Review

WAYS TO DETERMINE COLIFORM BACTERIA IN FOOD – A MINI REVIEW

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Abstract: *The presence/absence of coliform bacteria in food is a hygienic-sanitary indicator that provides valuable information about the hygienic conditions in which the food was processed. The determination of coliform bacteria in food using the classical method (multiple-tube method) is a laborious work, and the results take a long time. In recent years, modern techniques for the determination of coliform bacteria have emerged, which include enzyme tests, ELISA techniques, PCR techniques, and spectrophotometric techniques, with much faster results.*
In this paper, the use of ion mobility spectrometry for the determination of o-nitrophenol (ONP), a volatile marker generated by the E. coli species, is briefly described, discussing both the strengths and weaknesses of this method.

Keywords: coliform bacteria, classic and modern methods, ONP, food.

INTRODUCTION

Coliform bacteria are part of the *Enterobacteriaceae* family, and include the genera *Escherichia*, *Klebsiella*, *Enterobacter*, and *Citrobacter*.

Coliform bacteria are considered indicators of food safety, and are used to estimate the degree of hygiene and microbial innocuousness of food processing.

Physiologically and depending on the source, coliforms can be:

- faecal coliforms (FC) characterised by rapid growth in 16 hours, in a nutrient broth medium at 41-44°C (these are considered hygienic indicators that highlight the contamination of food with faecal matter)
- non-faecal coliforms (NFC) of aquatic or telluric origin multiply at 4°C in 2-4 days, are unable to grow at 41°C, being psychrotrophs (Dan, 1999).

In the past, the genus *Escherichia* was thought to have only one species, *E. coli*. Taxonomic research has shown that the genus *Escherichia* comprises 5 species: *E. coli*, *E. blattae*, *E. fergusoni*, *E. hermani*, and *E. vulneris* (Bârzoi&Apostu, 2002). *E. coli* represents the type species of the genus, is a Gram-negative, aerobic/facultative anaerobic, non-sporogenous, acapsulogenic, and motile coccobacillus. *E. coli* is an indicator of faecal pollution (it is eliminated in the same way as the pathogenic bacteria present in sick individuals), and it can develop in water and food. *E. coli* is sensitive to common disinfectant substances at the usual concentrations in current practice, which is why this bacterium is the main indicator for checking the efficiency of the sanitisation and disinfection operation in the food industry and public catering (Șerban&Călugăru, 2005).

Currently, *E. coli* serotypes can be classified into 4 main groups (Doyle&Padhye, 1989; Milon, 1993):

- group I enteropathogenic *E. coli* (EPEC) strains
- group II enterotoxigenic *E. coli* (ETEC) strains
- group III enteroinvasive *E. coli* (EIEC) strains
- group IV enterohemorrhagic *E. coli* (EHEC) strains

Food poisoning outbreaks have been particularly associated with VTEC (verotoxin) and, to a lesser extent, EPEC, ETEC and EHEC strains (Ramos *et al.*, 2020; Basak & Ahmet, 2017). Determining EPEC, ETEC and EIEC in food faces some difficulties due to the associated microflora. For this reason, the use of selective enrichment and isolation media is required.

The classic method for determining coliform bacteria includes presumptive tests, confirmatory tests, and biochemical tests based on the ability of coliform bacteria to ferment lactose (similar to lactic acid bacteria) at 35-

37°C for 48 hours, and to produce lactic acid, carbon dioxide, and hydrogen. The classic technique for determining coliform bacteria has the disadvantage of being laborious, with the final results known only after 5-6 days. Modern techniques for determining coliform bacteria are more demanding in terms of equipment, are less laborious, and the results are much faster.

CLASSIC METHOD

In the classic technique for the determination of coliform bacteria (multiple-tube method), lactose broth is used as enrichment media, and BGBL (Brilliant Green Bile Lactose) or Lauryl Sulphate Broth as liquid confirmatory media (Dan *et al*., 1991; Oprean, 2002). Mac Conkey agar (VRBL - Violet Red Bile Agar with Lactose), Levine EBM medium (Eosin Methylene Blue Agar), or Rambach medium is used as solid identification media (Manafi, 2000).

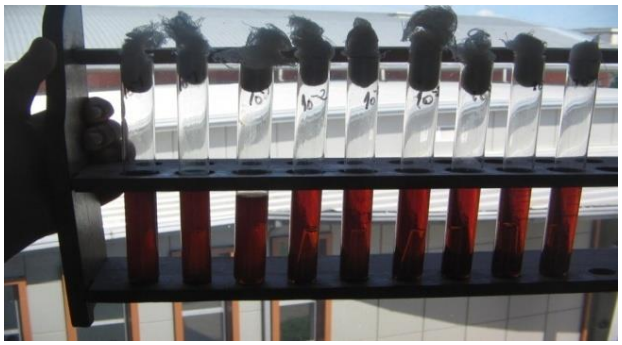

Figure 1. Durham tubes with BGBL medium for the determination of coliform bacteria in milk (personal archive photo)

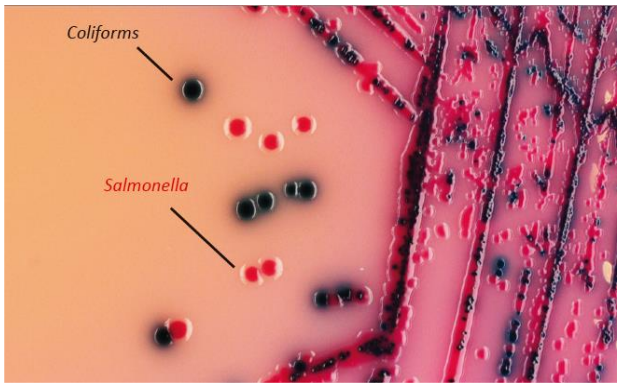

Figure 2. Differentiation of coliform colonies on Rambach medium (Rambach catalogue – instructions for use)

After confirmation, the formula used for the biochemical differentiation of *E. coli* from *Enterobacter aerogenes* is the IMVC formula described in Table 1:

Table 1. Biochemical differentiation of the *E. coli* species (Dan *et al*., 1991)

Species	I	M	V	C	Mobility
Escherichia coli	+	+	-	-	+
Enterobacter aerogenes	-	-	+	+	-

I – production of indole from tryptophan
M – reaction with methyl red
V – the Voges Proskauer reaction for the production of acetoin
C – the use of citrate

MODERN METHODS

Most of the modern determination techniques of the coliform bacteria include enzyme tests (determination of β-galactosidase as the main metabolite), ELISA techniques (determination of verotoxins), PCR techniques, spectrophotometric techniques, (Bouvet&Vernozy-Rozand, 2000; Bellin *et al.*, 2001). Another modern techniques use a microbial photoelectric detection system which quantifies microorganisms by detecting the light signal generated by the measured sample during growth (Cui *et al.*, 2023).

In immunoenzymatic analysis methods, different marker enzymes can be used depending on the metabolite to be dosed. Table 2 lists the enzymes most commonly used as markers together with the main chromogens and the wavelengths at which maximum absorbance is measured.

Table 2. Main marker enzymes used in immunoenzymatic techniques (Cojocaru *et al.*, 2007)

Marker enzyme	Chromogenic substrate	Product and wavelength corresponding to maximum absorbance
Peroxidase	o-phenylenediamine	o-nitroaniline (λ = 492 nm)
Alkaline phosphatase	p-nitrophenyl phosphate	p-nitrophenol ((λ = 405 nm)
β-D-galactosidase	o-nitrophenyl-β-D-galactopyranose	o-nitrophenol (λ = 405 nm)
β-D-galactosidase	4-methyl-umbelliferyl-β-D-galactopyranose	4-methyl-umbelliferone with fluorescent properties ($\lambda_{emission}$ = 448 nm)
Glucose oxidase	o-phenylenediamine	o-nitroaniline (λ = 492 nm)
Glucose-6-phosphate dehydrogenase	glucose-6-phosphate	$NADPH+H^+$ (λ = 340 nm)

Table 2 shows that marker enzyme β-D-galactosidase can be determined using two methods, both by measuring the absorbance, and by measuring the emission of the product resulting from the reaction.

One of the modern methods for determining coliform bacteria is based on the reaction of the extracellular enzyme β-galactosidase with o-nitrophenyl β-D-galactopyranose (ONPG). The enzyme cleaves the substrate generating a colourless saccharide (galactopyranose) and o-nitrophenol (yellow ONP compound); this coloured compound is detected spectrophotometrically at wavelengths λ = 405 nm (Rațiu *et al.*, 2017)

In a series of works (Bocoș-Binținţan, 2004; Peter-Snyder *et al.*, 1991a; Strachan *et al.*, 1995; Peter-Snyder *et al.*, 1991b), the use of ion mobility spectrometry was described to investigate the bacterial enzyme/substrate reaction by investigating the analyte produced in an unconventional manner. Thus, the property of ONP to have a relatively high vapour pressure (0.54 torr at 40°C) is used, which allows the direct analysis of these vapours using ion mobility spectrometry (IMS).

ONP is a universal volatile marker for several bacterial species as shown in Table 3:

Table 3. Markers generated by various bacterial species (Strachan *et al.*, 1995)

Microorganisms	Substrate used	Generated volatile marker
E. coli	o-nitrophenyl β-D-galactopyranose (ONPG)	ONP
Yersinia	Urea	Ammonia
Aeromonas	o-nitrophenyl β-D-galactopyranose (ONPG)	ONP
Listeria	o-nitrophenyl β-D-glucopyranose (ONPGluco)	ONP
Staphylococcus aureus	o-nitrophenyl β-D-galactoside-6-phosphate	ONP

In their research, Snyder et al. used a CAM (Chemical Agent Monitor) ion mobility spectrometer (IMS) operated in negative mode (negative ions produced by ONP were detected). *E. coli* suspensions were prepared by growing in a nutrient solution for 48 hours to which 0.5% lactose was added to induce the β-galactosidase enzyme; the ONPG solution had a concentration of 2 mg/mL in sterile pH 7.4 phosphate buffer solution. The procedure was as follows: 2 µL of the ONPG solution and 2 µL of the *E. coli* suspension in phosphate buffer were added to a 15 mm diameter sterile filter paper disc. After a short incubation period at 40-42°C, the headspace atmosphere in the glass vial containing the filter paper disc was sampled using the IMS apparatus (Peter-Snyder *et al.*, 1991a).

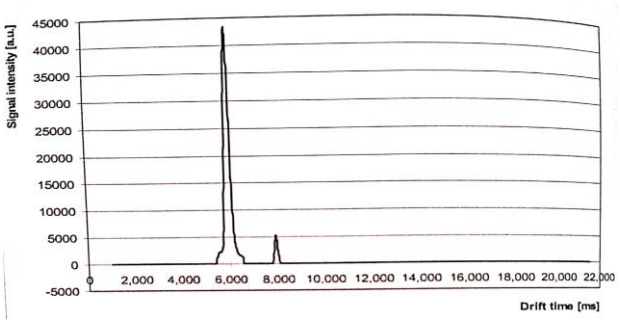

Figure 3. The ion mobility spectrum of ONP following the development of coliform bacteria at a temperature of 42°C (Bocoș-Binținţan&Rațiu, 2009)

The ion mobility spectrum shown in Figure 3 shows two distinct peaks: the 5.96 millisecond RIP peak, and the 7.92 millisecond peak generated by ONP (the target analyte), of much lower intensity.

CONCLUSIONS

The determination of coliform bacteria in water and food by ion mobility has both advantages, and disadvantages. Among the advantages we have identified:

- determination takes place in real time (drift times are a few milliseconds)
- the sensitivity is extremely high (the ONP analyte is detected to the order of parts per billion-ppb, or even parts per trillion-ppt)
- the device is portable, the cost of the instrument is quite low.

Among the disadvantages we identified:

- ONP is a volatile marker that is also generated by other types of bacteria, other than coliform bacteria (g. *Aeromonas*, g. *Listeria*, g. *Staphylococcus*)
- the linear range of response in ion mobility spectrometry (IMS) is limited, having negative consequences on the

quantitative determinations of the analyte (ONP), and implicitly on the determination of coliform bacteria.

In conclusion, the ion mobility spectrometry technique lends itself to the determination of coliform bacteria in food when their number is low and there are no other ONP-generating microorganisms in the product that would affect the result.

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