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Use of exogenous volatile organic compounds to detect *Salmonella* in milk

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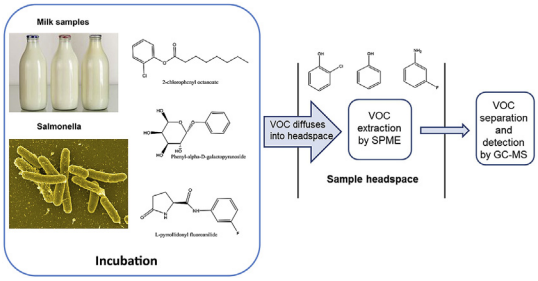
HIGHLIGHTS

- Detection of *Salmonella* in milk samples.
- Detection of exogenous volatile organic compounds by HS-SPME-GC-MS.
- Use of enzyme substrates to target C8 esterase and α-galactosidase activity.
- Detection of *Salmonella* species within 5 h.

GRAPHICAL ABSTRACT



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ABSTRACT

Rapid, sensitive, and selective detection and identification of pathogenic bacteria is required in terms of food security. In this study, exogenous VOCs liberated by *Salmonella* strains have been identified and quantified *via* head space-solid phase microextraction gas chromatography mass spectrometry (HS-SPME-GC-MS) in milk samples. The specific enzymes targeted for detection and/or differentiation of *Salmonella* were C8 esterase, α-galactosidase and pyrrolidonyl peptidase using the following enzyme substrates: 2-chlorophenyl octanoate, phenyl α-D-galactopyranoside and L-pyrrollidonyl fluoroanilide, respectively. Detection of the exogenous VOCs, 2-chlorophenol, phenol and 3-fluoraniline was possible with typical limits of detection of 0.014, 0.045 and 0.005 μg/mL, respectively and correlation coefficients >0.99. The developed methodology was able to detect and identify *Salmonella* species within a 5 h incubation at 37 °C by the detection of the liberated VOCs. It was found that the milk samples tested were *Salmonella* free.

1. Introduction

The detection of pathogenic bacteria is important to protect consumers and prevent human foodborne illnesses, as well as for effective treatment of patients and to reduce high medical and economical costs. *Salmonella* can cause serious illness in infants, older adults and people with chronic diseases and can lead to high mortality rates [1]. *Salmonella*, within the genus *Enterobacteriaceae*, are Gram-negative rod-shaped bacteria [2]. Most *Salmonella* can grow over the temperature range 6–48 °C with an optimum temperature range of 32–37 °C; most *Salmonella* are not particularly heat resistant and can be readily destroyed at the pasteurization temperature (71.7 °C for 15 s) [3–5]. However, *Salmonella* are often resistant to adverse conditions [6] and this allows them to persist in the environment and interfere with the food chain i.e. via animals

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for human consumption or plants that are fertilized with animal manure. This is since all *Salmonella* strains can grow with or without oxygen (facultative anaerobes) and in atmospheres containing high levels of carbon dioxide (up to 80%) [7].

Although food safety practices have been improved (e.g. pasteurization) to reduce the risks from Salmonellosis associated with consuming milk and its associated products (e.g. butter, yoghurt) it has not been eliminated. In addition, incidences of Salmonellosis have been reported due to consumption of pasteurised milk [8–11]. Pasteurised milk does have the potential to transfer *Salmonella* from infected farm animals to humans due to the occurrence of improper pasteurization. In addition, milk can be also contaminated by unsanitary handling after the completion of the pasteurization process.

Generally, *Salmonella* detection methods can be categorized into two groups, conventional *Salmonella* detection methods (e.g. culturing) and rapid *Salmonella* detection methods (e.g. immunology-based assays, nucleic acid-based assays, miniaturized biochemical assays, and biosensors) [12]. Ultimately however, the time required for the conventional and rapid analysis methods depends on the cell enrichment steps to reach minimal cell concentration (at least 10^4 CFU/mL) for *Salmonella* detection.

For detection of *Salmonella* cultural methods have been established using nutrient acquisition, biochemical characteristics, and metabolic products unique to *Salmonella* spp [13]. To detect/identify *Salmonella* in food samples the species need to be isolated selectively as possible from the sample. Therefore, the isolation procedure contains several steps, such as nonselective pre-enrichment of a defined weight or volume of the food sample, followed by a selective enrichment, and then testing on an agar medium usually by plating onto selective agars, and biochemical and serological confirmation of suspect colonies. The most common media in pre-enrichment step are buffered peptone water (BPW) and lactose broth [12]. Enrichment (selective) media have been evaluated and developed to increase the sensitivity and the specificity of *Salmonella* detection. This is done by addition of two or more inhibitory reagents such as bile salts, brilliant green, thiosulphate, deoxycholate, malachite green, novobiocin, tetrathionate, cycloheximide, nitrofurantoin, and sulphacetamide [12–14]. The function of these inhibitors, in a selective media, is to suppress bacteria present in the sample and allow continuous growth of *Salmonella* [15]. Rappaport-Vassiliadis (RV) medium and tetrathionate (TT) broth has been used as *Salmonella* enrichment media in approved standard methods such as FDA Bacteriological Analytical Manual (BAM) and FERN *Salmonella* methods [12]. Plating media have also been developed for isolation of *Salmonella* (and include *Salmonella*-Shigella agar (SS), brilliant green agar (BGA), bismuth-sulfite agar (BSA), Hektoen enteric (HE), and xylose-lysine-deoxycholate agar (XLD). However, due to some *Salmonella* serotypes not being distinctive and even missed on those media, yielding false negatives and increasing the cost for additional tests [16], and presumptive *Salmonella* colonies isolation, resulting in false positives [17] chromogenic and fluorogenic media have been developed to improve the detection. These include SM-ID agar, Rambach agar, ABC Medium and BBL CHROM agar *Salmonella*. The use of these media directly on the isolation plate for detection, enumeration, and identification of *Salmonella* has made improvements to the conventional methods as these media have been shown to be convenient, reliable, and more specific and selective than conventional media [12,18–20].

Rapid methods for *Salmonella* spp. detection have been developed [12] to overcome the competing flora in food samples and reduce the interference of the food matrix and increase the sensitivity of detection. Generally, the rapid test protocols include a selective enrichment stage, and then apply concentration and/or rapid detection techniques to replace culture on selective agars and further confirmatory tests. The rapid detection techniques can be divided into three categories based on the principle used: Immunology-based techniques, nucleic acid-based technique and diagnostic biosensors. Immunoassays include immunofluorescence, immunoimmobilization, enzyme-linked immunosorbent assay (ELISA) and Immunomagnetic separation (IMS) methods [12]. The major disadvantage of all immunoassays is the difficulty of getting good quality antibodies, as the accuracy of the entire reaction process depends upon the binding specificity of the antibody to all *Salmonella* cells. This is critical to prevent false-negative results as all *Salmonella* strains can cause disease in humans, leaving holes in this method if it is used to screen the food supply [21]. The nucleic acid-based detection methods are genetic methods that include hybridization and the most popular method is the polymerase chain reaction (PCR) technique [22,23]. The development and advancement of the PCR technique improves the specificity and sensitivity for detecting *Salmonella* in very low concentration (one molecule of target DNA) in a defined sample however, there is concern over the detection of live versus dead cells because DNA may linger for prolonged periods after the death of the cell. A modification of the polymerase chain reaction has resulted in an efficient method for selective detection of live *Salmonell*a cells using quantitative PCR (qPCR) [24]. Many rapid identification and confirmation methods of these techniques have been, validated, standardized and developed into commercial products by several manufacturers to be used in a simple and easy way [12]. The other rapid detection technique is the biosensor technology. Biosensors are detection/identification methods that do not require complicated and expensive assay steps. In this method a recognition signal is generated when a specific analyte binds to the biological recognition element. The signal can be a change in mass, oxygen consumption, potential difference, refractive index, pH, current, and other parameters [12]. Various pathogen-detecting biosensors have been developed, among these, optical sensors, especially colorimetric sensors, allow easy-to-use, rapid (within 15 min), portable, and cost-effective diagnosis [25]. Several reviews for different methods used to detect *Salmonella* in food samples have been published [12,16,23,26,27].

The extensive literature concerning the use of VOC analysis for identification of pathogens is already the subject of various articles [28–33]. The introduction of new analytical approaches and technological developments in instrumentation has enabled the detection of low concentrations of VOCs generated through hydrolysis of an enzymatic substrate. Analysis of volatile compounds in foods is complicated due to the presence of highly complex mixtures of the VOCs. However, GC-MS has become the first choice for analysis of volatile compounds in food samples due to its high performance in the separation and identification of complicated and similar compounds [34]. The volatile analysis using this technique requires a prior sampling step, in which volatiles are isolated from the matrix and, if possible, pre-concentrated. Headspace solid phase microextraction (HS-SPME) is a popular method of sampling and pre-concentration of volatiles and semi-volatiles, which is being routinely used in combination with GC-MS [35].

Chemical analysis of bacterial metabolites has been introduced as bacterial differentiation and detection methods [36]. Volatile organic compounds (VOCs) are produced as parts of microorganism's metabolic pathways. VOCs are a large and highly diverse group of carbon-based molecules which are naturally volatile in ambient temperature with a minimum evaporate pressure of 1 kPa [37,38]. Bacteria produce a wide range of VOCs that can be characterized in several groups including fatty acids, aromatic compounds, nitrogen containing compounds and sulphur volatile compounds [39,40]. Recent advances in ionization technologies

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