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A novel chromatographic approach to distinguish Gram-positive from Gram-negative bacteria using exogenous volatile organic compound metabolites

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Highlights

- Differentiation of Gram-positive and Gram-negative bacteria
- Exogenous volatile organic compound metabolites
- Novel enzyme substrates for VOC
- Static headspace multicapillary column gas chromatography ion mobility spectrometry
- Headspace solid phase microextraction gas chromatography mass spectrometry

Abstract

This paper utilized L-alanine aminopeptidase activity as a useful approach to distinguish between Gram-negative and Gram-positive bacteria. This was done using two enzyme substrates, specifically 2-amino-N-phenylpropanamide and 2-amino-N-(4-methylphenyl)propanamide which liberated the volatile compounds aniline and p-toluidine, respectively. Two complementary analytical techniques have been used to identify and quantify the VOCs, specifically static headspace multicapillary column gas chromatography ion mobility spectrometry (SHS-MCC-GC-IMS) and headspace solid phase microextraction gas chromatography mass spectrometry (HS-SPME-GC-MS). Superior limits of detection were obtained using HS-SPME-GC-MS, typically by a factor of x6 such that the LOD for aniline was 0.02 μg/mL and 0.01 μg/mL for p-toluidine. In addition, it was also possible to determine indole interference-free by HS-SPME-GC-MS at an LOD of 0.01 μg/mL. The approach was applied to a range of selected bacteria: 15 Gram-negative and 7 Gram-positive bacteria. Use of pattern recognition, in the form of Principal Component Analysis, confirmed that it is possible to differentiate between Gram-positive and Gram-negative bacteria using the enzyme generated VOCs, aniline and p-toluidine. The exception was *Stenotrophomonas maltophilia* which showed negligible VOC concentrations for both aniline and p-toluidine, irrespective of the analytical techniques used and hence was not characteristic of the other Gram-negative bacteria investigated. The developed methodology has the potential to be applied for clinical and food applications.

Keywords: Volatile organic compounds; enzyme substrate; L-alanine aminopeptidase; static headspace multicapillary column gas chromatography ion mobility spectrometry (SHS-MCC-GC-IMS); headspace solid phase microextraction gas chromatography mass spectrometry (HS-SPME-GC-MS).

Introduction

The development of more rapid and efficient bacterial identification methods is a continuing area of research. One of the alternate methods used to identify bacteria involves the detection of characteristic bacterial volatile organic compounds (VOCs) [1]. However, bacterial VOC profiles are influenced by the microbiological sample preparation procedures e.g. choice of culture media, as well as the analytical sampling, extraction and chromatographic procedures e.g. choice of separation column, all of which can create many inconsistencies. A modification of this approach is to intentionally add an enzyme substrate into the culture media which, in the presence of a specific enzyme, will liberate a unique VOC which can be sampled and analysed [2-4]. Bacteria can be divided into two major groups, called Gram-positive and Gram-negative. The cell wall of Gram-positive bacteria consists of mainly a thick layer of peptidoglycan, but lacks the lipopolysaccharide outer membrane characteristic of Gram-negative cells. The original distinction between both groups was based on the Gram-stain, which depends on differences in the structure of the cell wall [5]. Clinical outcome in patients with bacterial infections is strongly related to the early administration of an appropriate antibiotic therapy. Confirmation of the adequacy of the antibiotic therapy initially chosen still depends on the results of conventional microbiological diagnostic methods. By using these methods, this important information is available after 48 to 96 h [6]. Clinical studies have shown a dramatic increase in hospital mortality associated with the delay of adequate antibiotic therapy for more than 24 h [7]. Unfortunately, the most active agents against Gram-negative bacteria are not very effective against Gram-positive bacteria [8]. Several species display variable Gram-stain reactions which often make identification difficult. Some factors, such as the age of the culture, excessive decolourisation with powerful solvents such as acetone, exposure to antimicrobial agents or the composition of the growth medium, can influence the Gram-stain reaction. Additionally, errors in determining the Gram reaction have been observed, since strains appear to be Gram variable. These errors may lead to misidentification [9].

It is well known that microbial species produce a range of volatile compounds including alcohols, aliphatic acids, and terpenes [10]. There have been many studies related to the production of volatile compounds, for example, production of volatile compounds by *Coryneform* bacteria in liquid cultures [11], comparison of volatile compounds in yogurt made with milk and soymilk [12], volatile compounds produced by acid bacteria to enhance aroma in sourdough bread [13], identification of the bacteria responsible for the spoilage of smoked salmon [14], characterization of microorganisms

for their production of odor-active volatile compounds and evaluation of their utility as ripening cultures in cheese manufacture [15].

The detection of characteristic bacterial VOC profiles could potentially be a useful diagnostic tool in the identification of bacteria in clinical and food samples [3]. A major advantage of VOCs methods is simplified sample preparation. VOCs can be extracted directly from the head space (HS) of the clinical or food sample, whereas traditional methods used in a microbiology laboratory often rely upon time-consuming, laborious culturing, enrichment broths, and biochemical methods for bacterial identification [3]. The appearance of a characteristic volatile compound profile is attributable to the specific metabolism or metabolic pathway(s) that are active in the bacteria. Depending on the growth media and growth conditions, the bouquet of released compounds can vary, e.g. the growth of *Stenotrophomonas rhizophila* P69 on nutrient broth with and without glucose results in qualitatively and quantitatively different GC profiles [16].

For selective isolation and differentiation of food-associated spoilage and pathogenic bacteria of interest, a variety of chromogenic and fluorogenic culture media are available. Incorporation of enzyme substrates into selective media may expedite (or eliminate) follow up biochemical confirmation of bacterial identity. Fluorogenic enzyme substrates consist of a sugar or amino acid conjugated to a fluorogen [17]. These substrates have been used with the purpose of studying the kinetics of specific enzymes according to the following general principle: the substrate is hydrolysed by the specific enzyme, yielding free chromo- or fluorophores. These detection principles have since been found useful in microbiological assays [17]. The detection of VOCs liberated following enzyme activity should increase the specificity of bacterial VOC profiles, as these liberated VOCs would act as markers for a particular species, hence aiding identification of bacteria [3].

The L-alanine aminopeptidase enzyme has been suggested for differentiating Gram-negative and Gram-positive bacteria [18] and has been correlated well with the Gram reaction [19]. The L-alanine aminopeptidase enzyme is generally located in the cell wall of Gram-negative bacteria [20]. Enzyme substrates are also available to purchase separately but these substrates generally incorporate compounds that are chromogenic on release and are often unsuitable for VOC analysis due to the non-volatility of the cleaved compounds [21]. Chromogenic enzyme substrates are mainly phenol derivatives, such as 2-nitrophenol [22]. James *et al.* [20] reported a potential application for detecting and identifying Gram-positive and Gram-negative bacteria with L-alanine-based substrates as the selected chromogenic L-alanine aminopeptidase substrates inhibit the growth of several Gram-positive bacteria including *Enterococcus faecalis*. In this paper two enzyme substrates were designed and synthesized to allow differentiation of Gram-negative from Gram-positive bacteria. The substrates are 2-amino-N-phenylpropanamide and 2-amino-N-(4-methylphenyl)propanamide

which liberate aniline and *p*-toluidine, respectively, in the presence of L-alanine aminopeptidase enzyme activity [23]. The generated bacterial VOCs were sampled and chromatographically analysed using two different approaches. The use of headspace solid phase microextraction coupled to gas chromatography mass spectrometry (HS-SPME-GC-MS) allows the volatiles in the headspace of the broth samples to be pre-concentrated prior to separation and analysis by GC-MS. An alternate approach uses a static headspace approach coupled to a multi capillary column gas chromatography with ion mobility spectrometry detection (SHS-MCC-GC-IMS) to analyse the samples. Ultimately the use of headspace sampling of gaseous exogenous volatiles, by either approach, is advantageous over the direct sampling of products in the liquid nutrient broth as it provides a 'cleaner' extract with the associated inherent 'cleaner' chromatogram. This not only allows a longer column life-time but also provides the potential for interference-free sampling.

Experimental

Chemicals and reagents

All reagents used were, at least, of analytical grade. N-methyl-2-pyrrolidone (99%), aniline (≥99%), p-toluidine (99%), Boc-L-alanine-OH (≥99.0%), N-methyl morpholine (98%) isobutyl chloroformate (98%), citric acid (99%), $NaHCO_3$ (≥99 %), hydrochloric acid (0.1M), $MgSO_4$ (≥99%), and tetrahydrofuran (THF) (99%) were purchased from Sigma Aldrich (Poole, UK). All other solvents (dichloromethane (DCM), ethyl acetate, and acetone) were of analytical reagent grade, and purchased from Fisher Scientific (Loughborough, UK).

Stock solutions (10,000 ppm) were prepared using N-methyl-2-pyrrolidone. Ultra-164 pure water of conductivity 18.2 MΩ.cm was produced by a direct QM Millipore system 165 (Mosheim, France) and was used in all dilution steps. Brain heart infusion (BHI) broth and Tryptone Soya Agar (TSA) were purchased from Oxoid (Basingstoke, UK). A SPME fibre, 85 µm polyacrylate (PA), for extracting bacterial VOCs, was purchased from Supelco Corp. (Bellefonte, PA, USA). The fibre was conditioned in the GC injection port prior to use as directed by manufacturer's guidelines and was used with a manual holder.

Instrumentation

Gas chromatography mass spectrometry (GC-MS) analysis was performed on a Thermo Finnigan Trace GC Ultra and Polaris Q ion trap mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) with Xcalibur 1.4 SR1 software. Separation of VOCs was carried out using a 30 m x 0.25 mm x 0.25 µm VF-WAXms polar GC column (Varian, Agilent Technologies, Stockport, UK). Separation of bacterial VOCs was achieved using the following temperature program: initial 50 °C with a 2 min hold, ramped to 220 °C at 15 °C/min and then held for 6 min. The split-splitless injection port was held at 230 °C for desorption of volatiles in split mode at a split ratio of 1:10. Helium was used as the

carrier gas at a constant flow rate of 1.0 mL/min. MS parameters were as follows: full-scan mode with scan range 50 – 650 amu at a rate of 0.58 scans/s. The ion source temperature was 250 °C with an ionising energy of 70 eV and a mass transfer line temperature of 250 °C. Identification of VOCs was achieved using the National Institute of Standards and Technology (NIST) Mass Spectral Reference Library (NIST Mass Spectral Library, version 2.0a, 2001) as well as the comparison with the retention times and mass spectra of authentic standards.

A FlavorSpec SHS-MCC-GC-IMS instrument manufactured by G.A.S.-Gesellschaft für Analytische Sensorsysteme mbH (Dortmund, Germany) was used throughout this project. The SHS-MCC-GC-IMS was fitted with an automatic sampler unit (CTC-PAL, CTC Analytics AG, Zwingen, Switzerland) and utilised a heated air-tight syringe. An MCC (Multichrom, Novosibirsk, Russia) was used for the chromatographic separation. The MCC comprises a stainless steel tube, 20 cm x 3 mm ID, containing approximately 1000 parallel capillary tubes, 40 µm ID, coated with 0.2 µm film thickness of stationary phase (Carbowax 20M). Atmospheric pressure ionisation is generated by a Tritium (^{3}H) solid state bonded source (β-radiation, 300 MBq with a half-life of 12.5 years). The IMS has a drift tube length of 50 mm. Separation in the IMS drift tube is achieved by applying an electric field of 2 kV to the ionized volatiles in a pulsed mode using an electronic shutter opening time of 100 µs. The drift gas was N_2 (99.998%) with a drift pressure of 101 kPa (ambient pressure). All data are acquired in the positive ion mode and each spectrum is formed with the average of 42 scans. All data, determined as peak height, against x and y co-ordinates per VC, are processed using the LAV software (version 2.0.0 from G.A.S). The software package enables both two- and three-dimensional data visualisation plots.

Separation of bacterial VOCs was achieved using the following temperature program. Sampling: incubation temperature at 50 °C for 5 minutes. Injection: the syringe temperature was set at 85°C, then 0.5 mL was injected at 80 °C, the column was at 70 °C and the carrier gas flow at 100 mL/min. IMS conditions: 60 °C in drift tube temperature and 500 mL/min drift gas flow.

Microbiology

A set of 15 Gram-negative strains comprising of 12 different species and 7 Gram-positive bacteria were obtained from the National Collection of Type Cultures (NCTC), Colindale, UK. Gram-negative bacteria: *Acinetobacter baumannii* NCTC 12156; *Burkholderia cepacia* NCTC 1222; *Citrobacter freundii* NCTC 9750; *Enterobacter cloacae* NCTC 11936; *Escherichia coli* NCTC 8007, 9001, 10418, and 12486; *Klebsiella pneumoniae* NCTC 13438; *Proteus mirabilis* NCTC 10975; *Providencia rettgeri* NCTC 7475; *Pseudomonas aeruginosa* NCTC 10662; *Serratia marcescens* NCTC 10211; *Stenotrophomonas maltophilia* NCTC 10257; and *Yersinia enterocolitica* NCTC 11176. Gram-positive bacteria: *Bacillus subtilis* NCTC 9372; *Corynebacterium diphtheriae* NCTC 10356; *Enterococcus faecalis* NCTC 775;

Enterococcus faecium NCTC 7171; *Staphylococcus aureus* NCTC 6571; *Staphylococcus epidermidis* NCTC 11047; and *Streptococcus pyogenes* NCTC 8306. All are hazard group 2 organisms. Bacteria were stored and sub-cultured weekly on Tryptone Soya Agar.

Synthesis of enzyme substrates

Synthesis of 2-amino-N-phenylpropanamide: Tert-butyl N-[1-(phenylcarbamoyl)ethyl]carbamate, an intermediate compound (Figure 1(A)) was synthesised from Boc-L-alanine (1.0683 g, 5.65 mmol) in dry THF (5 mL), to this N-methyl morpholine (0.59 mL, 5.38 mmol) was added and reaction was cooled to -10 °C and stirred for 30 minutes. After which isobutyl chloroformate (0.70 mL, 5.38 mmol) was added, mixture was stirred for a further 2 minutes, then an ice-cooled solution of aniline (0.43 mL, 5.38 mmol) dissolved in THF (5 mL) was added and the mixture was stirred at -10 °C for 2 hours, then allowed to heat to room temperature with stirring overnight. The resulting mixture was concentrated, then extracted into DCM, washed with citric acid (0.1 M, 20 mL), $NaHCO_3$ (10%, 20 mL), water (20 mL), dried ($MgSO_4$) and concentrated to give white crystals (1.1836, 83.27%).
The synthesis of 2-amino-N-phenylpropanamide HCl was made from the intermediate (Figure 1(A)) (0.2958 g, 1.12 mmol) by stirring in hydrochloric acid saturated ethyl acetate (25 mL) for 3 hours at room temperature, the resulting mixture was concentrated to give desired product as white crystals (0.179 g, 79.72%).

Synthesis of 2-amino-N-(4-methylphenyl)propanamide: Tert-butyl N-{1-[(4-methylphenyl)carbamoyl]ethyl}carbamate, an intermediate compound, was synthesised from Boc-L-alanine (Figure 1(B)) (1.5018 g, 7.94 mmol) in dry THF (7 mL), to this N-methyl morpholine (0.83 mL, 7.56 mmol) was added and reaction was cooled to -10 °C and stirred for 30 minutes. After which isobutyl chloroformate (0.99 mL, 7.56 mmol) was added, mixture was stirred for a further 2 minutes, then an ice-cooled solution of p-toluidine (0.81 mL, 7.56 mmol) dissolved in THF (7 mL) was added and the mixture was stirred at -10 °C for 2 hours, then allowed to heat to room temperature with stirring overnight. The resulting mixture was concentrated, then extracted into DCM, washed with citric acid (0.1 M, 20 mL), $NaHCO_3$ (10%, 20 mL), water (20 mL), dried (MgSO4) and concentrated to give crude product as yellow solid, recrystalisation in ethanol give desired product as white crystals (0.8051 g, 38.26%). Synthesis of 2-amino-N-(4-methylphenyl)propanamide HCl was synthesised from its intermediate (Figure 1(B)) (0.2608 g, 0.94 mmol) with stirring at room temperature in hydrochloric acid saturated ethyl acetate (20 mL) for 3 hours. The resulting mixture was concentrated to give the desired product as white crystals (0.1648 g, 81.94%). The synthesized enzyme substrates were confirmed by NMR and that data are reported in the Supplementary Material file.

Growth of bacteria and sample preparation

All bacteria were sub-cultured overnight at 37 °C on Tryptone Soya Agar one day prior to the preparation of samples for VOC analysis. After overnight incubation on blood agar at 37 °C, bacteria were inoculated in sterile BHI broth and incubated at 37 °C. Media were made up according to manufacturer's guidelines. Sterilisation of media was achieved by autoclaving at 126 °C for 11 minutes. Samples were prepared by measuring the absorbance of the incubated bacterial suspension at OD 600 nm. At an absorbance reading of 0.132 (equivalent to 0.5 McFarland units / 1.5 x 10^8 organisms per mL) an aliquot of 100 µL of bacterial suspension (1.5 x 10^7 organisms) was added to a 20 mL clear vial with PTFE septum and screw cap containing 10 mL sterile BHI broth with enzyme substrate.

Media with enzyme substrate was prepared by dissolving 5 mg of enzyme substrate in 500 µL of BHI broth then transferring aliquots aseptically to BHI broth (10 mL total volume). All samples were incubated overnight at 37 °C. Inoculated and uninoculated broths were incubated for 18 hours at 37 °C and then subjected to volatile profiling via HS-SPME-GC-MS or SHS-MCC-GC-IMS.

Procedure for HS-SPME-GC-MS

Bacterial VOCs were extracted from the headspace of inoculated and uninoculated broths and concentrated via SPME before desorption in the hot GC injection port. All samples and blanks were held at 37 °C in a water bath for 15 minutes prior to VOC extraction and kept at this temperature throughout sampling. A fused-silica SPME fibre with polyacrylate (PA) coating, pierced the PTFE septum, was exposed in the headspace of the vial for 10 minutes. Immediately after VOC extraction the SPME fibre was exposed in the hot GC injection port for 3 minutes for desorption of bacterial VOCs. VOCs liberated by bacteria inoculated with the enzyme substrates (2-amino-N-phenylpropanamide and 2-amino-N-(4-methylphenyl)propanamide) were separated on the polar GC column using the Trace GC and DSQ quadrupole mass spectrometer.

Procedure for SHS-MCC-GC-IMS

For IMS measurement, after the HS-SPME procedure, 1 mL of the samples were put into 20 mL glass vials that were closed with PP caps with PTFE/silicon septa, and immediately connected with the sample inlet system. All samples were incubated at 50 °C for 5 minutes prior to VOC extraction and 0.5 mL of the head space was injected.

Data Analysis

Calibration graphs of all VOCs were prepared by spiking standards of known concentrations into water. VOCs were quantified using external calibration and the values for limit of detection (LOD) and limit of quantification (LOQ) were determined as the peak area 3 times the signal to noise ratio

and 10 times the signal to noise ratio, respectively. Principal Component Analysis of the VOC data was done using R version 3.2.3 [24].

Results and Discussion

Analytical Data

The analytical performance of the two enzyme substrate exogenous VOCs, i.e. aniline and p-toluidine, was done by SHS-MCC-GC-IMS (Figure 1). The retention times of aniline and p-toluidine were 359.3 ± 1.4 s and 551.4 ± 1.7 s, respectively. Both compounds had a monomer and a dimer; aniline had drift times of 7.89 ± 0.02 and 9.82 ± 0.02 ms, respectively (Figure 1(B)) while p-toluidine had drift times of 8.35 ± 0.02 and 10.65 ± 0.01 ms, respectively (Figure 1(C)). In addition, it was also possible to calculate the relative drift time ($t_r$drift) for each VOC using the following equation (1) [25].

$$t_r\text{drift} = t_d / t_d\text{RIP} \qquad (1)$$

where t_d is the measured drift time of the VOC and t_dRIP is the drift time of the reactant ion peak (RIP) (Table 1). Additionally, the normalised reduced ion mobility (Ko, $cm^2\ V^{-1}\ s^{-1}$) can be calculated for aniline and p-toluidine. Firstly, this involves calculating the normalised reduced ion mobility for the RIP (Ko(RIP)) (using equation 2):

$$Ko(RIP) = [(L^2/ E\ .\ t_D(RIP_)) .\ (P / P_o) .\ (T_o / T)] \qquad (2)$$

Where L is the length of the drift region (cm), E is the electrical field strength (V), t_D(RIP) is the drift time (s) of the RIP, P is the pressure of the drift gas (hPa), P_o is the standard atmospheric pressure (1013.2 hPa), T is the temperature of the drift gas (K), and T_o is the standard temperature (273 K).

The normalised reduced ion mobility for the RIP (Ko(RIP) was experimentally determined to be 1.56 ± 0.02 $cm^2\ V^{-1}\ s^{-1}$ (n = 20). Finally, it is possible to determine the normalised reduced ion mobility (Ko) for aniline and p-toluidine (equation 3):

$$K_0(VOC) = F_{IMS} / t_D(VOC) \qquad (3)$$

Where F_{IMS} is the IMS factor ($cm^2\ V^{-1}$) derived as follows: $F_{IMS} = K_0(RIP)\ .\ t_D(RIP)$; and, t_D(VOC) is the drift time (ms) of the VOC. The derived normalised reduced ion mobilities for aniline and p-toluidine for their respective monomers and dimers are shown in Table 1.

The calibration data for aniline and p-toluidine using SHS-MCC-GC-IMS was determined (Table 2). Non-linearity was determined for both aniline and p-toluidine over the concentration range 0 – 30 µg/mL. However, a linear calibration graph was obtained for aniline over the concentration range 0-6 µg/mL with a correlation coefficient, R^2, of >0.99; similarly, p-toluidine produced a linear

calibration graph over the concentration range 0-15 µg/mL with a correlation coefficient, R^2, of >0.99 (Table 2).

Analytical data were also determined for aniline and p-toluidine, as well as indole, by HS-SPME-GC-MS with retention times of 10.34, 10.85 and 15.38 min, respectively (Figure 2). Calibration graphs were also obtained for all three VOCs over the concentration range 0.5-30 µg/mL with correlation coefficients, R^2, of >0.99 in all cases. The limit of detection (LOD) and limit of quantitation (LOQ), based on 3 or 10 x standard deviation of the blank, were determined for each VOC. The inclusion of a pre-concentration step, using SPME, allowed superior LOD and LOQ to be obtained using GC-MS (Table 2). Typical inter-day precision data for analysis by HS-SPME-GC-MS was as follows: aniline 4.2% RSD, p-toluidine 3.0% RSD, and indole 6.9% RSD, respectively, for a 10 ppm standard. Whereas, typical inter-day precision data for SHS-MCC-GC-IMS was as follows: aniline 14.3% RSD and p-toluidine 10.7% RSD, respectively, for a 5 ppm standard.

Analysis of selected Gram-positive and Gram-negative bacteria

L-alanine aminopeptidase activity was tested in 15 Gram-negative and 7 Gram-positive bacteria by both SHS-MCC-GC-IMS and HS-SPME-GC-MS using two synthesised enzyme substrates: 2-amino-N-phenylpropanamide and 2-amino-N-(4-methylphenyl)propanamide. An example chromatogram showing the evolution of the VOCs from *Escherichia coli* NCTC 9001 and detection by SHS-MCC-GC-IMS is shown in Figure 3, with detection by SPME-GC-MS in Figure 4. Table 3 outlines the concentration of aniline and *p*-toluidine produced by Gram-negative and Gram-positive bacteria tested by each analytical technique. In addition, indole a naturally occurring VOC resulting from tryptophan activity [26] was determined by HS-SPME-GC-MS.

In the case of SHS-MCC-GC-IMS aniline and *p*-toluidine were generated by all Gram-negative bacteria after 18-24 hours of incubation at 37 °C, but based on the determined LOQ (0.5 ppm for aniline and 0.18 ppm for *p*-toluidine) the amount of both compounds is negligible for *Stenotrophomonas maltophilia*; this could be because its optimum growth temperature is 35 °C [27]. The amounts of both aniline and *p*-toluidine liberated varied between Gram-negative species. The highest amount of aniline and *p*-toluidine produced was by *Escherichia coli* NCTC 10418 and the amounts of VOCs liberated between *E. coli* strains varied. Variation in substrate activity is not uncommon within the same species, and it has been reported that substrates targeting the same enzymes can exhibit differences in activity with the same species [28]. Aniline was detected in three Gram-positive bacteria under the LOQ: *Enterococcus faecalis, Staphylococcus aureus*, and *Streptococcus pyogenes*. *p*-Toluidine was only detected in *S. pyogenes* with a concentration of 0.24 ppm ± 0.06 ppm. This is not unusual. For example, researchers [9] have found L-alanine aminopeptidase activity in 3 out of

35 Gram-positive bacteria tested while others [19] reported similar results for *E. faecalis, E. faecium, S. pyogenes*, and *B. subtilis*.

By analysing the same bacteria by HS-SPME-GC-MS it was found that aniline and *p*-toluidine were detected in all Gram-negative and Gram-positive bacteria tested. All VOC concentrations were above the LOQ (0.05 ppm for aniline, 0.04 ppm for *p*-toluidine, and 0.01 ppm for indole). However, comparing Gram-positive results with those obtained in Gram-negative bacteria it is noted that the concentrations are considerably lower, except for *S. maltophilia*. These results indicate that L-alanine aminopeptidase activity is low in Gram-positive bacteria. *C. diphtheriae* has not previously been shown to have L-alanine aminopeptidase activity, although other *Corynebacterium* species have previously demonstrated L-alanine aminopeptidase activity [19]. All bacteria demonstrated uninhibited growth in the presence of both enzyme substrates that is important because selected chromogenic L-alanine aminopeptidase substrates were reported to inhibit the growth of several Gram-positive bacteria including *E. faecalis* [21]. In addition, indole was found in all *E. coli* strains [29] and in two more bacteria: *Yersinia enterocolitica* and *Providencia rettgeri*. *Y. enterocolitica* is indole-variable [30] and *P. rettgeri* is known to produce indole [31].

It is noted (Table 3) in all cases that HS-SPME-GC-MS detects a statistically significantly higher concentration ($p = 0.05$) of both aniline and p-toluidine (typically by a factor of x2), as compared to SHS-MCC-GC-IMS. Statistical significance was tested using the t-distribution based on the comparison of two means, with 4 degrees of freedom, and a critical value of 2.78 at the 95% confidence interval ($p = 0.05$). This was expected given that SPME provides a pre-concentration of the available volatiles in the headspace above the BHI broth. However, and often in clinical and food applications, diagnosis of bacterial infection or contamination is required rapidly then the speed of analysis is important. In this situation, identification and analysis of the exogenous VOCs is known within approximately 16 min (including sampling (5 min) and analysis times (10.85 min for p-toluidine)) using SHS-MCC-GC-IMS as compared to approximately 22 min (including sampling (10 min), fibre desorption (3 min) and analysis times (9.2 min for p-toluidine)) using HS-SPME-GC-MS. It is suggested that these overall speed of analysis differences are marginal considering that the bacterial growth in BHI broth is done over 18 hours.

To demonstrate the capabilities of this approach for clinical and food samples based on the use of enzyme substrates, and the detection of exogenous VOCs to differentiate between Gram-negative and Gram-positive bacteria it was necessary to establish a visual representation protocol. This was done using radial plots for both HS-SPME-GC-MS and SHS-MCC-GC-IMS for aniline and p-toluidine (Figure 5). It is clearly demonstrated that in all cases the Gram-negative bacteria produced a significant concentration of both aniline and p-toluidine in all cases, except *Stenotrophomonas*

maltophilia NCTC 10257. In addition, and based on the amounts of aniline and *p*-toluidine generated, multivariate analysis of the data using principal component analysis (PCA) was carried out using R (version 3.2.3) [24]. Figure 6 shows the results of the PCA, based on the mean VOC concentration by both analytical techniques, with respect to Principal Component (PC) 1 and PC2. PC1 identified 97.2% while PC2 2.1% of the data variance. PCA identified two visually distinct clusters; one cluster that included all Gram-negative bacteria, except *Stenotrophomonas maltophilia* NCTC 10257, while the other cluster included all Gram-positive bacteria.

Conclusion

This paper highlights the benefits of the incorporation of enzyme substrates into culture media, e.g. broth, as an aid to bacterial VOC analysis in terms of increasing specificity between different pathogenic bacteria. Two approaches for the sampling and detection of exogenous VOCs have been investigated, specifically HS-SPME-GC-MS and SHS-MCC-IMS. It was found that the use of SPME, with its inherent sample pre-concentration approach, allowed lower limits of detection for both aniline and p-toluidine. This approach has the potential to be of use as a diagnostic tool in the detection of pathogenic bacteria in both clinical and food laboratories. However, it is recognised that in polymicrobial samples the use of antibiotics, to reduce or eliminate competing bacteria species, will be necessary. Specifically, the approach could be used as an effective first step to eliminate Gram-negative pathogenic bacteria in an initial screening of sample types.

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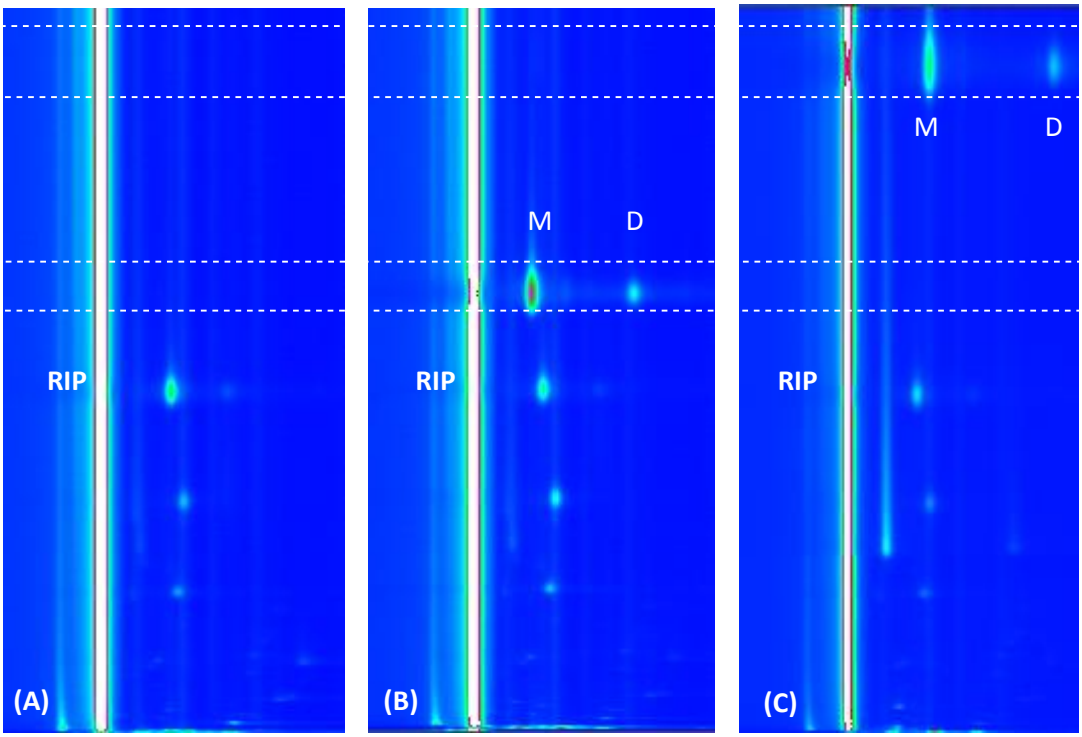
References

[1] C. Tiebe, H. Miessner, B. Koch, T. Hübert, Detection of microbial volatile organic compounds (MVOCs) by ion-mobility spectrometry, Anal. Bioanal. Chem. 395 (2009) 2313–2323.
[2] E. Tait, K.A. Hill, J.D. Perry, S.P. Stanforth, J.R. Dean, Development of a novel method for detection of *Clostridium difficile* using HS-SPME-GC-MS, J. Appl. Microbiol. 116 (2013) 1010-1019.
[3] E. Tait, J.D. Perry, S.P. Stanforth, J.R. Dean, Use of volatile compounds as a diagnostic tool for the detection of pathogenic bacteria, Trends Anal. Chem. 53 (2014) 117-125.
[4] E. Tait, S.P. Stanforth, S. Reed, J.D. Perry, J.R. Dean, Analysis of pathogenic bacteria using exogenous volatile organic compound metabolites and optical sensor detection, RSC Advances 5 (2015) 15494–15499.
[5] A. Popescu, R.J. Doyle, The Gram stain after more than a century, Biotech. Histochem. 71 (1996) 145-51.
[6] N. Kunze, J. Gopel, M. Kuhns, Detection and validation of volatile metabolic patterns over different strains of two human pathogenic bacteria during their growth in a complex medium using multi-capillary column-ion mobility spectrometry (MCC-IMS), Appl. Microbiol. Biotech. 97 (2013) 3665-3676.

[7] J. Garnacho-Montero, T. Aldabo-Pallas, C. Garnacho-Montero, A. Cayuela, R. Jiménez, S. Barroso, C. Ortiz-Leyba, Timing of adequate antibiotic therapy is a greater determinant of outcome than are TNF and IL-10 polymorphisms in patients with sepsis, Critical Care 111 (2006) 1-12.
[8] G. Gessoni, G. Saccani, S. Valverde, F. Manoni, M. Caputo, Does flow cytometry have a role in preliminary differentiation between urinary tract infections sustained by gram positive and gram negative bacteria? An Italian polycentric study, Clin. Chim. Acta 440 (2015) 152-156.
[9] A. Bamarouf, A. Eley, T. Winstanley, Evaluation of Methods for Distinguishing Gram-Positive from Gram-Negative Anaerobic Bacteria, Anaerobe 2 (1996) 163-168.
[10] A. Turner, N. Magan, Electronic noses and disease diagnostics, Nat. Rev. Microbiol. 2 (2004) 161-166.
[11] N. Jollivet, M. Bezenger, Y. Vayssier, J. Belin, Production of volatile compounds in liquid cultures by six strains of coryneform bacteria, Appl. Microbiol. Biotech. 36 (1992) 790-794.
[12] T. Murti, C. Bouillanne, M. Landon, M. Desmazeaud, Bacterial Growth and Volatile Compounds in Yoghurt-Type Products from Soymilk Containing *Bifidobacterium ssp,* Food Sci. 58 (1993) 153-157.
[13] B. Hansen, A. Hansen, Volatile compounds in wheat sourdoughs produced by lactic acid bacteria and sourdough yeasts, Lebens. Unters. Forsch. 198 (1994) 202-209.
[14] J. Joffraud, F. Leroi, C. Roy, J. Berdague, Characterisation of Volatile Compounds Produced by Bacteria Isolated From the Spoilage Flora of Cold-Smoked Salmon, Food Microbiol. 66 (2001) 175-184.
[15] T. Pogacic, M. Maillard, A. Leclerc, C. Herve, V. Chuat, A. Yee, F. Valence, A. Thierry, A methodological approach to screen diverse cheese-related bacteria for their ability to produce aroma compounds, Food Microbiol. 46 (2015) 145-153.
[16] M. Kai, M. Haustein, F. Molina, A. Petri, B. Scholz, B. Piechulla, Bacterial volatiles and their action potential, Appl. Microbiol. Biotechnol. 81 (2009) 1001-1012.
[17] K. Gracias, J. McKillip, A review of conventional detection and enumeration methods for pathogenic bacteria in food, Can. J. Microbiol. 50 (2004) 883-890.
[18] G. Cerny, Studies on the aminopeptidase test for the distinction of gram-negative from gram-positive bacteria, Eur. J. Appl. Microbiol. Biotechnol. 5 (1978) 113-122.
[19] G. Carlone, M. Valadez, J. Pickett, Methods for Distinguishing Gram-Positive from Gram-Negative Bacteria, J. Clin. Microbiol. 16 (1983) 1157-1159.
[20] A. James, J.D. Perry, A. Rigby, S.P. Stanforth, Synthesis and evaluation of novel chromogenic aminopeptidase substrates for microorganism detection and identification. Bioorg. Med. Chem. Lett. 17 (2007) 1418-1421.
[21] S. Orenga, A. James, M. Manafi, J.D. Perry, D. Pincus, Enzymatic substrates in microbiology, J. Microbiol. Meth. 79 (2009) 139-155.
[22] M. Manafi, Fluorogenic and chromogenic enzyme substrates in culture media and identification tests, Int. J. Food Microbiol. 31 (1996) 45-58.
[23] Tait, E. The development and evaluation of unique volatile organic compound (VOC) profiles using enzyme substrates as a novel method for bacterial detection/identification. Northumbria University. PhD Thesis (2012).
[24] R Core Team. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (version 3.2.3) (2015). https://www.R-project.org/ (Accessed 12/09/2016).
[25] C. Denawaka, I.A. Fowlis, J.R. Dean, Source, impact and removal of malodour from soiled clothing, J. Chromatogr. A 1338 (2014) 136-148.
[26] W.A. Newton, Y. Morino, E.E. Snell, Properties of crystalline tryptophanase, J. Biol. Chem., 240 (1965) 1211–1218.
[27] M. Denton, K. Kerr, Microbiological and clinical aspects of infection associated with Stenotrophomonas maltophilia, Clin. Microbiol. Rev. 11 (1998) 57-80.
[28] M. Kilian, L. Mikkelsen, J. Henrichsen, Taxonomic Study of Viridans Streptococci: Description of *Streptococcus gordonii sp. nov.* and Amended Description of *Streptococcus sanguis* (White and

Niven, 1946), *Streptococcus oralis* (Bridge and Sneath, 1982), and *Streptococcus mitis* (Andrews and Horder, 1906), Int. J. System. Bacter. 39 (1989) 471-484.
[29] J.H. Lee, J. Lee, Indole as an intercellular signal in microbial communities, FEMS Microbiol. Rev. 34 (2010) 426–444.
[30] M. Fredriksson-Ahomaa, A. Stolle, R. Stephan, Prevalence of pathogenic Yersinia enterocolitica in pigs slaughtered at a Swiss abattoir, Food Microbiol. 119 (2007) 207-212.
[31] C. O'Hara, F. Brenner, M. Miller, Classification, identification, and clinical significance of Proteus, Providencia, and Morganella, Clin. Microbiol. Rev. 13 (2000) 534-546.
Figure Caption

Figure 1. SHS-MCC-GC-IMS chromatograms of (A) blank, (B) aniline standard (20 µg/mL) (M-monomer and D-dimer), and (C) *p*-toluidine standard (20 (µg/mL) (M-monomer, D-dimer).



RIP = Reactive Ion Peak

Figure 2. HS-SPME-GC-MS chromatogram of standards (10 ppm) of aniline (t_R 10.34 min), *p*-toluidine (t_R 10.85 min) and indole (T_R 15.38).

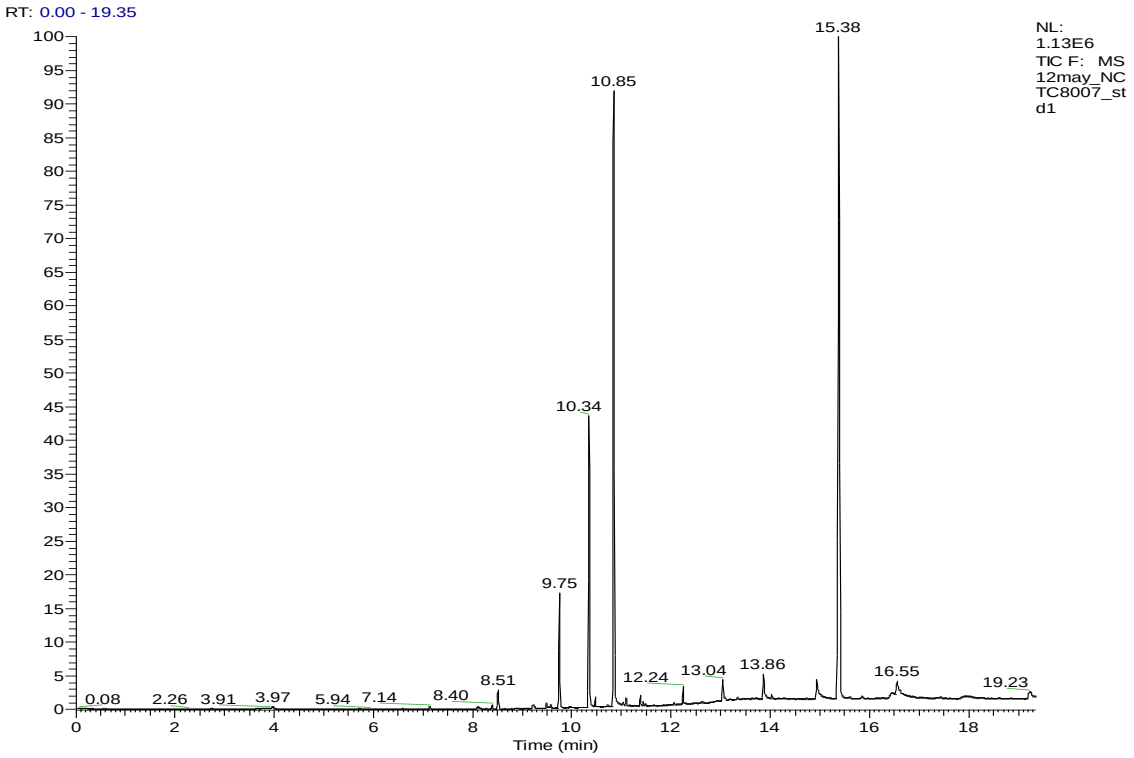
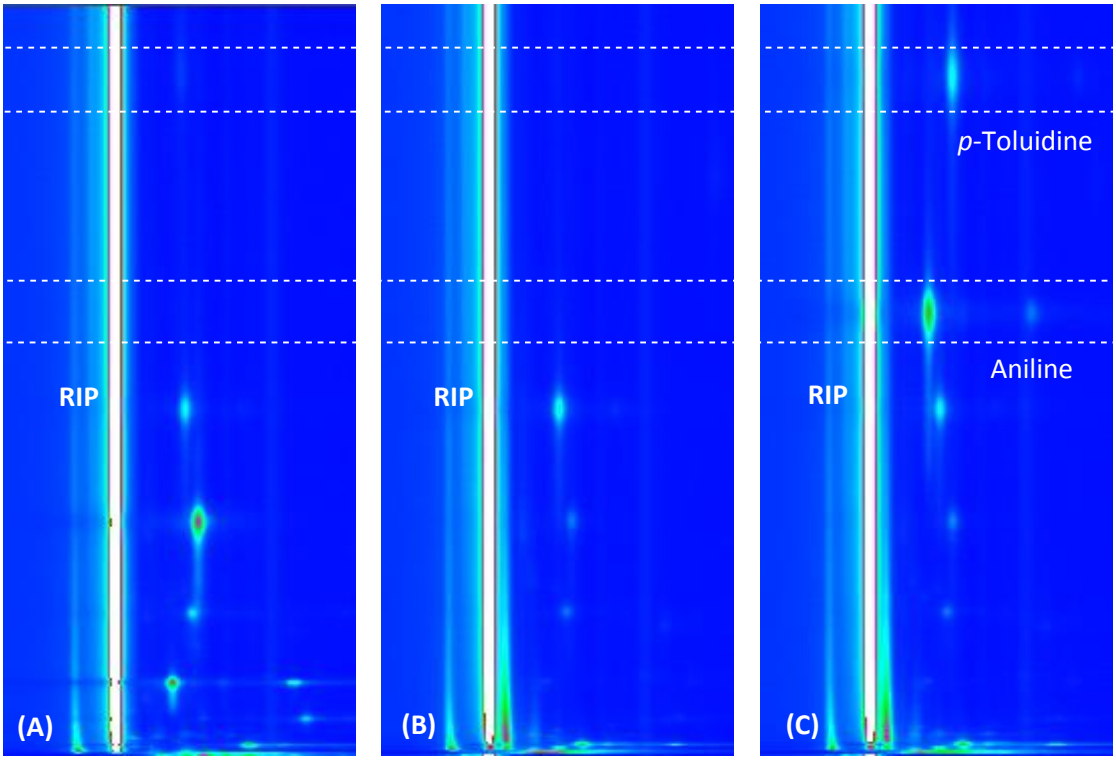
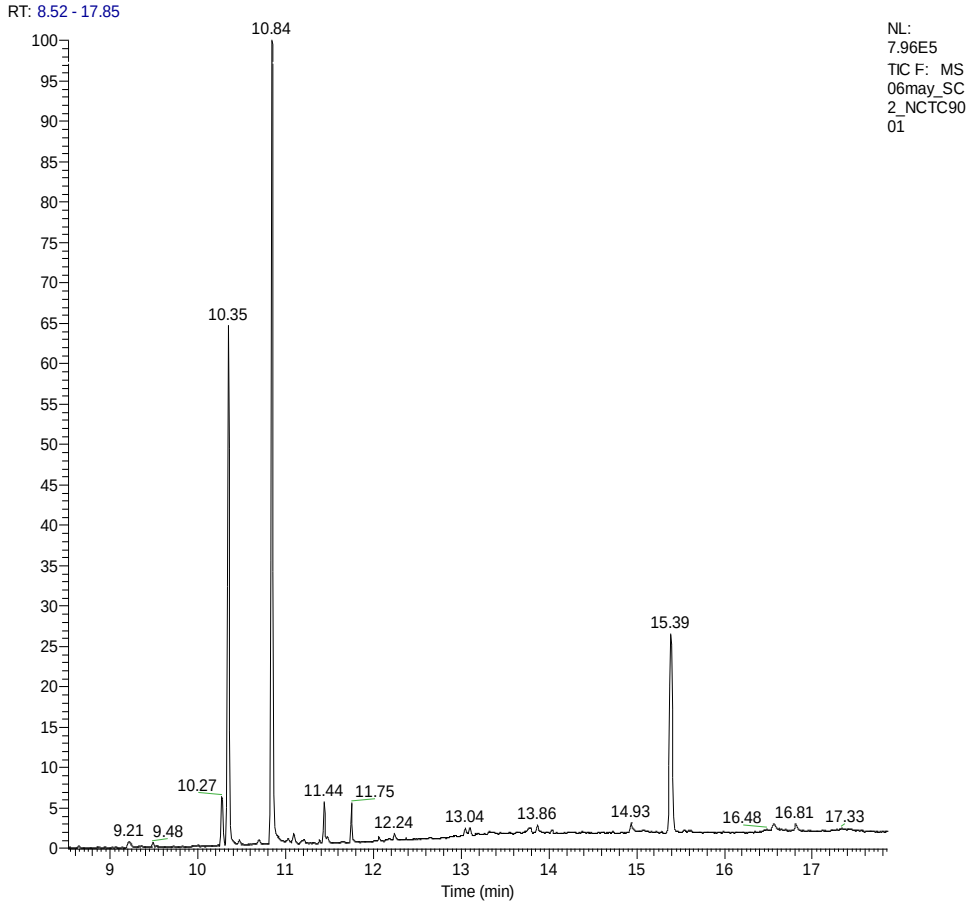


Figure 3. SHS-MCC-GC-IMS chromatograms of (A) blank, (B) culture of *Escherichia coli* NCTC 9001 and (C) addition of both enzyme substrates (50 µg/mL) to culture of *Escherichia coli* NCTC 9001.



RIP = Reactive Ion Peak

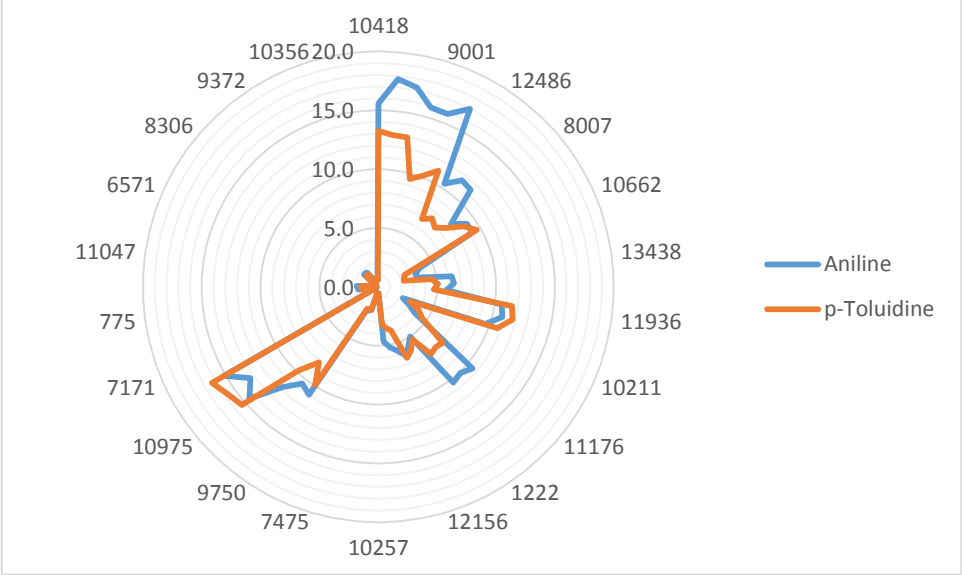
Figure 4. HS-SPME-GC-MS Retention time (s) chromatogram of *Escherichia coli* NCTC 9001 after addition of both enzyme substrates (50 µg/mL).



Note: aniline (t_R 10.35 min), *p*-toluidine (t_R 10.84 min) and indole (T_R 15.39).

Figure 5. The differentiation between Gram-negative and Gram-positive bacteria: VOC Data for Aniline and p-toluidine by (A) HS-SPME-GC-MS and (B) SHS-MCC-GC-IMS.

(A)



(B)

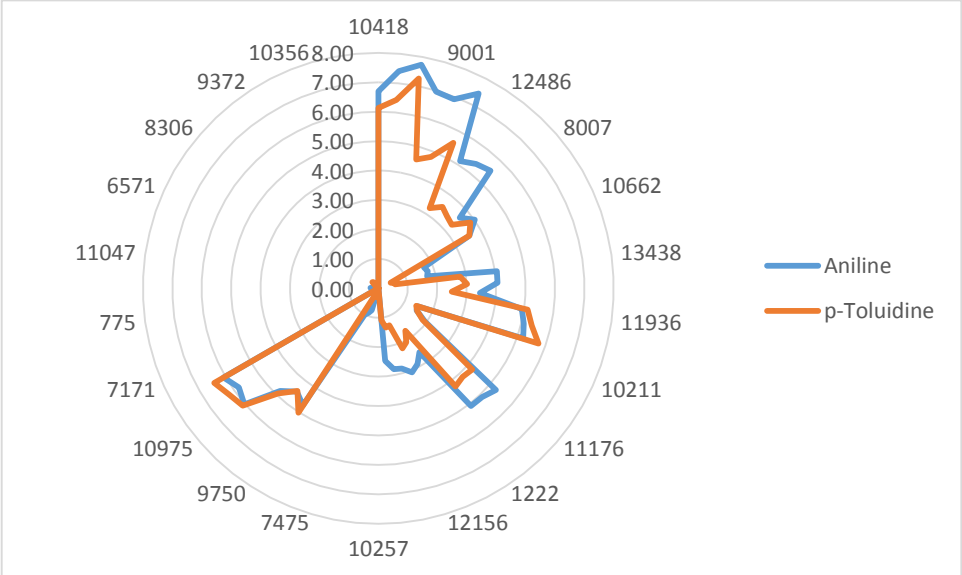
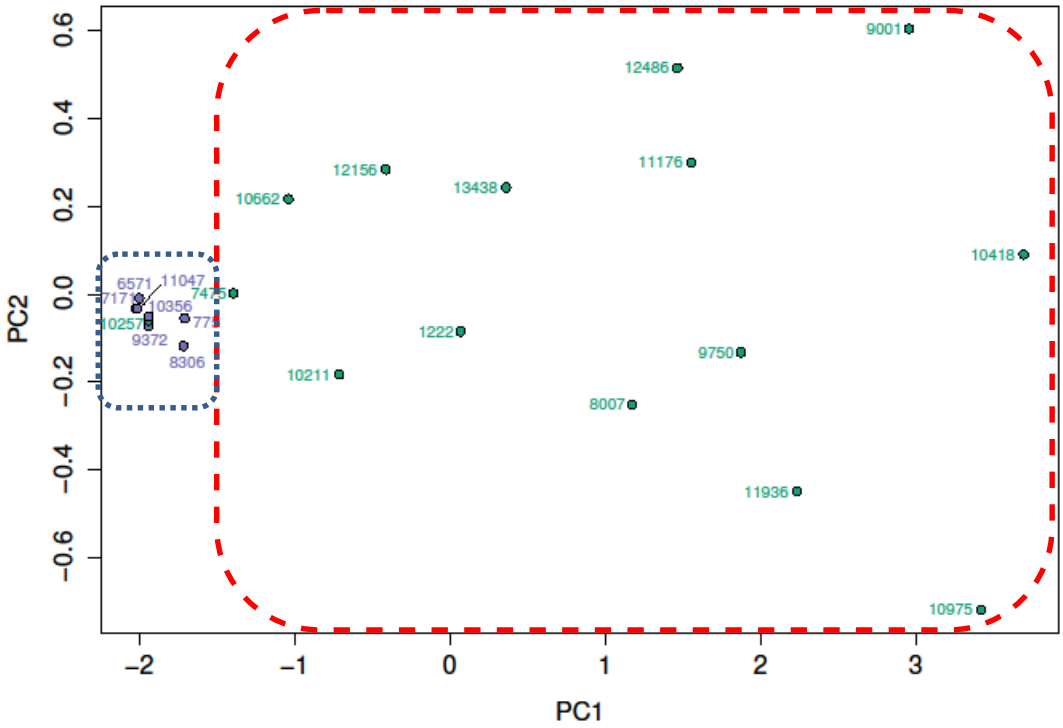


Figure 6. Principal Component Analysis of data from Aniline and p-Toluidine from Gram-negative and Gram-positive bacteria.



Key:

Gram-negative bacteria

Gram-positive bacteria

Table 1. Compound identification by SHS-MCC-GC-IMS and HS-SPME-GCMS

Compound	SHS-MCC-GC-IMS*					HS-SPME-GC-MS		
	Compound cluster	Retention time (s) Mean ± SD (n = 20)	Drift time (ms) Mean ± SD (n = 20)	Relative drift time Mean ± SD (n = 20)	Normalised reduced ion mobility ($cm^2V^{-1}S^{-1}$) Mean ± SD (n = 20)	Retention time (t_R; min)	Qualitative m/z	Quantita m/z
Aniline	Monomer	359.3 ± 1.4	7.89 ± 0.02	1.15 ± 0.00	1.34 ± 0.00	10.34	66	93
	Dimer		9.82 ± 0.02	1.43 ± 0.00	1.08 ± 0.00			
p-Toluidine	Monomer	551.4 ± 1.7	8.35 ± 0.02	1.22 ± 0.00	1.27 ± 0.00	10.85	77	106
	Dimer		10.65 ± 0.01	1.56 ± 0.00	0.99 ± 0.00			
Indole	NA	NA	NA	NA	NA	15.38	89	117

* Reactant Ion Peak (RIP): drift time 6.84 ± 0.01 ms (n = 20); normalised reduced ion mobility 1.54 ± 0.00 $cm^2V^{-1}S^{-1}$ (n = 20).
NA = not applicable

Table 2. Calibration Data for VOCs by SHS-MCC-GC-IMS# and HS-SPME-GC-MS

Compound Name	Analytical Technique	Non-Linear				Linear					
		Range µg/mL	N	Equation	R^2	Range µg/mL	N	Equation	R^2	LOD µg/mL	LO µg/
Aniline	SHS-MCC-GC-IMS	0 – 30	20	$y = -4E\text{-}06x^4 + 0.0004x^3 – 0.0168x^2 + 0.323x - 0.0144$	0.9984	0 - 6	10	$y = 0.2405x + 0.0368$	0.9924	0.15	0.
	HS-SPME-GC-MS	NA				0.5 - 30	9	$y = 53559x - 8326$	0.9965	0.02	0.
p-Toluidine	SHS-MCC-GC-IMS	0 – 30	20	$y = 4E\text{-}06x^3 - 0.0027x^2 + 0.1467x + 0.0442$	0.9983	0 - 15	14	$y = 0.102x + 0.031$	0.9982	0.06	0.
	HS-SPME-GC-MS	NA				0.5 - 30	9	$y = 121816x - 28306$	0.9966	0.01	0.
Indole	HS-SPME-GC-MS	NA				0.5-30	9	$y = 203804x + 56510$	0.9995	0.00_4	0.

analytical data is based on Σmonomer + dimer

NA = not applicable

N = number of determinations

Table 3. VOCs liberated by Gram-negative and Gram-positive bacteria (n = 3).

Bacteria	Growth	Aniline (µg/mL); Mean ± SD	*p*-Toluidine (µg/mL); Mean ± SD	Indole (µg/mL); Mean ± SD

		SHS-MCC-GC-IMS	HS-SPME-GC-MS	|t|-value	SHS-MCC-GC-IMS	HS-SPME-GC-MS	|t-value	HS-SPME-GC-MS
Gram-negative								
Acinetobacter baumannii NCTC 12156	+	2.70 ± 0.20	5.18 ± 0.48	*8.3*	1.25 ± 0.15	3.55 ± 0.32	*11.3*	ND
Burkholderia cepacia NCTC 1222	+	2.85 ± 0.25	5.63 ± 0.62	*7.2*	1.99 ± 0.25	5.96 ± 0.61	*10.4*	ND
Citrobacter freundii NCTC 9750	+	4.67 ± 0.18	11.0 ± 0.7	*15.2*	4.80 ± 0.32	9.30 ± 0.98	*7.6*	ND
Enterobacter cloacae NCTC 11936	+	5.08 ± 0.14	10.4 ± 0.7	*12.9*	5.41 ± 0.32	11.3 ± 0.5	*17.2*	ND
Escherichia coli NCTC 8007	+	3.75 ± 0.24	8.88 ± 0.60	*13.8*	3.57 ± 0.27	8.72 ± 1.02	*8.5*	0.12 ± 0.10
Escherichia coli NCTC 9001	+	7.10 ± 0.29	16.2 ± 0.7	*20.8*	4.97 ± 0.52	10.3 ± 0.8	*9.7*	1.71 ± 0.39
Escherichia coli NCTC 10418	+	7.28 ± 0.53	16.9 ± 1.1	*13.7*	6.61 ± 0.58	13.0 ± 0.2	*18.0*	5.86 ± 0.58
Escherichia coli NCTC 12486	+	5.34 ± 0.19	11.1 ± 0.6	*15.9*	3.38 ± 0.14	7.06 ± 0.29	*19.8*	0.04 ± 0.01
Klebsiella pneumoniae NCTC 13438	+	3.86 ± 0.35	6.18 ± 0.34	*8.2*	2.78 ± 0.26	4.80 ± 0.25	*9.7*	ND
Proteus mirabilis NCTC 10975	+	5.97 ±0.14	14.3 ± 0.9	*15.8*	6.25 ± 0.17	15.8 ± 0.5	*31.3*	ND
Providencia rettgeri NCTC 7475	+	0.90 ± 0.16	2.17 ± 0.19	*8.9*	0.33 ± 0.03	2.06 ± 0.01	*94.8*	0.05 ± 0.12
Pseudomonas aeruginosa NCTC 10662	+	1.73 ± 0.04	3.52 ± 0.29	*10.6*	0.53 ± 0.06	2.33 ± 0.11	*24.9*	ND
Serratia marcescens NCTC 10211	+	1.63 ± 0.27	3.04 ± 0.73	*3.1*	1.60 ± 0.22	3.83 ± 0.82	*4.6*	ND
Stenotrophomonas maltophilia NCTC 10257	+	< LOQ	0.55 ± 0.03	NA	< LOQ	0.60 ± 0.04	*NA*	ND
Yersinia enterocolitica NCTC 11176	+	5.16 ± 0.11	10.3 ± 0.2	*39.0*	4.20 ± 0.05	7.19 ± 0.10	*46.3*	0.30 ± 0.06
Gram-positive								
Bacillus subtilis NCTC 9372	+	ND	0.51 ± 0.05	NA	ND	0.67 ± 0.03	NA	ND

Corynebacterium diphtheriae NCTC 10356	+	ND	0.63 ± 0.03	NA	ND	0.57 ± 0.01	NA	ND
Enterococcus faecalis NCTC 775	+	< LOQ	1.76 ± 0.08	NA	ND	1.44 ± 0.04	NA	ND
Enterococcus faecium NCTC 7171	+	ND	0.15 ± 0.01	NA	ND	0.20 ± 0.01	NA	ND
Staphylococcus aureus NCTC 6571	+	< LOQ	0.17 ± 0.01	NA	ND	0.21 ± 0.01	NA	ND
Staphylococcus epidermidis NCTC 11047	+	ND	0.18 ± .01	NA	ND	0.23 ± 0.01	NA	ND
Streptococcus pyogenes NCTC 8306	+	< LOQ	1.58 ± 0.03	NA	0.24 ± 0.06	1.32 ± 0.06	*22.1*	ND

ND = not detected

+ = normal growth

<LOQ = concentration lower than limit of quantification (LOQ)

NA = not applicable

* t-distribution based on the comparison of two means, with 4 degrees of freedom, and a critical value of 2.78 at the 95% confidence interval (p = 0.05). All significant values are highlighted in *italics*.