of CFU mL^{-1}) obtained with the reference method with the time occurred for taken to change color with the identical samples analyzed with MBS method. A linear inverse relationship between the MBS method and the bacteria concentration, with a correlation factor (R^2) close to 1.00, confirming the linearity of the data can be observed. Using naturally and artificially contaminated food samples, bacteria concentrations (expressed as the log of CFU mL^{-1}) obtained with the reference method are plotted against the time taken to change color with the identical samples analyzed with MBS method. A linear inverse relationship between the time, taken to change color with the MBS method and the bacteria concentration could be observed with Enterobacteriaceae vials and *S. aureus* vials on three different food matrices: raw meat products, pastry and dairy products. The correlation factors (R^2) are 0.98 and 0.95 for Enterobacteriaceae and for *S. aureus*, respectively.

Accuracy is the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples (ISO 16140, 2003). Figure 2 show a perfect correlation between the bacteria number (expressed as log CFU mL^{-1}) obtained with the traditional counting method and the alternative MBS method. The straight lines obtained were close to the theoretical y = x (slope = 1,00), with values of correlation factor (R^2), which confirm the high equivalence between the reference method and the alternative. Using naturally and artificially contaminated food samples, bacteria numbers (expressed as the log CFU mL^{-1}) obtained with the reference method are plotted against the time taken to change color with the identical samples analyzed with MBS method. A good correlation between the bacteria numbers (expressed as log CFU mL^{-1}) obtained with the traditional counting method and the alternative MBS method could be observed. In fact the slopes are close to the theoretical value of 1.00 (i.e., 0,98 for Enterobacteriaceae and 0.93 for *S. aureus*). The correlation factors (R^2) are 0.97 and 0.94 for Enterobacteriaceae and for *S. aureus*, respectively.

Confirmation tests for *S. aureus* were performed on all the positive and negative results. Results obtained using coagulase test were in full agreement with results obtained by MBS method since all the MBS positive results were positive in the coagulase tests.

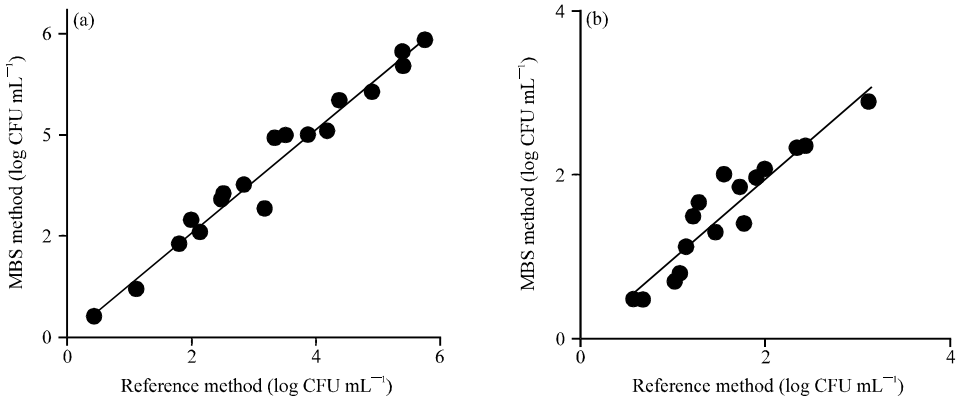

Fig. 2 (a-b): Accuracy: Correlation line between alternative MBS method and reference method (a) Enterobacteriaceae and (b) *S. aureus*

DISCUSSION

Rapid and reliable detection of microorganisms in food samples is essential for prevention of disease, but it is also important to save on the cost of storage and transportation of infected products, (Rathnayaka, 2011), therefore, the development of new method for detection and identification of microorganisms in food, water and environmental samples which give accurate results and are economically competitive, are always needed. With traditional count plate method bacteria replication can be observed with the naked eye, but greater expertise between the operators and operational complexity are required. On the other hand, alternative method often turn out to be very expensive and also require highly equipped laboratories (Settanni and Corsetti, 2007; Thacker *et al.*, 1996).

In this context, rapid colorimetric MBS method may play an important role. This is a fast colorimetric system for the detection and selective counting of bacteria in agro-food, in water and in environmental samples. Colorimetric method currently available are mainly based upon microorganisms secondary metabolism measuring. On the contrary, the MBS method measures the catalytic activity of redox enzymes of the main metabolic pathways of bacteria (Antonini *et al.*, 2007; Bottini *et al.*, 2011), allowing an unequivocal correlation between the observed enzymatic activity and the number of viable cells present in the samples. The time required for color change is inversely related to the log of bacterial concentration; like an enzymatic reaction, the greater the number of bacteria, the faster the color change (Bottini *et al.*, 2011). Alternative rapid analytical method, like the MBS method, are allowed by regulatory authorities once they have been validated against the reference method according to ISO 16140 (2003) and McGuinness *et al.* (2009). A previous study provided the evidence that the MBS method for TVC and *E. coli* gave similar results and is in agreement with reference method (Bottini *et al.*, 2011). The purpose of the present study was the primary validation of qualitative MBS method for *Listeria* spp. and *Salmonella* spp. and of quantitative MBS method for Enterobacteriaceae and *S. aureus*. Qualitative method are method of analysis whose responses are either the presence or absence of the analyte detected either directly or indirectly in a certain amount of sample as indicated by ISO 16140 (2003). Quantitative method are method of analysis whose responses are the amount of the analyte measured either directly or indirectly in a certain amount of sample as indicated by ISO 16140 (2003). Both the MBS qualitative and quantitative method were demonstrated to be very selective and all showed a high reliability and correlation with traditional count plate method. No interference due to food matrices was observed.

In particular, quantitative MBS method for Enterobacteriaceae and *S. aureus* demonstrated high linearity (results are in proportion to the amount of analyte present in the sample) and accuracy (correspondence between the results obtained by the reference method and the results obtained by the alternative method on identical samples). It should also be noted that results obtained using coagulase test were in full agreement with results obtained by the MBS method on *S. aureus*, demonstrating that the well known variability of the morphological and biochemical properties of naturally occurring *S. aureus* strains did not influence the exact quantification by MBS method of *S. aureus* cells present in the food samples.

For qualitative analysis, a perfect correspondence between the MBS method and the reference method for *Listeria* spp. and *Salmonella* spp. was observed when ATCC reference strains were inoculated into different food matrices. On the contrary, a limited number of discrepancies between MBS method and reference method were observed when the same food matrices were contaminated by naturally occurring strains of *Listeria* and *Salmonella*. This phenomenon may lead to either false positive or false negative results; however these discrepancies may be attributed either to the MBS method or to the reference method. It should be kept in mind that the apparent false negatives or false positives become real false negatives or false positives only when an independent reference method has been proven to be true. The paired values for relative accuracy, sensitivity and specificity for MBS method here reported are lower than the same parameters reported for the reference method (ISO 11290-1:1996/Adm 1, 2004; ISO 6579:2002/COR 1, 2004). As a matter of fact, a small percentage of false negatives and false positives were observed using the reference method in a inter-laboratory experiments (ISO 11290-1:1996/Adm 1, 2004; ISO 6579:2002/COR 1, 2004). For these reasons, the very same ISO documents stated that when the reference method indicates a positive result, supplementary analysis to prove whether there is a real presence or not of pathogenic bacteria should be carried out (ISO 11290-1:1996/Adm 1, 2004; ISO 6579:2002/COR 1, 2004).

Although, we have here demonstrated that relative accuracy, sensitivity and specificity for MBS method for *Listeria* spp. and *Salmonella* spp. are more reliable than the respective reference method, an independent analysis should be carried out when a positive result is found.

CONCLUSIONS

The validations of the MBS method for *Listeria* spp., *Salmonella* spp., Enterobacteriaceae and *S. aureus* give similar results and are in agreement with the reference method according to ISO rules. MBS method could therefore become a valid support for the control procedures for all the food farming companies willing to do a microbiological screening of their products to ensure complete hygienic production.

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