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New tool for Gram-negative and Gram-positive bacterial strains differentiation through GC-MS direct identification of exogenous VOC metabolites in headspace vials

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Abstract
In this paper, a chromatographic method for the determination of volatile organic compounds (VOCs) as a tool for Gram-positive and Gram-negative bacterial strains identification was described. Direct Headspace gas chromatography coupled with mass spectrometry (Headspace-GC-MS) has been used for the qualitative determination of VOCs emitted by certain bacterial strains (Salmonella Typhimurium, Escherichia coli, Serratia rubidae, Staphyloccocus aureus, Enterococcus casseliflavus, and Enterococcus faecalis). In the first stage of the study, pathogenic bacteria were cultured in broth-specific liquid culture medium directly (lauryl sulfate broth) into headspace vials. Subsequently, the VOC emissions were analyzed by airborne screening in the headspace vials. Thus, the objective was to analyze all the resulting volatile organic compounds in order to select only those compounds, which can be exclusively associated with specific pathogenic bacteria. Qualitative analysis by Headspace-GC-MS has proven to be a non-invasive, accurate, and fast method for identifying certain bacterial strains, based on VOCs emission. A considerable number of volatile organic compounds have been determined in the headspace air, a significant difference being observed between the VOCs emitted by bacterial cultures compared to the culture medium but also between the types of bacterial cultures themselves.
The study presents preliminary results, which prove that the identification of the studied pathogenic bacteria is possible, based on the determination of certain types of VOCs in the headspace air of these cultures. This method can be used successfully for the rapid identification of bacterial culture types compared to classical methods.

Keywords: *VOC metabolites, GC-MS, screening, bacterial strains differentiation*

INTRODUCTION

It is well known that bacteria produce a large number of volatile organic compounds (VOCs). Studies in the field have shown that VOCs are products or by-products of metabolic pathways [1-3]. For example, many studies have reported indole as being associated with the presence of *Escherichia coli* [4-6].
Recent research tends to quickly identify bacteria with major implications for intra-hospital infections. Usually, the identification of these microorganisms involves a sequence of biochemical tests, but they require a long analysis time, up to 48 hours, to determine a bacterial species [7]. The identification of VOCs emitted by pathogenic bacteria can allow their formation to be used as markers to identify the presence or absence of pathogenic bacteria.
Thus, it has been shown that the presence of alcohols can be associated with the presence of Gram-negative bacteria, while the emission of hydrogen cyanide can be a marker for the presence of

Gram-positive bacteria [8-11]. The analytical determination of VOCs emitted by bacteria allows much faster identification of pathogenic bacteria compared to the usual methods.
Many VOCs emitted by bacteria have been identified by the headspace method. It was observed that their profile differs a lot depending not only on the bacterial culture used but also on the culture medium used, their emission being closely related to the bacterial metabolism [12-14]. For this reason, the VOCs profile determined for the same bacterial species proved to be very different using different culture medias.
Qualitative analysis of volatile organic compounds emitted by certain microorganisms can lead to early identification of pathogenic bacteria in expired air [1]. VOCs are released mainly by bacteria as signaling molecules for intercellular communication, as metabolic products during growth, but also as by-products for protection against competitors and antagonists. Recent research has shown the usefulness of VOC analysis in assessing bacterial growth in vitro [15-17]. However, based on the detection of a single volatile organic compound, no distinction can be made between specific strains or species. For this reason, well-defined combinations of VOCs can provide greater certainty regarding the identification of different pathogenic bacteria [18-22].
The aim of this paper was to determine which VOC profiles are emitted by certain pathogenic bacteria in order to easily identify these cultures. In addition, we present a rapid method for the determination of VOCs emitted by various Gram-positive and Gram-negative bacterial strains by growing them in vitro, directly in the vial followed by VOCs emission determination by qualitative screening analysis, using headspace gas chromatography method coupled with mass spectrometry.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Bacterial strains obtained from our bacterial data bank collection (Table 1), were seeded on a solid nutrient medium (soy agar tryptone) (Oxoid, UK) and incubated at 37°C for 24 hours. After incubation, one colony of each bacterial strain was growth in broth-specific liquid culture medium, namely lauryl sulfate broth (Himedia Laboratories Pvt. Ltd.). Incubation in liquid medium was performed for 24h in an incubator with gentle rotation (130rpm) and temperature control (37°C) (New Brunswick Scientific, Innova 44). The headspace vials used in this study were 75.5 × 22.5mm, 20 mL, screw neck, clear glass vials (Agilent Technologies) with magnetic screw caps with blue silicone/PTFE septa. Gram-negative and Gram-positive bacterial strains were grown in autoclaved headspace vials in 5 ml culture media and incubated in sterile condition for another 24h at 37°C.

Table 1. Bacterial strains

Species	Bacterial strains	Codification
Salmonella typhymurium	Gram-negative	B1
Escherichia coli	Gram-negative	B2
Serratia rubidae	Gram-negative	B3
Staphyloccocus aureus	Gram-positive	B4
Enterococcus casseliflavus	Gram-positive	B5
Enterococcus faecalis	Gram-positive	B6

Headspace-GC-MS screening method and peak identification

VOCs screening analysis were performed using an Agilent 7890B GC (split/splitless injector) coupled to a triple quadrupole mass spectrometer (Agilent 7010B) (Agilent Technologies, Waldbronn, Germany). Samples were run through a polar cyanopropyl low bleeding column (14% cyanopropylmethylpolysiloxane column, 30 m × 0.25 mm i.d., 1.0 μm film thickness (TG-17MS from Thermo Scintific) using He 6.0 (1.0 mL/min). MS parameters were as follows: full-scan mode with scan range 30 – 300 amu at a rate of 100 ms. Detection was made in EI mode at 70 eV. Split/splitless inlet was operated in split mode (1:5) at a constant temperature of 230°C. GC oven

temperature program started at 35ºC (15 min), raised to 145ºC (in 5 min), and then ramped to 185ºC (in 20 minutes). A 10-minute final isothermal plateau was used to elute all sample components (185°C). Identification of VOCs was achieved using the National Institute of Standards and Technology (NIST) Mass Spectral Reference Library (NIST Mass Spectral Library). Background-subtracted mass spectra were used for the representation of unidentified VOC metabolites. A recorded, full-scan-mode chromatogram was used for qualitative analysis via library-assisted matching. The compounds were identified by comparing the mass spectra with the database spectra. Based on the probability-based match, the mass spectra of the obtained peaks were automatically assigned and reviewed manually by an experienced analyst. Finally, the appropriate reference spectra contained in the spectral libraries were assigned. In order to assess if the detected volatile organic compounds are present only in the samples containing bacterial strains, the chromatograms obtained were compared with those recorded for the blank sample, which contains only the culture medium.

RESULTS AND DISCUSSION

Headspace analysis

The first step in data analysis was to create the average chromatograms for all six microorganisms and for medium control to visually examine if it is clear differences in the VOCs patterns. These average chromatograms are showed in Fig. 1 and Fig. 2 and clearly show that each type of culture group tested resulted in a distinct peak pattern, which suggest that different VOC compounds are present in the headspace air of the tested bacteria.

As it can be seen in the chromatograms registered for *Salmonella typhymurium*, *Escherichia coli* and *Serratia rubidae*, the volatile organic compounds profiles detected in the headspace air samples proved to be very similar (Fig 1).

Fifteen volatile organic compounds were determined by GC-MS analysis of Gram-negative bacterial cultures, but only a few of them were not determined in the blank sample. Thus, 4 substances (alkenes, amide and aldehydes) were detected in the headspaces of the investigated cultures with a probability higher than 50% (Fig. 1 and Table 2).

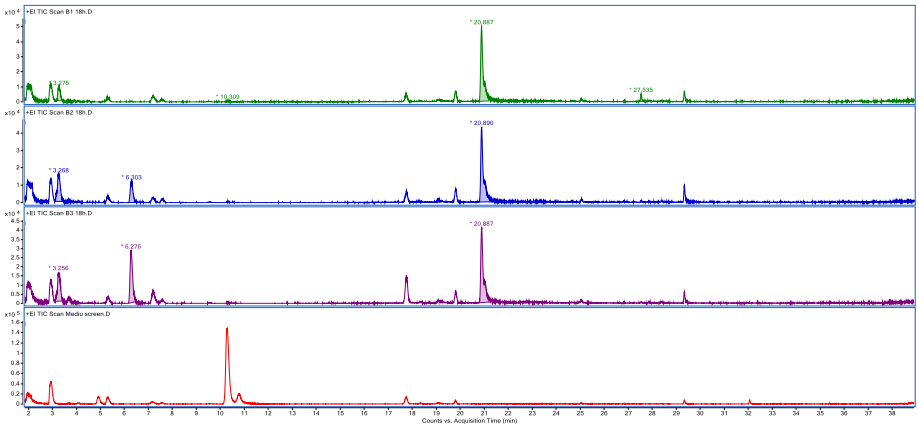


Fig. 1. Chromatograms registered for gram-negative bacteria culture and blank medium

For *Staphyloccocus aureus*, *Enterococcus casseliflavus*, *Enterococcus faecalis*, eighteen volatile organic compounds were observed, but only seven of them were detected only in the bacterial culture samples and not in the medium. The VOCs that were increased in the samples with Gram-positive bacterial strains comparing to the culture medium and of which the presence is thus positively indicative of this class are alkenes, alcohols and cyclic compounds (Table 3).

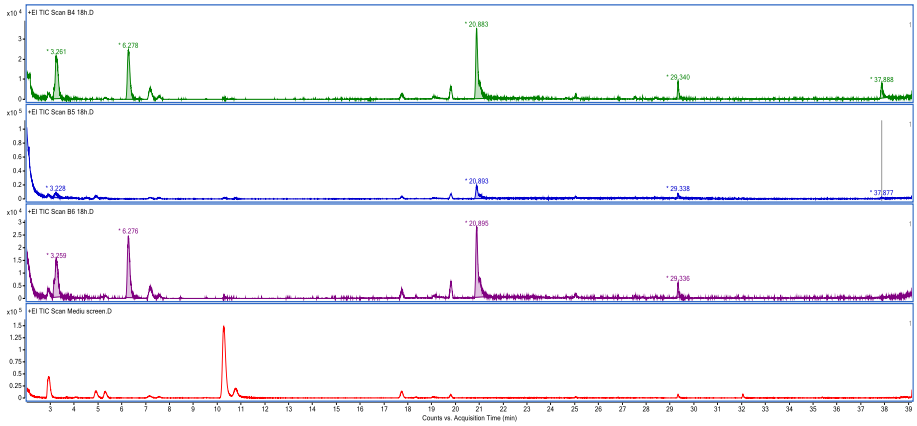
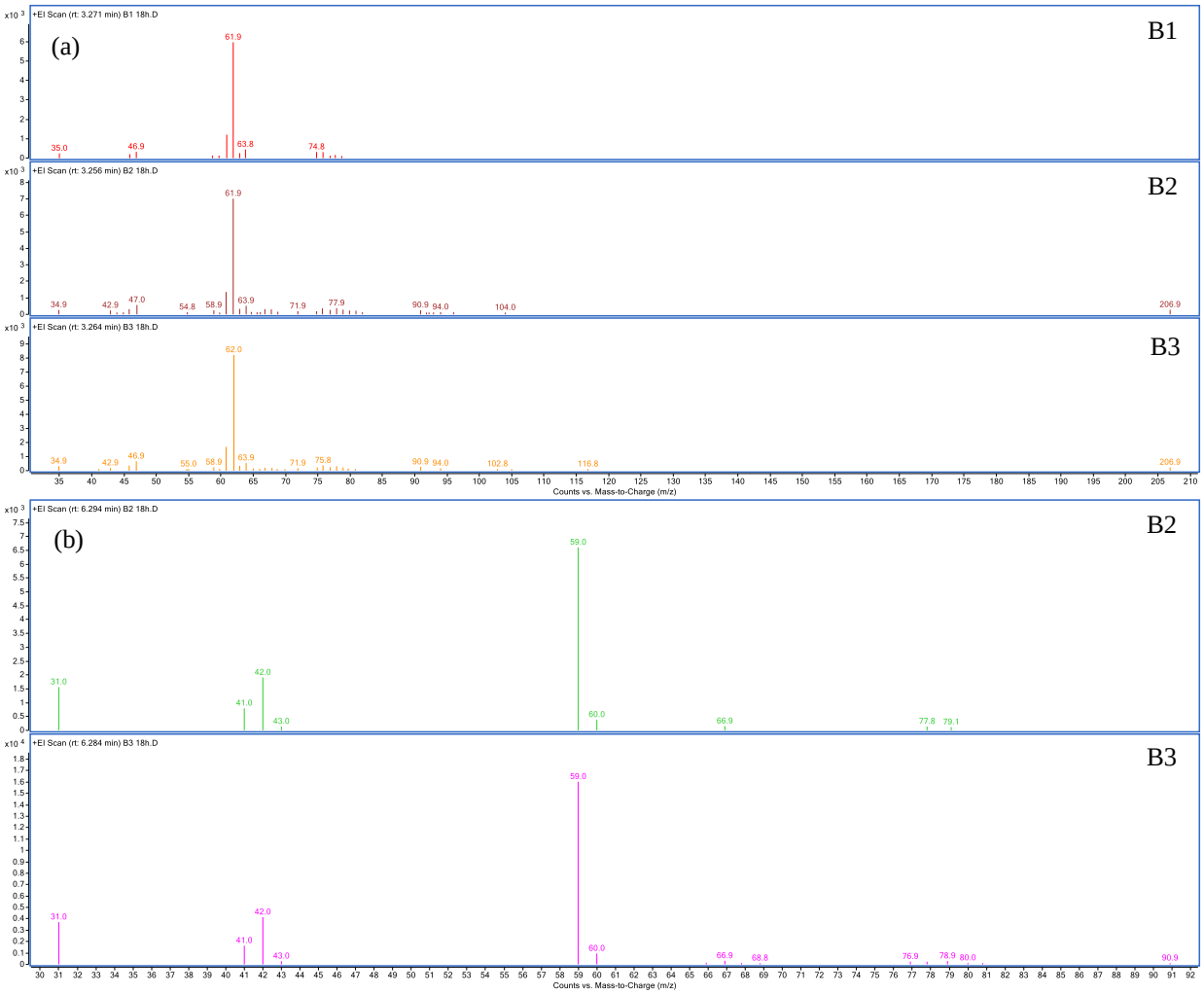


Fig. 2. Chromatograms registered for gram-positive bacteria culture and blank medium

VOCs profiling

The scope of the present experiments was to identify the volatile organic compounds profiles of three Gram-negative (*Salmonella typhymurium*, *Escherichia coli* and *Serratia rubidae*) and three Gram-positive (*Staphyloccocus aureus*, *Enterococcus casseliflavus*, *Enterococcus faecalis*) bacterial strains. The emission signatures obtained in the headspace air were distinctive for all six bacteria and permitted their differentiation.



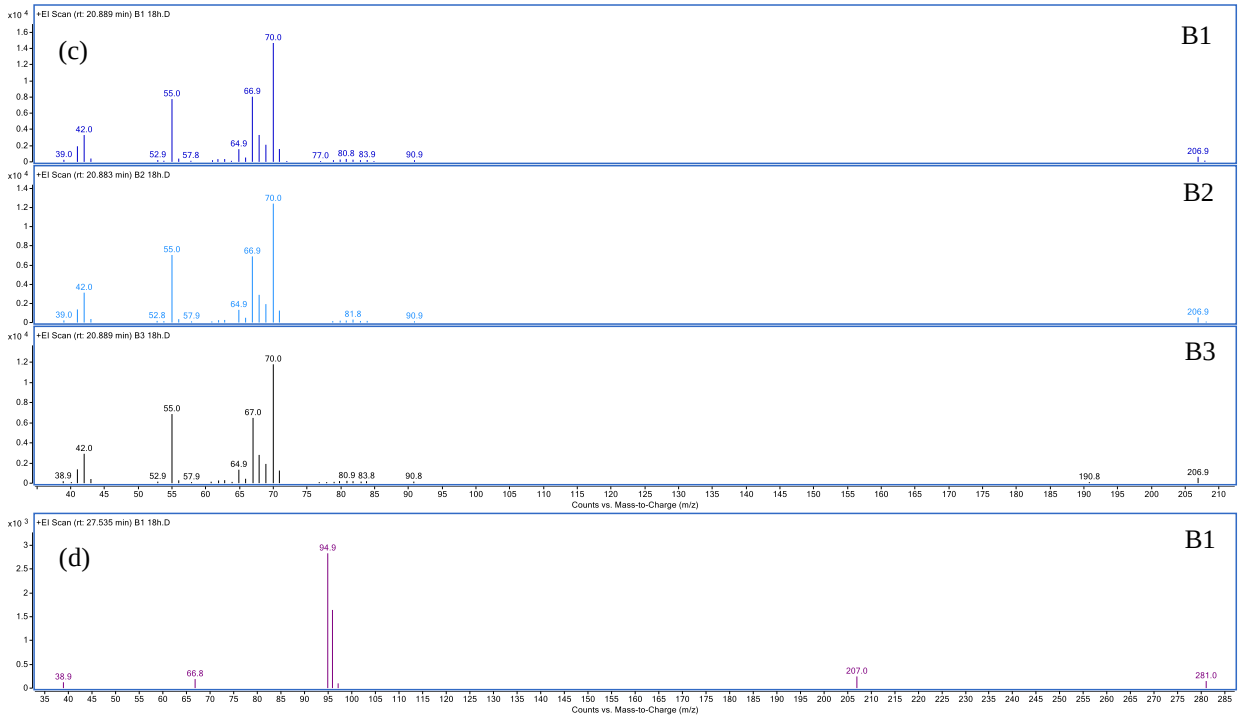


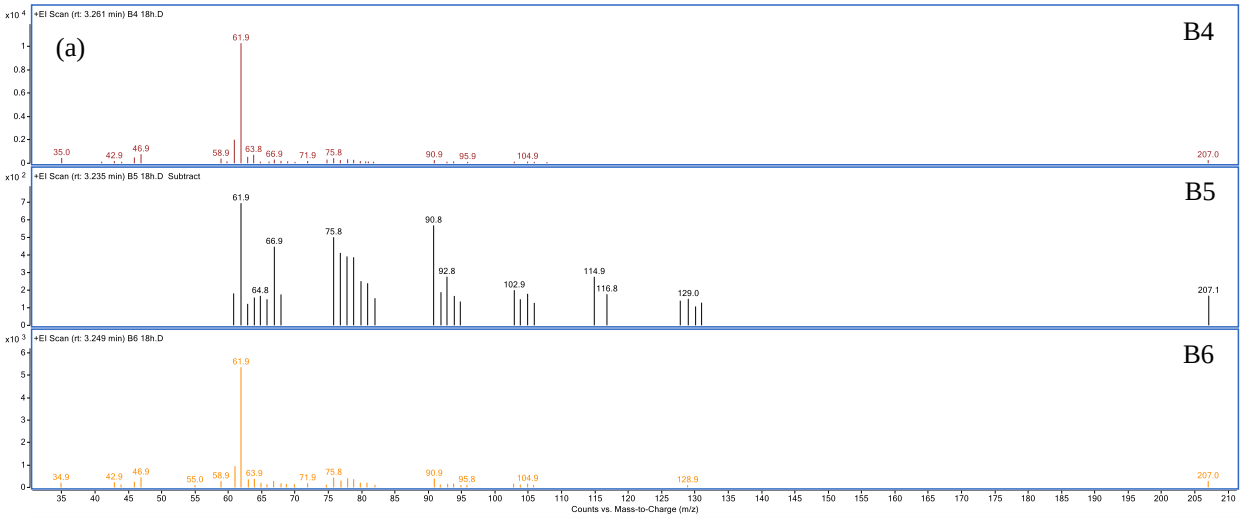
Fig. 3. MS spectra obtained for VOC compounds detected in headspace air of gram-negative bacterial cultures at retention times: (a) 3.2 min, (b) 6.2 min, (c) 20.8 min and (d) 27.5 min

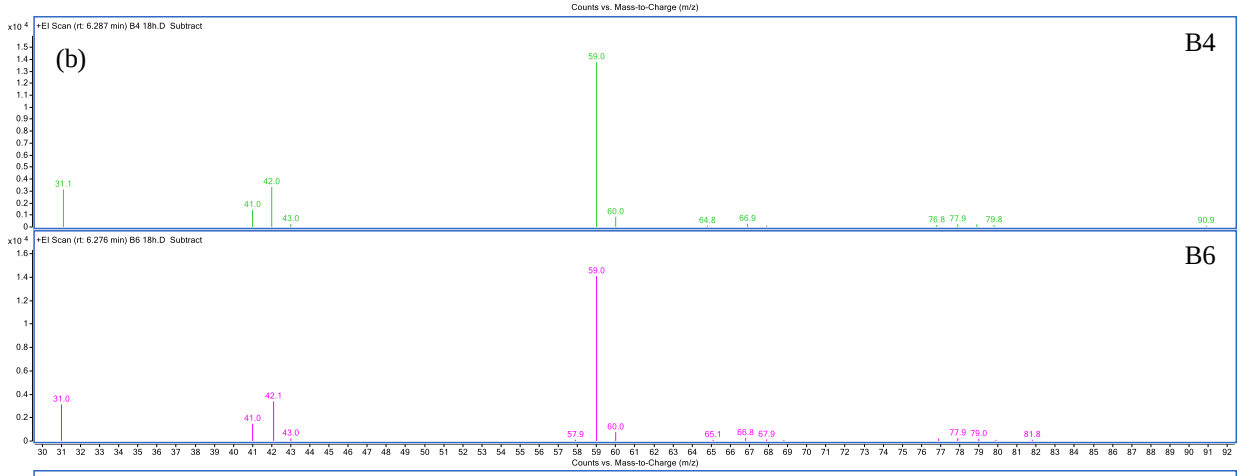
Thus, for *Salmonella typhymurium,* the MS spectra substracted from the chromatograms revealed specific molecular ions with m/z 60.9, 70.0 and 94.1 respectively, at 3.27 min, 20.89 min and 27.53 min retention times (Fig. 3). Using NIST MS program were identified three VOC compounds, namely Chloroetene, 2-Pentene and 3-Furaldehyde. Similar with results obtained for *Salmonella typhymurium*, Chloroetene and 2-Pentene were also identified for *Escherichia coli* and *Serratia rubidae*. Furthermore, Methylimino-bis-formaldehyde was observed only for the last two Gram-negative bacterial strains, while the formation of 3-Furaldehyde was observed only for *Salmonella typhymurium.*

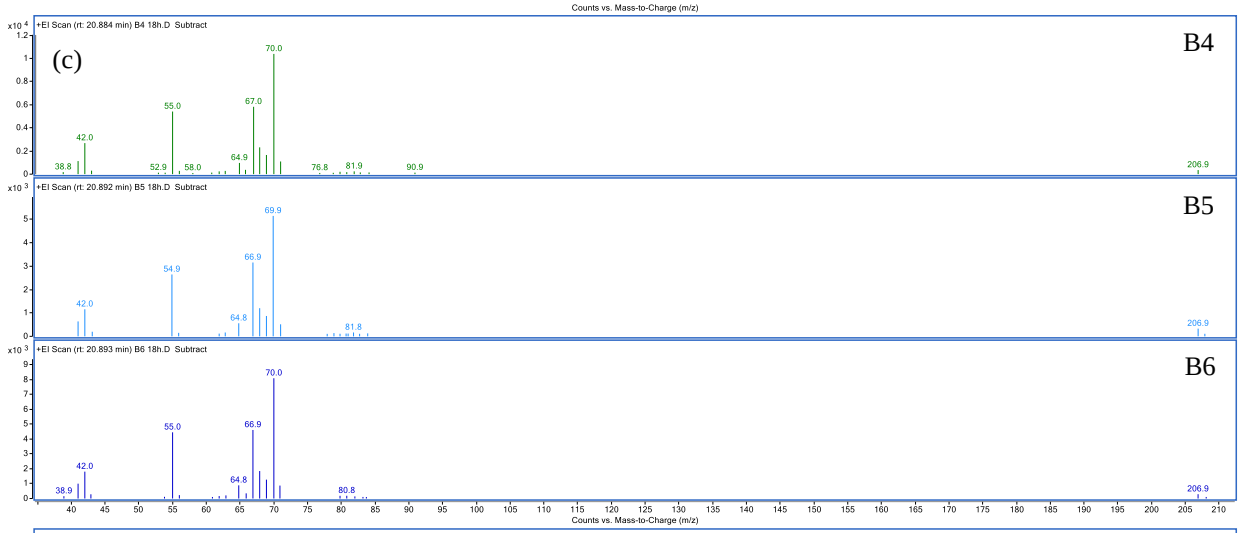
Table 2. The identified VOCs in headspace air of Gram-negative bacterial cultures

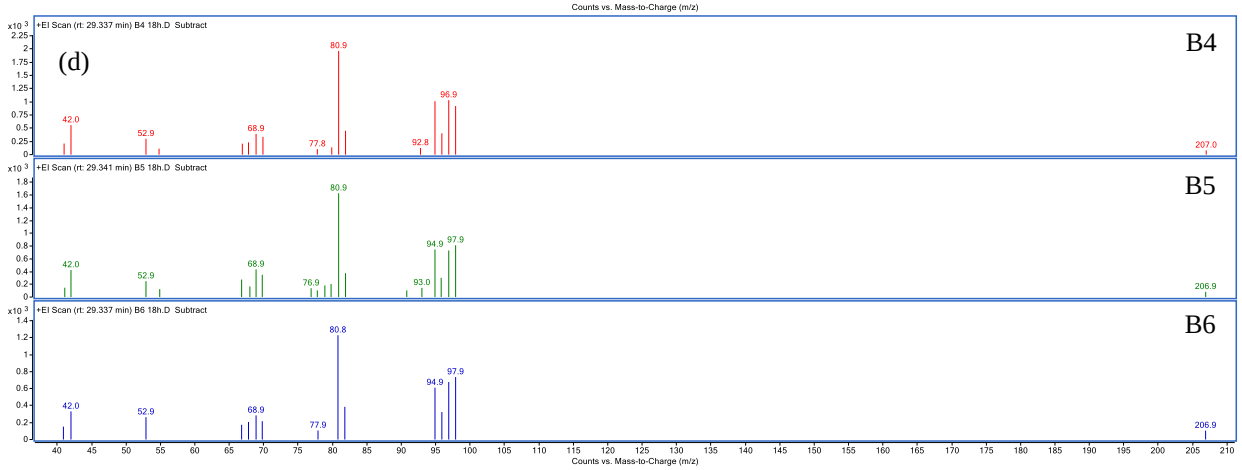
tR	Compound	CAS No.	Probability (%)		
			Salmonella typhymurium (B1)	*Escherichia coli* (B2)	*Serratia rubidae* (B3)
3.27	Chloroetene	75-01-4	71	76	71
6.28	Methylimino-bis-formaldehyde	18197-25-6	-	55	54
20.89	2-Pentene	109-68-2	56	57	57
27.53	3-Furaldehyde	498-60-2	65	-	-

The MS spectra subtracted from characteristic chromatograms, using NIST MS program, highlighted the occurrence of seven VOC compounds in the headspace air of the Gram-positive bacteria cultures (Fig. 4). The dominant molecular ions, with m/z 61.9, 59.0, 70.0, 80.9 and 66.9/90.1 could be classified into three classes of volatile organic compounds, such as alkenes, alcohols and cyclic compounds.


(a)
B4
+EI Scan (rt: 3.261 min) B4 18h.D
61.9
35.0
42.9
46.9
58.9
63.8
66.9
71.9
75.8
90.9
95.9
104.9
207.0
B5
+EI Scan (rt: 3.235 min) B5 18h.D Subtract
61.9
64.8
66.9
75.8
90.8
92.8
102.9
114.9
116.8
129.0
207.1
B6
+EI Scan (rt: 3.249 min) B6 18h.D
61.9
34.9
42.9
46.9
55.0
58.9
63.9
71.9
75.8
90.9
95.8
104.9
128.9
207.0
Counts vs. Mass-to-Charge (m/z)


(b)
B4
+EI Scan (rt: 6.287 min) B4 18h.D Subtract
59.0
31.1
41.0
42.0
43.0
60.0
64.8
66.9
76.8
77.9
79.8
90.9
B6
+EI Scan (rt: 6.276 min) B6 18h.D Subtract
59.0
31.0
41.0
42.1
43.0
57.9
60.0
65.1
66.8
67.9
77.9
79.0
81.8
Counts vs. Mass-to-Charge (m/z)


(c)
B4
+EI Scan (rt: 20.884 min) B4 18h.D Subtract
70.0
55.0
67.0
42.0
38.8
52.9
58.0
64.9
76.8
81.9
90.9
206.9
B5
+EI Scan (rt: 20.892 min) B5 18h.D Subtract
69.9
66.9
54.9
42.0
64.8
81.8
206.9
B6
+EI Scan (rt: 20.893 min) B6 18h.D Subtract
70.0
55.0
66.9
42.0
38.9
64.8
80.8
206.9
Counts vs. Mass-to-Charge (m/z)


(d)
B4
+EI Scan (rt: 29.337 min) B4 18h.D Subtract
80.9
96.9
42.0
52.9
68.9
77.8
92.8
207.0
B5
+EI Scan (rt: 29.341 min) B5 18h.D Subtract
80.9
94.9
97.9
42.0
52.9
68.9
76.9
93.0
206.9
B6
+EI Scan (rt: 29.337 min) B6 18h.D Subtract
80.8
97.9
94.9
42.0
52.9
68.9
77.9
206.9
Counts vs. Mass-to-Charge (m/z)

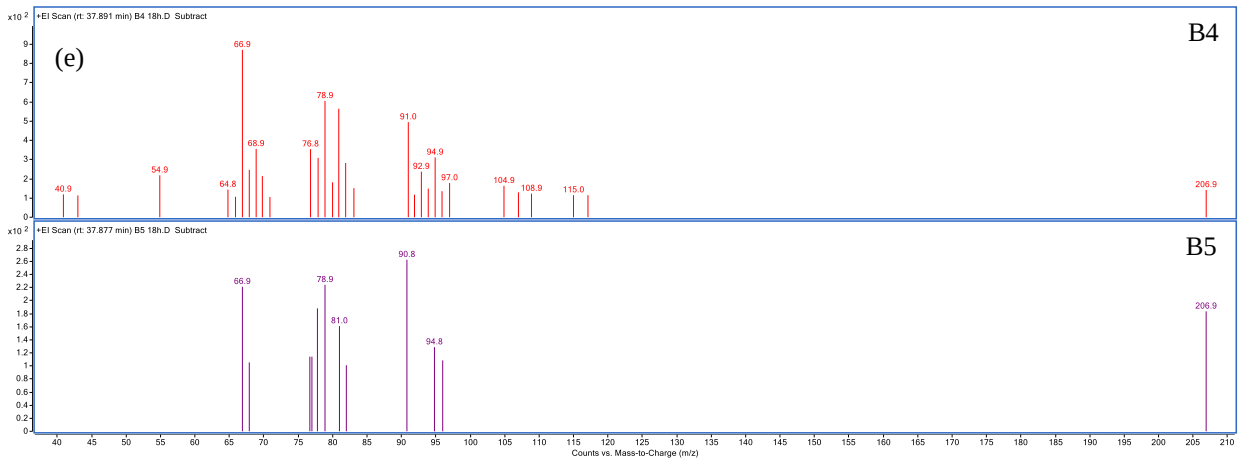


Fig. 4. MS spectra obtained for VOC compounds detected in headspace air of Gram-positive bacterial cultures at retention times: (a) 3.2 min, (b) 6.2 min, (c) 20.8 min, (d) 29.3 min and (3) 37.8 min

Thus, tert-butanol was identified for *Staphyloccocus aureus* and *Enterococcus faecalis*. 2-Pentene was observed for all three bacteria cultures, while ethanethiol and 3-oxiranyl-7-oxabicyclo [4.1.0] heptane were detected only for *Staphyloccocus aureus*. Tricyclo[4.4.1.1(3,8)]dodeca-4,9-diene and 2,5-cyclooctadien-1-ol were detected only for *Enterococcus casseliflavus*. 8-methylene-bicyclo[5.1.0]octane was identified only in the headspace air of *Enterococcus faecalis* culture (Table 3).

Table 3. The identified VOCs in headspace air of Gram-positive bacterial cultures

tR	Compus	CAS No.	Probability (%)		
			Staphyloccocus aureus (B4)	*Enterococcus casseliflavus* (B5)	*Enterococcus faecalis* (B6)
3.26	Ethanethiol	75-08-1	78	-	-
3.26	Tricyclo[4.4.1.1(3,8)]dodeca-4,9-diene	39840-95-4	-	57	-
3.26	Bicyclo[5.1.0]octane, 8-methylene-	54211-15-3	-	-	70
6.29	Tert-butanol	75-65-0	54	-	55
20.89	2-Pentene	109-68-2	62	51	60
37.9	7-Oxabicyclo[4.1.0]heptane, 3-oxiranyl-	106-87-6	63	-	-
37.9	2,5-Cyclooctadien-1-ol	10054-74-7	-	52	-

Due to their oxidative actions and metabolic, a variety of VOC compounds was also reported in the literature for many microorganisms. Recent studies have shown that distinct VOCs produced by many pathogenic microorganisms could be divided into groups: hydrocarbons and alcohols produced by the oxidation of fatty acids; acids and aldehydes due to anaerobic or oxidative metabolism. In addition, cyclic compounds whose biological origin is unknown, compounds with S whose origin is also unknown and compounds with N resulting from the breakdown of amino acids [23-25] could be produced. According to recent studies, it has been shown that different VOCs can be considered specific to some bacterial cultures [23]. Thus, it has been observed that the following volatile organic compounds are characteristic for *Staphyloccocus aureus*: dimethyl sulfide, isovaleric acid, 2-methyl-2-butanal and ammonia [15, 23, 26], while for *Escherichia coli* the emission of indole [26, 27] and octane-1-ol and ethanol are specific [26]. Moreover, it has been reported that some VOCs are not specifically produced by a bacterial strain, but are shared by different pathogens. Thus, compounds such as 2-pentanone, formaldehyde, methyl mercaptan and trimethylamine are emitted by all pathogens studied so far and can serve as biomarkers [23]. Overlapping VOCs emission by different bacteria suggests that a single VOC does not have enough

differentiating power to separate specific strains of microorganisms, requiring unique and distinct combinations of volatile organic compounds to differentiate between various bacterial cultures or between bacterial strains and other microorganisms [28].

CONCLUSIONS

Identifying microorganisms such as pathogenic bacteria using Headspace-GC-MS by analyzing VOC profiles can be a quick and accurate method. This study presented preliminary results on the detection and identification based on mass spectra of volatile organic compounds emitted by certain bacterial strains. The types of VOCs were different and specific both for the classes of pathogenic bacteria analyzed and compared to those determined only in the culture medium. For Gram-negative bacteria, three common volatile organic compounds could be identified for all three bacterial strains tested, namely alkenes, amide and aldehydes, while for Gram-positive bacteria the major VOCs compound were alkenes, alcohols and cyclic compounds. Nevertheless, a direct comparison of the determined VOCs emitted in headspace air by the studied bacterial strains with those reported in other similar studies is not always appropriate, because depending on the working conditions (bacterial culture medium or chromatographic column used) the type of VOCs emitted may be completely different. In conclusion, the results of this study demonstrate the ability to identify specific pathogenic bacteria based on the profile of VOCs emitted into the headspace air of these cultures.

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