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**Detection of Exogenous VOCs as a Novel *in vitro* Diagnostic Technique  
for the Detection of Pathogenic Bacteria**

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## **Abstract**

The evolution of volatile organic compounds (VOCs) provides an opportunistic approach for the detection of pathogenic bacteria. This approach can be enhanced by the application of VOC-labelled enzyme substrates that target specific enzyme activities of the bacteria under investigation.

Detection of the VOCs provides a novel, specific and sensitive approach for the detection of pathogenic bacteria. This review highlights the importance of this approach alongside a range of alternative detection techniques for the identification of pathogenic bacteria.

**Keywords:** Volatile organic compounds; detection; enzymes; markers; diseases

## **Introduction**

The rapid and specific detection of a pathogen within a polymicrobial culture can be achieved by the analysis of volatile organic compounds (VOCs) present in the atmosphere above both agar and broth cultures [1,2]. Many cultures produce a number of VOCs naturally (endogenous VOCs) as a result of metabolism of substrates available in the growth media provided, thus the methodology can be broadly applied to culture methods currently in use assuming the bacteria of interest produces VOCs. A number of studies have described the ability to identify bacteria based on the VOCs detected in the atmosphere surrounding a culture [2-5]. The potential methods for VOC detection are shown in Figure 1.

It has long been known that cultures of certain species of bacteria will produce a characteristic odour, which can often be used as a crude means of presumptive species identification; for example the odour associated with an *Escherichia coli* culture, partly caused by metabolism of tryptophan to indole by the enzyme tryptophanase [6]. In reality the odour experienced by the human olfactory system is caused by a complex mixture of VOCs. There has been a significant volume of research conducted into the elucidation of the odour causing compounds in a search for species specific

profiles of volatile metabolites, which could act as biomarkers and serve as a means of diagnosis [3, 5, 7-11].

Solid phase microextraction (SPME) is the most commonly used headspace sampling technique; frequently coupled with gas chromatography-mass spectrometry (HS-SPME-GC-MS) as a means of separating and identifying culture VOCs. In initial evaluation of this approach it is important to consider and optimize various sampling parameters, such as the fibre coating type, extraction time, temperature equilibration time, extraction temperature and desorption temperature [5]. There is a large body of published data regarding the identification of *in vitro* volatile microbial metabolites by HS-SPME-GC-MS and other techniques such as ion mobility spectrometry (IMS), which have been extensively reviewed in the literature by various authors [12-15], much of which has been compiled in the online database mVOC [16]. Therefore, while some work has identified some degree of specificity in terms of key VOCs it is apparent that many of the VOCs identified are found to be common in pathogenic species of interest due to shared metabolic pathways. Often studies employ pattern recognition techniques, such as principal component analysis of the profile of volatiles, to distinguish between species. However, many experimental parameters must be controlled to obtain reproducibility of VOC profiles for this type of statistical analysis to be relevant in terms of identification of bacteria. Many factors can affect the VOCs detected, such as the growth medium, sampling technique, strain tested, and the initial and final sampling time.

The commonality of reported VOCs between different species, and difficulties encountered in corroborating findings due to differing methodologies, suggests that the profiling of endogenous volatile metabolites cannot provide a robust means of detecting specific pathogens of interest. Therefore, other means of generating specific VOC biomarkers must be investigated which have the potential to be more robust and specific. This review specifically focuses on the use of enzyme substrates that will liberate exogenous VOC biomarkers for the detection of pathogenic bacteria. By

considering examples from the literature allows a range of pathogenic bacteria to be investigated against a diverse range of analytical techniques for detection of VOCs.

### **Use of enzyme substrates to liberate exogenous VOCs**

To increase the diagnostic specificity of VOCs, present in a culture headspace, it is possible to modify a given growth medium by the addition of a substrate that will liberate a unique VOC upon enzymatic metabolism, which is ideally non-biogenic, in response to the presence of enzyme activity exhibited by a target pathogen. Such metabolic enzyme substrates are commonly used in selective growth medium for the isolation of bacteria [17-19]. However, the liberated label compound will contain either a chromophore or fluorophore, which will result in the formation of coloured/fluorescent colonies upon growth of the target bacteria; the colour/fluorescence pertaining to the presence of a specific enzyme produced by the target microorganism. In order to apply this concept to VOC diagnostics, a pathogen with known enzyme activity is identified. Then, a specific substrate is selected that will be metabolised to liberate a compound with a sufficiently high vapour pressure that can be detected in the gas phase. Many commercially available substrates used in biochemical assays are labelled with 2-nitrophenol, such as 2-nitrophenyl- $\beta$ -D-glucopyranoside which is hydrolysed by the enzyme  $\beta$ -glucosidase (Figure 2). 2-Nitrophenol has a sufficiently high vapour pressure that it can be detected in the headspace above a culture (Figure 3). Detection of 2-nitrophenol in biological assays is usually determined by visual inspection (yellow colour formation) or spectrophotometry, detection in the headspace provides a significant advantage as there is no potential for interference in the measurement by the assay medium.

Ion mobility spectrometry (IMS) has been utilized as an analytical technique for detection of enzymatically produced volatile label compounds by Snyder et al. [20] IMS separates analytes in the gas phase, based on their charge, mass, and cross-sectional area. This technique is particularly

advantageous for making rapid measurements of vapours of complex composition, as separations occur on the time scale of milliseconds thus improving sample throughput capabilities.

Snyder et al. [20] deployed a hand-held IMS, in a proof of concept study, designed to detect *E. coli* contamination with potential applications of waste water monitoring and detection from clinical samples. A solution of the enzyme substrate 2-nitrophenyl- $\beta$ -D-galactopyranoside was dispensed onto a filter paper disc placed in the bottom of a 5 ml glass vial. After incubation of the substrate, with pure cultures of *E. coli*, in phosphate-buffered saline solution (PBS) at 40-42 °C detection by IMS in the culture headspace revealed 2-nitrophenol as a volatile metabolite within ten minutes. The presence of 2-nitrophenol in the sample headspace can be attributed to the production of the  $\beta$ -galactosidase enzyme by the target microorganism *E. coli*. However this enzyme is not specifically produced by *E. coli*, therefore there is a high likelihood that environmental, food, and clinical samples may contain other  $\beta$ -galactosidase producing bacteria, such as lactic acid bacteria [21] and *Bacillus* spp. [22], which could lead to the false positive detection of *E. coli* thereby limiting the applicability of the assay.

The lack of specificity of the  $\beta$ -galactosidase enzyme as a biomarker for *E. coli* was recognized [23] in an assay for enumeration of coliforms in processed foods. Instead, the enzyme  $\beta$ -glucuronidase was chosen as a more specific marker for *E. coli* [24]; the ability of bacteria to produce the enzyme  $\beta$ -glucuronidase is less prevalent in other species, thus decreasing the potential number of false positive results. Application of the assay to the detection of *E. coli* in various cooked and raw meats, after addition of the enzyme substrate, demonstrated a capability to detect  $1.4 \times 10^1$  CFU ml<sup>-1</sup> of *E. coli* after just 9 hours of incubation [24]. Interestingly it was shown that control samples of liver also produced the VOC label 2-nitrophenol, after just one hour of incubation. This was attributed to the inherent  $\beta$ -glucuronidase activity of the food matrix, as opposed to a  $\beta$ -glucuronidase producing contaminating species of bacteria.

This concept was further extended to encompass a larger range of foodborne pathogens [25], by attributing a VOC labelled enzyme substrate to each target pathogen based on known enzyme production (Table 1). The assay was applied to pure cultures of each pathogen in standard enrichment broths commonly used to isolate each species from food samples. While each of the enzymes targeted as biomarkers are produced by the target species, other species of bacteria can also produce the enzymes required to hydrolyse the substrates, a potential source of false positives. The number of potential false positives varies depending on the targeted enzyme activity; however, it is possible to guard against false positives by the inclusion of antibiotics to suppress the growth of non-target species which may produce the targeted enzyme.

The detection of *Listeria monocytogenes* in milk samples with HS-SPME-GC-MS as the detection method was investigated [26]. This approach utilized substrates targeting  $\beta$ -glucosidase and hippuricase enzyme production (2-nitrophenyl- $\beta$ -D-glucopyranoside and 2-[(3-fluorophenyl) carbamoylamino] acetic acid), that could be used successfully to differentiate milk samples spiked with *L. monocytogenes* from non-inoculated controls in a selective *Listeria* enrichment broth. Although *L. monocytogenes* could be successfully identified in pasteurized milk samples, the chosen analyte matrix is expected to contain an inherently low number of competing microflora, due to milk pasteurization. It was also demonstrated that pure cultures of *Enterococcus faecalis* and *Lactobacillus acidophilus* could hydrolyse the enzyme substrates in the presence of the selective antimicrobials included in the broth, and therefore could give false positive results were they to survive pasteurization [26].

Another study, by the same group, involved the development of a novel VOC assay for the detection of *Clostridium difficile* in stool samples by inclusion of VOC liberating enzyme substrates and selective antibiotics in a liquid growth medium [27], in conjunction with a sample pre-treatment step to eliminate competing microflora. By subjecting the stool samples to alcohol (ethanol) shock the

large number of microflora present in a stool sample were killed leaving only spores of *C. difficile*. Subsequent removal of the ethanol and inoculation of the stool sample in cooked meat broth allowed the spores to germinate, and *C. difficile* to grow and produce volatile metabolites detectable by HS-SPME-GC-MS. Previously it has been noted [28] that volatiles such as, *p*-cresol and short chain fatty acids, are known to be associated with stool samples of patients suffering gastrointestinal disease, and have been proposed as biomarkers for *C. difficile* infection. However, they are not specifically produced by *C. difficile*, whereas the metabolic pathway to production of *p*-cresol is almost unique to *C. difficile*. The *p*-cresol production by *C. difficile* is due to the metabolism of *p*-hydroxyphenylacetic acid (a product of L-tyrosine metabolism) by decarboxylation to *p*-cresol. Use of a halogen-labelled version of this substrate i.e. 3-fluoro-4-hydroxyphenylacetic acid, resulted in the production of the VOC 2-fluoro-4-methylphenol, thus facilitating differentiation of the production of *p*-cresol by other species. In a blind study of 100 stool samples, 100 % of *C. difficile* positive stool samples could be correctly identified by this methodology [27].

The use of VOC-liberating enzyme substrates has also been applied as a detection method in an immunoassay [29]. In this instance, an antibody specific to the target pathogen was used with an enzyme of choice. Upon addition of a compatible VOC labelled enzyme substrate to the assay medium the pathogen was detected by VOC analysis by IMS. This type of assay known as enzyme-linked immunosorbent assay (ELISA) usually employs an enzyme substrate labelled with a chromophore / fluorophore. The use of VOC labelled enzyme substrates in ELISA, was first developed as an assay for detection of *Bacillus cereus* [29]. The developed assay employed a polystyrene paddle, as a solid immunosorbent support, for the adherence of the *B. cereus* antigen. Further binding of a biotinylated secondary antibody enzyme-linked to  $\beta$ -galactosidase, and subsequent addition of the enzyme substrate 2-nitrophenyl- $\beta$ -D-galactopyranoside, facilitated *B. cereus* detection within 8 mins by IMS detection of the 2-nitrophenol hydrolysis product. A similar methodology has been applied to the detection of *Salmonella typhimurium* following a standard



ELISA protocol in a 96 well plate [30]. The CSA-1 antibody conjugated to alkaline phosphatase was used for the antigen-antibody capture of *S. typhimurium*, which once bound was incubated with phenyl phosphate.

It has been reported that antibody labelled magnetic particles can increase the chance of antibody-antigen interactions occurring in comparison to a standard ELISA due to their higher surface area to volume ratio and dispersion in solution [31, 32]. In this approach *E. coli* cells were mixed in a solution of the goat anti *E. coli* 0157-H7 antibody-labelled magnetic particles, and one of 3 enzyme-linked antibodies. Once *E. coli* had bound to the particles enzyme-linked antibody, an assembled magnetic apparatus consisting of a rod shaped NdFeB magnet was inserted into the microcentrifuge tube and the immunomagnetic particles of immobilised *E. coli* were collected as a pellet. The pellet was then removed and placed in a solution of enzyme substrate corresponding to the enzyme-linked antibody. The pellet was then incubated at 37 °C for up to 10 mins to produce the substrate metabolite which was detected by IMS (Figure 4).

### **Gel-based optical VOC sensors**

Efforts to remove the necessity for analytical instrumentation for the detection of VOCs, and produce a simple device for culture and detection of exogenous VOCs have involved the doping of polymeric gel matrices with chemo selective indicator compounds. In this situation, the indicator compound can react with a VOC label to produce a coloured product which can be detected spectrophotometrically or by the unaided eye. Chemically doped gel matrices provide a low cost alternative to instrument based methods of VOC detection; and gel matrices, such as agarose and most commonly silica, have been used for this purpose due to their ease of preparation, ability to tailor gel properties for optimum detection of analyte, ease of doping with chemical sensor molecules, transparency to UV/visible electromagnetic radiation permitting conjunction with UV/vis

based detection methods, and ability to be cast as monoliths, powders or thin films for different sensor applications [33].

Silica xerogels have been employed in optical gas sensing devices for the detection of toxic air pollutants for environmental monitoring [34, 35]. However, it is only recently this methodology has been applied to the detection of VOCs produced by enzymatic reactions of bacteria. Silica xerogels can be prepared via the 'sol-gel' process, in which a silica alkoxide precursor such as tetramethyl orthosilicate (TMOS) is mixed with a solution of organic solvent (usually methanol or ethanol) and water, known as a sol. TMOS is hydrolysed by water to produce a silicon alkoxide with the organic solvent acting to solubilize the immiscible reactants in the same phase; the silicon alkoxide generated *in situ* readily undergoes polycondensation to form a polymeric network of siloxane nanoparticles. These nanoparticles grow in size and agglomerate to eventually form a nanoporous gel which must be aged and dried to form the silica xerogel. Addition of a chemical indicator compound when preparing the sol provides a simple means by which the xerogel can be doped, as the indicator compound remains within the pores of the gel once the gel is formed. In a proof of concept study [36] a doped silica xerogel based sensor was developed, surface-functionalized with (3-aminopropyl)triethoxysilane (APTES) for the detection of indole, with the dopant compound *p*-dimethylaminocinnamaldehyde (*p*-DMACA) acting as an indicator. Upon enzymatic production of indole by bacteria and diffusion into the pores of the xerogel, indole reacts with the *p*-DMACA to produce a green azafulvenium chloride salt. Using this sensor and a modified petri dish with a suspended compartment to house the sensor, as few as 100 cells of *E. coli* could be detected when cultured on LB agar or DEV tryptophan agar within 17 hours of incubation by observing the gel colour change from orange to green. A similar APTES functionalized silica xerogel was also produced by the same group [37] for the detection of 4-nitrophenol produced by the enzymatic hydrolysis of 4-nitrophenyl- $\beta$ -D-glucuronide by *E. coli*. In this system [37], the xerogel is cast into the recess of a cuvette, above which an *E. coli* culture containing the substrate (i.e. 4-nitrophenyl- $\beta$ -D-glucuronide)

was placed in a microcentrifuge tube. The whole apparatus was placed in a sealed vessel, such that the evolved 4-nitrophenol diffused into the nanoporous xerogel. The basic environment within the pores led to the deprotonation of p-nitrophenol yielding the yellow coloured phenolate anion.

A similar methodology was used for the detection of 2-nitrophenol [38], using agarose as the gel matrix cast into a spectrophotometry cuvette. The preparation of agarose gel sensors can be achieved by simply microwaving a solution of agarose in distilled water, followed by addition of aqueous solutions of the dopant indicator compounds, in this case NaOH for the detection of 2-nitrophenol, or the sodium salt of 1,2-naphthoquinone-4-sulphonic acid (NQS) for the detection of aniline. These doped agarose gels were used to study enzyme production by some common pathogenic bacteria. Production of  $\beta$ -glucosidase by *E. faecalis* and *Klebsiella pneumoniae* could be optically observed by deprotonation of 2-nitrophenol to form the yellow phenolate anion within the NaOH-doped agarose gel upon metabolism of 2-nitrophenol- $\beta$ -D-glucopyranoside. Similarly, production of  $\beta$ -alanine aminopeptidase by *Pseudomonas aeruginosa* resulted in hydrolysis of the enzyme substrate 3-amino-N-phenylpropanamide, liberating aniline which subsequently became entrapped in the NQS-doped agarose gel, reacting to produce an orange colouration. The production of the coloured products produced could be followed by observation of absorbance at 415 nm for the 2-nitrophenolate and 470 nm for the NQS/aniline conjugate. By this method approximately  $1-1.5 \times 10^4$  CFU ml<sup>-1</sup> *Enterococcus faecium* and *P. aeruginosa* could be detected after 18 h of incubation.

### **Optoelectronic colorimetric sensor arrays**

A promising development in the production of a low cost VOC based *in vitro* diagnostic test for pathogen identification is the optoelectronic colorimetric sensor array. The array consists of numerous 'chemo responsive' dyes which change colour on exposure to the headspace of a broth or agar culture of bacteria, and can achieve species level recognition of bacteria based on the measured colour changes of each dye. The device is fabricated by printing an array of chemo

responsive dyes onto a polymer film, each dye is added as a dopant to a silica gel sol mixture which is specifically designed for optimal entrapment of the respective dye. The sol mixtures are printed onto the polymer film using an automated microarray printer which spots each sol mixture onto the film which is then allowed to slowly age under a stream of nitrogen over 3 days. These microarrays were initially deployed to detect toxic industrial air pollutants [39]; thus the technology is transferrable as a tool for diagnosis of bacteria by measuring its response to the headspace of a microbial culture. Although the specific interactions of volatile headspace metabolites and the indicator dye compounds were not investigated, the dyes selected for the microarray were selected such that they should respond to potential volatile metabolites. Various iterations of the microarray have been produced with differing numbers of dye indicators, however the same general groups of indicators are used: metal ion containing dyes to sense Lewis basicity (amines), pH indicators to sense Brønsted acidity/basicity (amines, fatty acids), dyes with large permanent dipoles to sense local polarity (alcohols), metal salts to sense redox reactions (sulphides), and nucleophilic indicators to detect electrophilic analytes (aldehydes) [40]. The microarray works on a pattern recognition basis, such that the culture headspace of each species of bacteria grown in a particular growth medium will produce a different colour response on the array. The response of the array is measured by image scanning of the array at numerous time intervals, and measuring the difference in the red, green and blue (RGB) components of each spot in each image. The specific pattern of RGB difference can be attributed to a species of bacteria, and can be used to achieve a species level differentiation. The optoelectronic microarray sensor has been successfully applied to the detection of the potential bioterrorism agents *Bacillus anthracis* and *Yersinia pestis*, numerous sepsis causing bacteria in blood culture, pathogenic fungi, and species level detection of 15 common clinical isolates on blood agar [1, 40-42].

### **Mass spectrometry VOC fingerprinting**

Deficiencies in standard methodologies for microbial VOC analyses employing analytical instrumentation such as GC-MS include: the requirement for sample pre-concentration usually by SPME, requirement for skilled operatives to maintain and run equipment, low sample throughput due to lengthy analysis times, and potential systemic discrimination against analytes of low molecular mass, and analytes containing moieties which are strongly adsorbed in the injection port or on the column which require derivatization before analysis. Recently several mass spectrometric techniques have been applied to the analysis of microbial VOCs which directly sample the atmosphere above microbial cultures, eliminating the need for sample pre-concentration often providing near real-time and continuous measurements of the VOCs present, allowing dynamic changes in the VOC profile to be monitored.

A number of experiments performed *in vitro* have demonstrated the ability to discriminate between inoculated and non-inoculated growth media based on the presence or absence of molecular ions in the mass spectra of culture headspace [43-45]. Proton Transfer Reaction Mass-spectrometry (PTR-MS) has been used to differentiate between cultures of *E. coli*, *Salmonella enterica*, *Shigella flexneri*, and the yeast *Candida tropicalis* [43] which were cultured according to Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) recommended conditions. VOCs were assigned tentative identities based on  $m/z$  values corresponding to the molecular mass of the compound plus that of a proton due to the ionization method of proton transfer reaction (addition of a proton to the analyte by reaction with a hydronium ion), and VOCs expected to be produced as described in the scientific literature. Compounds with the same expected  $m/z$  could be differentiated based on the relative isotopic abundance of  $^{13}\text{C}$ . Using this methodology, several unique ions could be detected in the headspace of all tested species. The dynamic nature and necessity for continuous analysis of the headspace was observed, as the ion assigned as acetone could only be detected within the first 100 minutes of culture. Similarly, the application of atmospheric pressure chemical ionization mass-spectrometry (APCI-MS) could be applied to the detection of *K. pneumoniae*, *Acinetobacter*

*baumannii*, *E. coli*, *Staphylococcus aureus*, *P. aeruginosa*, and three species of *Candida* [44] grown in Mueller-Hinton medium. Mass spectra from cultures were obtained in the positive and negative ionization mode, and signals obtained from sterile culture media were subtracted from the spectra. An increase in a signal with  $m/z$  of 70 was associated with growth of *P. aeruginosa*. *Candida* species showed increased production of an  $m/z$  signal of 140 whereas a  $m/z$  signal of 101 was only observed in cultures of *S. aureus*, among the bacteria tested, and was tentatively attributed to the production of isovaleric acid which has been reported previously as a biomarker for *S. aureus* [46]. *E. coli* cultures also produced a  $m/z$  signal of 116 attributed to the deprotonated form of indole, with *E. coli* being a well-known indole producing species [6]. Overall *S. aureus* cultures produced the highest total number of signals in the VOC fingerprint (25), and *A. baumannii* the lowest (6) [44].

Selected ion flow tube mass spectrometry (SIFT-MS) and APCI-MS have been used to detect bacterial growth and potential species identification of bacteria in a blood culture [45, 47], as well as determining antibiotic susceptibilities of *E. coli* to gentamicin, and *S. aureus* to flucloxacillin during culture. Using SIFT-MS the VOC profiles of *Streptococcus pneumoniae*, *P. aeruginosa*, *E. coli*, *S. aureus*, and *Neisseria meningitidis* were determined in fastidious antimicrobial neutralization BacT/ALERT<sup>®</sup> bottles, the effect of antibiotics on VOC production was determined for *E. coli* with Gentamicin and *S. aureus* with Flucloxacillin using standard aerobic bottles [46]. Blood samples taken from healthy individuals were added to the BacT/ALERT<sup>®</sup> bottles, and inoculated with 10 CFU of test species to simulate a bacteraemia ( $10^3$  CFU for susceptibility experiments) with incubation at 37 °C. As opposed to the previously described study in which all ions over a specified mass range were observed for headspace analyses, in this study a number of VOCs were identified as suitable biomarkers for the test species including: 2-aminoacetophenone, ammonia, dimethyldisulfide (DMDS), dimethylsulfide (DMS), formaldehyde, ethanol, hydrogen sulfide, indole, methanethiol, pentanols, and propanol [47]. The SIFT-MS was set up in single ion monitoring (SIM) mode to

specifically detect ions relating to these compounds, with acetaldehyde, acetic acid, ethanol, acetone, ammonia, hydrogen sulfide, DMS, and DMDS concentrations quantified. Using this methodology, a profile of the concentrations of identified biomarkers could be determined for each species, which could potentially be used to differentiate between the species of bacteria tested. It was also found for cultures of *E. coli* and *S. aureus* that addition of gentamicin and flucloxacillin, respectively above and below the reported minimum inhibitory concentration (MIC) resulted in a reduction in VOC concentrations measured with the most significant reduction found above the MIC; this could provide a potential means of determining antibiotic susceptibility before pathogens are isolated.

Using APCI-MS, VOCs were determined for the detection of bacteraemia caused by *S. aureus*, *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*, with these species reported to be responsible for 50 % of cases of bacteraemia [45]. Blood samples from 180 healthy individuals were mixed with tryptic soya broth, and split into sub-samples each inoculated at  $10^4$  CFU ml<sup>-1</sup> of *S. aureus*, *E. coli*, *K. pneumoniae*, *A. baumannii*, or *P. aeruginosa*. VOC production was measured continuously by APCI-MS at 35 °C. The determined VOC profiles were used to diagnose bacteraemia caused by the aforementioned pathogens. Thirty three cases of bacteraemia were successfully identified as being caused by the tested pathogens using APCI-MS as a detection method and corroborated by conventional methods.

VOC determination via mass spectrometry has also been proposed as a means of detecting lung infections *in vitro*. SIFT-MS analyses performed on 21 *P. aeruginosa* isolates obtained from cystic fibrosis patients, whose lungs were colonized with the bacteria, found that detection of hydrogen cyanide as a VOC was highly associated with *P. aeruginosa* infection [47]. Cough, swab, or sputum samples provided by the patient were inoculated onto blood agar, and *Pseudomonas* selective medium. Plates were sealed inside a plastic bag and incubated at 37 °C for 48 h before SIFT-MS

analysis [48]; hydrogen cyanide could be detected in the headspace of 15/22 culture positive samples indicating its suitability as a biomarker for *P. aeruginosa*. Further studies have shown that VOC fingerprinting by SESI-MS could potentially be used as an *in vivo* diagnostic test to analyse breath for the detection of *P. aeruginosa* and *S. aureus* infection in a murine model by secondary electrospray ionization mass-spectrometry (SESI-MS) [49].

## Conclusions

The practical use of VOC detection as an *in vitro* diagnostic technique is ultimately governed by the specificity of VOCs produced as markers for the bacteria of interest, and robustness of VOC production across numerous strains of the species. The use of VOC labelled enzymatic substrates targeting specific enzyme activities of the target bacteria, requires use of selective growth medium, or cell separation methodologies to negate potential false positive results which may be caused by the microflora of the sample matrix. The use of higher cost analytical instrumentation must also be replaced by development of low cost sensors; optical gel sensors have been proposed for this purpose, however implication of other sensor technologies may be possible. Methods employing enzymatic substrates must be extensively validated for use with specific sample matrices, and the ability to achieve comparable specificity and sensitivity with conventional methods, as well as improved time to detection must be demonstrated. High specificity and sensitivity appears to be achievable when employing methods involving the detection of VOC metabolite profiles using colorimetric sensor arrays, and mass spectrometry. Although the application of these methods is seemingly limited to monomicrobial samples, specific applications such as bacteria identification in a blood culture bottle pose great advantages over conventional methods, chiefly a significant reduction in time to diagnosis and therefore patient treatment. A comprehensive summary of the suitability of each analytical technique for exogenous VOC detection is reported in Table 2. Table 2 also considers the advantages and limitations of each approach. There are still significant challenges to overcome in implementing this methodology, however promising progress is being



made, and even potential *in vivo* use has been described. Other avenues to investigate include determination of antibiotic susceptibility which could eventually lead to commercial products. Ultimately these methods may be used as a sample screening technique to provide ahead of time diagnoses, which can influence decisions on therapeutic treatment of infections and food safety. Initial VOC screening results can later be confirmed by culturing and biochemical testing.

### **Conflict of interest:**

The authors have no conflict of interest to declare.

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**Table 1. Foodborne illness causing bacteria and enzyme activities used as biomarkers**

<b>Microorganism</b>	<b>Enzyme</b>	<b>Enzyme substrate</b>	<b>VOC</b>
<i>E. coli</i>	$\beta$ -glucuronidase	2-nitrophenyl- $\beta$ -D-glucuronide	2-nitrophenol
<i>Yersinia</i> spp.	Urease	Urea	ammonia
<i>Aeromonas</i> spp.	$\beta$ -galactosidase	2-nitrophenyl- $\beta$ -D-galactoside	2-nitrophenol
<i>Listeria</i> spp.	$\beta$ -glucosidase	2-nitrophenyl- $\beta$ -D-glucoopyranoside	2-nitrophenol
<i>Staphylococcus aureus</i>	phospho- $\beta$ -galactosidase	2-nitrophenyl- $\beta$ -D-galactoside-6-phosphate	2-nitrophenol

**Table 2. A comparison of the analytical techniques used for detection of exogenous VOC biomarkers**

VOC	Sample Matrix	Analytical Technique	Microorganism	Time to Detection	Sensitivity	Advantages	Limitations	Reference
2-Nitrophenol	Cellulose filter paper	IMS (hand held)	<i>E. coli</i>	10 – 30 min	$2.0 \times 10^2$ CFU ml <sup>-1</sup>	Separation of complex VOC mixtures achieved on rapid timescales, portable instrumentation, simple bacteria detection based on observation of single VOC analyte	Requires 48 h pre-incubation to induce $\beta$ -D-galactosidase, presumptive VOC identification, bacteria detection based on enzyme activities which are non-specific	[20]
2-Nitrophenol	Cooked/raw meats	IMS	<i>E. coli</i>	9 hours	$1.4 \times 10^1$ CFU ml <sup>-1</sup>	Separation of complex VOC mixtures achieved on rapid timescales, simple bacteria detection based on observation of single analyte	Bacteria detection based on enzyme activities which are non-specific	[24]
Ammonia, 2-nitrophenol	Enrichment broth	IMS (automated)	<i>A. hydrophilia</i> , <i>E. coli</i> , <i>Listeria</i> spp., <i>S. aureus</i> , <i>Y. enterocolitica</i>	~ 10 – 20 hours	< 1.0 CFU ml <sup>-1</sup>	Automated sample processing, separation of complex VOC mixtures achieved on rapid timescales, provides estimation of colony count, simple bacteria detection based on observation of	Presumptive VOC identification, bacteria detection based on enzyme activities which are non-specific	[25]

2-Nitrophenol, 3-fluoroaniline	Milk	HS-SPME- GC-MS	<i>Listeria</i> spp.	24 hours	1 – 1.5 x 10 <sup>2</sup> CFU ml <sup>-1</sup>	single analyte Rapid detection in comparison to culture based methods, non-invasive sampling by SPME, electron ionization permits VOC identification by fragmentation pattern, greater analyte separation power compared to IMS	Bacteria detection based on enzyme activities which are non-specific, high-cost specialist instrumentation, instrument bias against low-weight and reactive analytes, selective bias of sampling technique	[26]
2-Fluoro-4-methylphenol, isocaproic acid, p-cresol	Stool sample	HS-SPME- GC-MS	<i>C. difficile</i>	24 hours	83.1 % culture-positive samples	Rapid detection in comparison to conventional methods, non-invasive solventless sampling by SPME, sample processing selective for growth of <i>C. difficile</i> , electron ionization permits VOC identification by fragmentation pattern, greater analyte separation power compared to IMS	Some samples present false negative VOC profile, high-cost specialist instrumentation, low sample throughput, instrument bias against low-weight and reactive analytes, selective bias of sampling technique	[27]

2-Nitrophenol	Phosphate-buffered saline	IMS (hand held)	<i>B. cereus</i>	8 mins	$< 1 \times 10^3$ CFU ml <sup>-1</sup>	Separation of complex VOC mixtures achieved on rapid timescales, portable instrumentation, VOC production based on specific antibody-antigen recognition	Laborious sample preparation, presumptive VOC identification	[29]
Phenol	Alkaline phosphatase buffer	IMS (hand held)	<i>S. typhimurium</i>	< 2 hours	30 – 50 ng phenol, $\sim 10^6$ CFU ml <sup>-1</sup>	Separation of complex VOC mixtures achieved on rapid timescales, portable instrumentation, VOC production based on antibody-antigen recognition, favourable performance in comparison to standard ELISA protocol, simple bacteria detection based on observation of single analyte	Laborious sample preparation, requires high inoculum of bacteria, presumptive VOC identification	[30]
2-Nitrophenol, 8-hydroxyquinoline	PBS buffer	IMS	<i>E. coli</i>	10 mins	$10^6$ CFU ml <sup>-1</sup>	Separation of complex VOC mixtures achieved on rapid timescales, selective separation of bacteria from sample matrix by antibody-labelled magnetic particles, reduction in	Requires high inoculum of bacteria, use of expensive assay reagents	[32]

						number of sample preparation steps in comparison to standard ELISA, simple bacteria detection based on observation of single analyte		
Indole	DEV tryptophan agar	Optical silica gel sensor	<i>E. coli</i>	17 hours	92 CFU	Low-cost VOC sensing device, Silica gel is tunable for optimal entrapment/exclusion of VOCs, chemical doping of gel is easily achieved, naked eye detection, potentially compatible with conventional diagnostic products	Requires indicator compound which reacts specifically with VOC analyte, potential for reaction of non-target VOCs with sensor	[36]
2-Nitrophenol, aniline	Brain heart infusion broth	Optical agarose gel sensor	<i>E. faecium</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i>	18 hours	$1 - 1.5 \times 10^4$ CFU ml <sup>-1</sup>	Low-cost VOC sensing device, chemical doping of gel is easily achieved, naked eye detection, potential for integration into commercial product, potentially compatible with conventional diagnostic products	Requires indicator compound which reacts specifically with VOC analyte, potential for reaction of non-target VOCs with sensor	[37]
Not identified	Blood agar	Colorimetric	<i>A. baumannii</i> , <i>B. anthracis</i> ,	6.8 – 11.2	10 <sup>2</sup> CFU	Sensitive and specific detection of pathogenic	Non-specific detection of VOCs,	[1, 39]



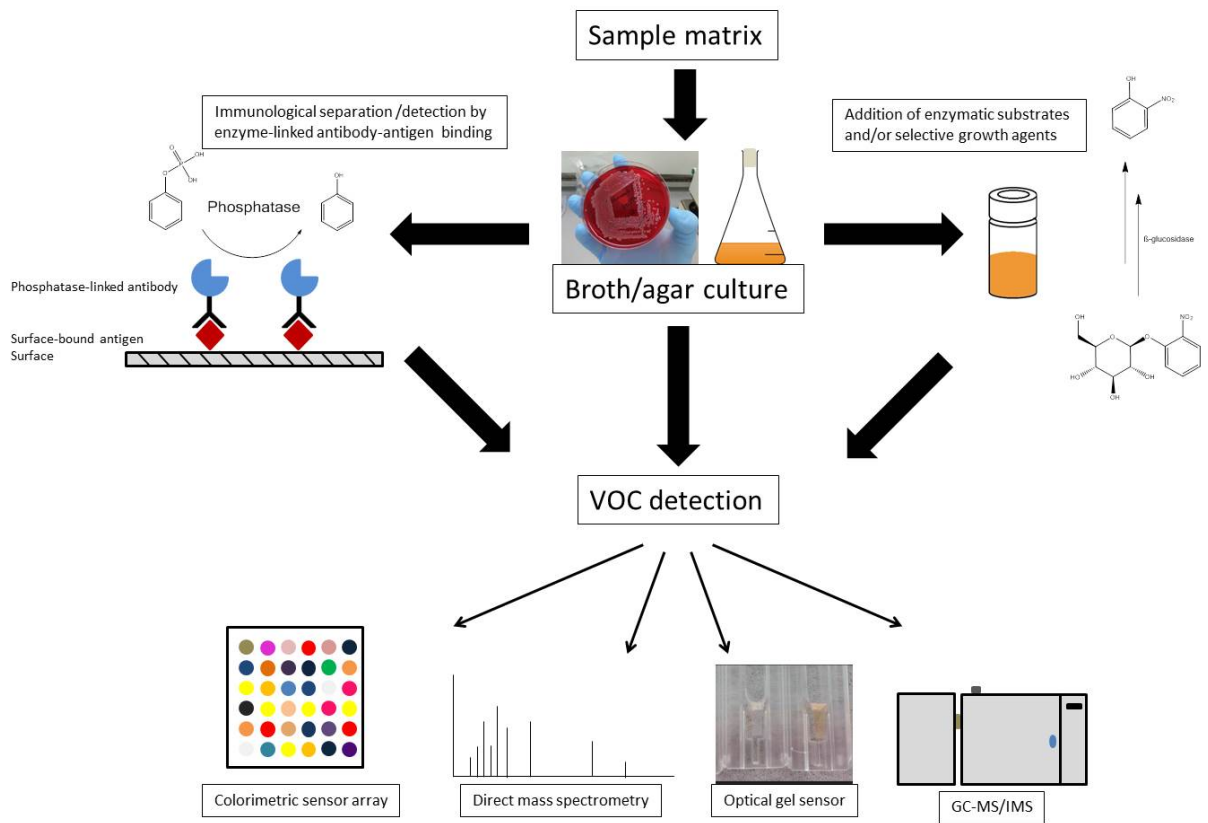
		sensor array	<i>E. cloacae</i> , <i>E. coli</i> , <i>E. faecalis</i> , <i>E. faecium</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> , <i>P. mirabilis</i> , <i>S. agalactiae</i> , <i>S. aureus</i> , <i>S. epidermis</i> , <i>S. lugdunensis</i> , <i>S. maltophilia</i> , <i>S. marcescens</i> , <i>S. pneumoniae</i> , <i>Y. pestis</i>	hours		bacteria before MALDI-TOF-MS is possible, low cost printable sensor technology with potential for mass production	limited applicability to monomicrobial sample matrix, potential for misclassification of species producing similar VOC profiles	
Not identified	Blood culture	Colorimetric sensor array	<i>A. baumannii</i> , <i>E. aerogenes</i> , <i>E. cloacae</i> , <i>E. coli</i> , <i>E. faecalis</i> , <i>E. faecium</i> , <i>K. oxytoca</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> , <i>P. mirabilis</i> , <i>S. agalactiae</i> , <i>S. aureus</i> , <i>S. enterica</i> , <i>S. epidermis</i> , <i>S. lugdunensis</i> , <i>S. marcescens</i> , <i>S. pneumoniae</i> ,	9.7 – 16.4 hours	10 CFU ml <sup>-1</sup>	Consolidation of bacteria detection and identification steps into one test, sensitive and specific detection of pathogenic bacteria before MALDI-TOF-MS is possible, significant reduction in identification time compared to culture based methods, reduction in sample processing requirements	Non-specific detection of VOCs, limited applicability to monomicrobial blood stream infections, potential for misclassification of species producing similar VOC profiles	[41]

Acetaldehyde, acetic acid, acetone, 1-butanol, 2-butanone, ethanol, methanethiol, methanol, 2-methyl-1-butanol, indole	DSMZ media	PTR-MS	<i>S. pyogenes</i> <i>C. tropicalis</i> , <i>E. coli</i> , <i>S. enterica</i> , <i>S. flexneri</i>	24 hours	OD <sub>660</sub> 0.01	'On-line' monitoring of temporal changes in VOC profile for more specific bacteria identification, no requirement for VOC pre-concentration or chromatographic separation	Presumptive VOC identification based on M+1 ion, cannot differentiate between isomers, potential for variable VOC production by different strains	[42]
Benzonitrile, 1,2-ethanediol, ethyl acetate, ethyl butanoate, indole, isovaleric acid, propionic acid, trimethylamine	Mueller-Hinton medium, Chine blue agar	APCI-MS	<i>A. baumannii</i> , <i>E. coli</i> , <i>C. albicans</i> , <i>C. tropicalis</i> , <i>C. parapsilosis</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> , <i>S. aureus</i>	Not specified	Not specified	no requirement for VOC pre-concentration, or chromatographic separation, non-invasive sampling, positive and negative ionization modes gives greater coverage of detectable VOCs	APCI has lower sensitivity compared with other ionization techniques, presumptive VOC identification, potential for variable VOC production by different strains	[43]
Acetic acid, butyric acid, indole, isovaleric acid, trimethylamine, 1-vinyl aziridine	Human blood culture	APCI-MS	<i>A. baumannii</i> , <i>E. coli</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> , <i>S. aureus</i>	16 hours	10 <sup>4</sup> CFU ml <sup>-1</sup>	No requirement for VOC pre-concentration, or chromatographic separation, non-invasive sampling, positive and negative ionization modes gives greater coverage of	APCI has lower sensitivity compared with other ionization techniques, presumptive VOC identification	[44]

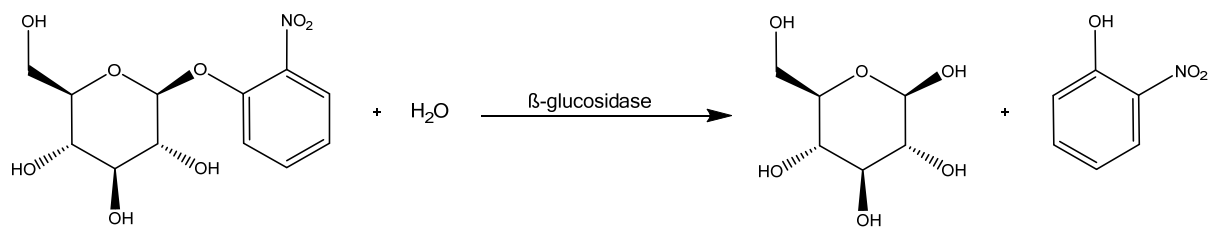
						detectable VOCs, particularly high sensitivity for species such as <i>E. coli</i> and <i>P. aeruginosa</i> , short analysis times of 10 – 20 seconds, reported reduction in cost per test		
Acetaldehyde, acetic acid, acetone, 2-aminoacetophenone, ammonia, ethanol, formaldehyde, hydrogen sulfide, dimethylsulfide, dimethyldisulfide, methanethiol, propanol	Inoculated human blood culture	SIFT-MS	<i>E. coli</i> , <i>N. meningitidis</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>S. pneumoniae</i>	6 hours	10 CFU	Potential to determine antibiotic susceptibility during culture, 'real time' VOC quantification possible, rapid analysis times of 30 seconds, simultaneous analysis of chemically diverse analytes	presumptive VOC identification, potential for variable VOC production by different strains	[46]
Acetonitrile, ammonia, dimethyldisulfide, ethanol, hydrogen cyanide	Human sputum culture	SIFT-MS	<i>P. aeruginosa</i>	48 hours	68 % of <i>P. aeruginosa</i> cultures detected on basis of HCN production, HCN production specific for cultures of <i>P. aeruginosa</i>	Close to real time measurements, no requirement for VOC pre-concentration, 'real time' VOC quantification possible, simultaneous analysis of chemically diverse analytes	48 hour culture time, presumptive VOC identification, some <i>P. aeruginosa</i> strains do not produce HCN	[47]

Not identified	Murine exhaled breath	SESI-MS	<i>P. aeruginosa</i> , <i>S. aureus</i>	1 hour	1 x 10 <sup>5</sup> – 2 x 10 <sup>10</sup> CFU ml <sup>-1</sup> , Detection of VOCs at ppt concentration	<i>In situ</i> diagnosis, obviates specimen collection and culture, rapid analysis time (30 seconds)	High number of breath related volatiles reduces diagnostic specificity of detected VOCs, external factors such as patient diet can cause false positive results, SESI-MS limited to detection of compounds that can be protonated or deprotonated, high variation between <i>in vitro</i> and <i>in vivo</i> detected VOCs	[48]
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**Figure 1. Different techniques applied to *in-vitro* VOC diagnostics**

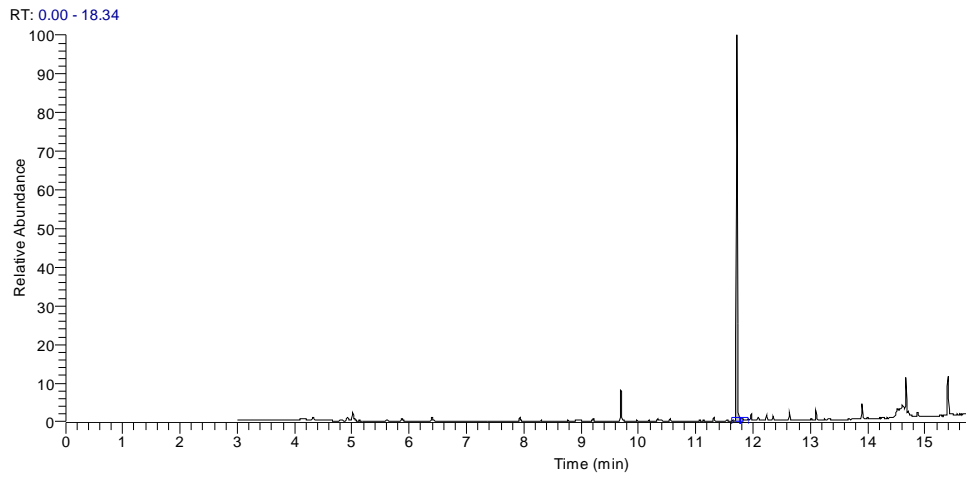


**Figure 2. Hydrolysis of 2-nitrophenyl- $\beta$ -D-glucopyranoside by  $\beta$ -D-glucosidase**



**Figure 3. Detection of 2-nitrophenol liberated from 2-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) by HS-SPME-GC-MS from a culture of *K. pneumoniae* (a) Chromatogram of 2-nitrophenol (11.7 mins, and (b) mass spectrum of 2-nitrophenol ( $M^+$  139 amu).**

**(a)**



**(b)**

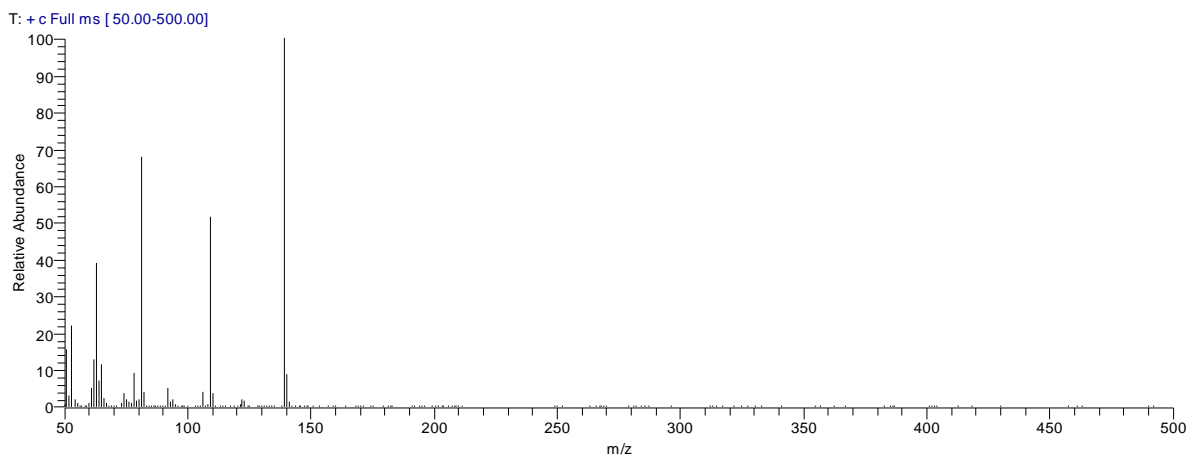


Figure 4. Immunomagnetic separation and VOC detection as an *in vitro* diagnostic technique

