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Bacteria detection based on the evolution of enzyme-generated volatile organic compounds: Determination of *Listeria monocytogenes* in milk samples

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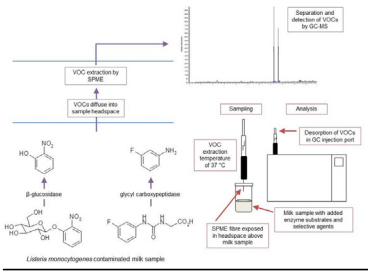
HIGHLIGHTS

- Rapid detection of *Listeria monocytogenes* contamination in food.
- Use of VOC liberating enzyme substrates.
- Analysis of VOCs by HS-SPME GC–MS.
- Use of selective agents to aid detection.

GRAPHICAL ABSTRACT



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ABSTRACT

The rapid detection of *Listeria monocytogenes* contamination in food is essential to prevent food-borne illness in humans. The aim of this study was to differentiate non-contaminated milk from milk contaminated with *L. monocytogenes* using enzyme substrates coupled with the analysis of volatile organic compounds (VOCs). The method is based on the activity of β-glucosidase and hippuricase enzymes and the detection of a specific VOC i.e. 2-nitrophenol and 3-fluoroaniline, respectively. VOCs were extracted, separated and detected by headspace-solid phase microextraction coupled to gas chromatography–mass spectrometry (HS-SPME GC–MS). This approach required the inclusion of the selective agent's cycloheximide, nalidixic acid and acriflavine HCl in the growth medium to inhibit interfering bacteria. The VOCs were liberated by *L. monocytogenes* provided that samples contained at least 1–1.5 × 10^2 CFU ml^{-1} of milk prior to overnight incubation. This approach shows potential for future development as a rapid method for the detection of *L. monocytogenes* contaminated milk.

1. Introduction

Listeria monocytogenes is a Gram-positive rod belonging to the genus *Listeria*, which also consists of nine further species: *Listeria ivanovii*, *Listeria welshimeri*, *Listeria seeligeri*, *Listeria grayi*, *Listeria innocua*, *Listeria marthii*, *Listeria rocourtiae*, *Listeria weihenstephanensis* and *Listeria fleischmannii*. *L. monocytogenes* causes food-borne infections in humans; symptoms of Listeriosis include gastroenteritis, blood poisoning and meningitis. All foods can potentially become contaminated with *L. monocytogenes*; foods most commonly associated with outbreaks include cooked meats, pâtés, shellfish and dairy products [1]. *L. monocytogenes* can survive and grow in extreme environmental conditions. It is able to grow over a temperature range of <0–45 °C which includes refrigeration temperatures, as well as extreme pH (for example, in excess of pH 9) and high salt concentrations [2]. However, identifying the source of *L. monocytogenes* contamination is often difficult. The ability of *L. monocytogenes* to survive under adverse conditions means that low levels of strains can persist in the environment for long periods of time [3]; for example, there is a

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risk that *L. monocytogenes* can proliferate if food products are refrigerated for a lengthy time period. In addition food samples are not always available to test for contamination due to the long incubation period often required for symptoms of food poisoning to develop [4]. Identifying the source of contamination is important to enable the rapid removal of foods from food suppliers and distributors, hence reducing the size of any outbreak or potential outbreak of Listeriosis [1].

The amount of *L. monocytogenes* detected in contaminated food samples varies considerably; enrichment methods are used to enable *L. monocytogenes* to grow to detectable levels. Enrichment broths incorporating selective agents such as nalidixic acid and acriflavin to inhibit background flora are used to isolate *L. monocytogenes* from contaminated foods. *L. monocytogenes* identification methods include culturing, biochemical tests and immunological assays [5]. These procedures are often time-consuming as sufficient bacterial growth is required for identification purposes. A rapid, high throughput method for the analysis of food samples that is able to detect *L. monocytogenes* contaminated foods would be highly desirable.

The hippuricase test can be one of several biochemical tests employed to identify *L. monocytogenes*. Hippuricase (glycyl carboxypeptidase) hydrolyses the peptide link in hippuric acid [6] and once hippuric acid has been hydrolysed into benzoic acid and glycine, glycine is detected by the development of a purple colour after the addition of ninhydrin [7]. *L. monocytogenes*, as well as other *Listeria* species, give positive results for the hippuricase test [8]. In addition, a VOC generating substrate targeting β-glucosidase used in conjunction with a glycyl carboxypeptidase substrate could form the basis of a simple and rapid method for the detection of *L. monocytogenes* contaminated food. The concept of using an enzyme substrate that liberates a specific VOC in the presence of bacteria has previously been reported [9–11]. In 1991 Snyder and co-workers published two papers [9,10] investigating the use of the enzyme substrate (2-nitrophenyl-β-D-galactopyranoside) in the presence of *Escherichia coli* in pure cultures to liberate the VOC, 2-nitrophenol with detection via a hand-held ion mobility spectrometer. This work, using an ion mobility spectrometer and enzyme substrates, was extended by others [11] and applied to bacteria of a food safety concern, specifically *E. coli* (using the substrate 2-nitrophenyl-β-D-glucuronide), *Aeromonas* spp. (using the substrate 2-nitrophenyl-β-D-galactoside), *Listeria* spp. (using the substrate 2-nitrophenyl-β-D-glucopyranoside) and *Staphylococcus aureus* (using the substrate 2-nitrophenyl-β-D-galactoside-6-phosphate) each liberating the VOC 2-nitrophenol. Alternative approaches for the detection of the emitted VOC from enzyme substrates are also being developed [12–16]. For example, Guillemot et al. (2013) have reported the detection of 4-nitrophenol liberated by *E. coli* in the presence of the substrate 4-nitrophenyl-β-D-glucuronide and detection by colorimetric analysis [12]. Whilst other approaches have investigated the detection of VOCs, using colorimetric analysis, emitted as a result of the metabolic growth of bacteria, and without the addition of enzyme substrates [13–16].

This study utilizes the inherent presence of bacterial enzymes to provide a rapid, non-invasive approach for the detection of listeria in food samples. The addition of specific enzyme substrates, one commercially available (2-nitrophenyl-β-D-glucoside) and the other specifically designed and synthesized for this study (2-[(3-fluorophenyl) carbamoylamino]acetic acid), to liberate unique, identifiable and quantifiable VOCs i.e. 2-nitrophenol and 3-fluoroaniline. The approach has been used for the identification of *L. monocytogenes* contaminated food samples. *L. monocytogenes* has been implicated in food-borne illness originating from various food types, but this work will focus on milk samples artificially contaminated with *L. monocytogenes*.

2. Materials and methods

2.1. Chemicals/reagents

2-Nitrophenol (98%) was purchased from Sigma–Aldrich (Poole, UK) and 2-nitrophenyl-β-D-glucoside was obtained from Apollo Scientific (Stockport, UK). 3-Fluoroaniline (99%) was obtained from Alfa Aesar (Morecambe, UK).

Columbia blood agar with 5% defibrinated horse blood, brain heart infusion broth, Listeria enrichment broth and Listeria selective enrichment supplement containing cycloheximide 25.0 mg, nalidixic acid 20.0 mg and acriflavine HCl 7.5 mg 500 ml^{-1} broth were purchased from Oxoid (Basingstoke, UK).

Types of milk used: whole milk (3.6% fat), semi-skimmed milk (1.8% fat), unhomogenised milk (5.2% fat), soya milk, whole goat's milk (3.6% fat), skimmed milk (0.1% fat) and unpasteurised milk.

2.2. Instruments

The GC–MS instrument used to separate and detect bacterial VOCs was a Trace GC Ultra fitted with a Polaris Q ion trap mass spectrometer with Xcaliber 1.4 SR1 software. Separation of VOCs was carried out using a 30 m × 0.25 mm ID × 0.25 μm VF-waxMS capillary column. The S/SL injector was set at 230 °C and operated in split mode with a flow rate of 10 ml min^{-1} and a split ratio of 10. The temperature program used was: 50 °C held for 2 min then increased at a rate of 10–220 °C min^{-1} with a final 2 min hold. The carrier gas was helium with a flow rate of 1.0 ml min^{-1}. Electron ionisation (EI) was used (70 eV), and the mass spectrometer was operated in total scan mode over an m/z range of 50–650 amu. The transfer line was held at 250 °C while the ion source temperature was maintained at 260 °C.

2.3. Procedure: SPME parameters

VOCs were extracted for 10 min at 37 °C (no stirring) prior to a desorption for 2 min at 230 °C in the injection port of the GC–MS prior to separation and identification. All fibres were conditioned in the GC injection port prior to use as directed by manufacturers guidelines. All fibres were used with a manual holder.

2.4. Procedure: microbiology

All bacteria were obtained from the National Collection of Type Cultures (NCTC) at the Microbiology Department, Freeman Hospital, Newcastle upon Tyne. Table 1 lists all strains used. Bacteria were stored and subcultured weekly on Columbia blood agar with 5% defibrinated horse blood.

Table 1
List of bacteria.

Listeria species	Gram-positive bacteria	Gram-negative bacteria
Listeria monoctyogenes NCTC 11994	*Corynebacterium striatum* NCTC 764	*Escherichia coli* NCTC 12079
Listeria monocytogenes NCTC 10357	*Corynebacterium xerosis* NCTC 9755	*Salmonella enteritidis* NCTC 6676
Listeria welshimeri NCTC 11857	*Lactococcus lactis* NCTC 662	
Listeria seeligeri NCTC 11856	*Lactobacillus acidophilus* NCTC 4504	
Listeria innocua NCTC 11288	*Bacillus licheniformis* NCTC 10341	
Listeria ivanovii NCTC 11846	*Bacillus subtilis* NCTC 3610	
Listeria grayi NCTC 10815	*Bacillus cereus* NCTC 7464	
	Enterococcus faecalis NCTC 775	
	Enterococcus faecium NCTC 7171	

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