Multiple stir bar sorptive extraction combined with gas chromatography-mass spectrometry analysis for a tentative identification of bacterial volatile and/or semi-volatile metabolites

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ABSTRACT

We propose a new approach combining the principles and advantages of stir bar sorptive extraction (SBSE) and headspace sorptive extraction (HSSE). Stir bars have so far never been used for the extraction of volatile/semi-volatile bacterial compounds. The effectiveness of two stir bars with polydimethylsiloxane (PDMS) or ethylene glycol/silicone (EGS) as sorbent was tested by performing sample extraction directly in gas chromatography (GC) vials containing bacterial cultures. Several combinations of desorption and extraction were tested at different growth times. When the extraction was carried out simultaneously with the EGS stir bar in headspace and the PDMS in the bacterial culture, the number of extracted compounds was significantly increased. Using both twisters increased the polarity range of the compounds found, and extraction at the end of the exponential phase of growth generated the best yields. This method was successfully applied to determine the production of 17 molecules by a strain of *Staphylococcus aureus*. In conclusion, this study paves the way for a new method for determining the volatile metabolite profile of bacteria, which can provide a promising innovative alternative in the identification of biomarkers.

1. Introduction

Analysis of volatile compounds is essential for environmental control (detection of micropollutants such as pesticides or endocrine disruptors) [1] and for the food industry (detection of aroma compounds, monitoring of food additives) [2,3].

More recently, the development of "omics" disciplines has paved the way for the use of metabolite biomarkers in diagnostics [4–7]. The principle is based on the observation that several diseases impact the metabolic profile of specific cells. The resulting metabolites can be used to track disease evolution [8].

Regardless of the field of application, the analysis of volatile compounds requires substantial consideration of the concentration and extraction of the compounds before their analysis. By using gas chromatography coupled to mass spectrometry (GC-MS), samples can be analysed from different matrices (gaseous, liquid or solid). For liquid matrices, liquid-liquid extraction has long been the method of choice. This extraction is based on the partition coefficient between two solvents, one of which is non-polar [9]. Due to its potential toxic effect, the use of such solvents has been progressively abandoned and alternative "solvent-free" extraction techniques have been developed.

Among the alternative techniques, Solid Phase Microextraction (SPME) consists of a small amount of sorptive phase coated on a solid support (fibre) exposed to a sample, either in headspace [10] or immersed in a liquid [11]. After a determined time, allowing the adsorption of compounds, the fibre can be inserted directly into the chromatograph for thermal desorption to analyse volatile compounds [12] or, for non-volatile compounds, desorbed by an organic liquid [13].

In 1999, Baltussen [14] introduced a new sorption technique called Stir Bar Sorptive Extraction (SBSE). Based on the same principle of SPME (partition of analytes between an aqueous matrix and a polymeric phase), SBSE increased the contact surface and improved the enrichment factor (defined as the analyte recovery in the extraction phase) [14].

SPME and SBSE proved to be more specific for volatile compounds

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by placing the sorptive phase (fibre or bar) in the headspace instead of immersing it in the liquid. A previous study showed that the stir bar (HeadSpace Sorptive Extraction (HSSE)) [15] exhibited a better concentration capacity than fibre [16].

Currently, two types of coating phases are mainly used: polydimethylsiloxane (PDMS) to retain non-polar compounds; and ethylene glycol/silicone (EGS) to absorb polar compounds, especially those forming hydrogen bonds [17]. SBSE or HSSE methods have been deployed in many fields and on a large variety of matrices [18–23].

With the exception of Ochiai et al. (2013), who described a multi SBSE for odour compounds analysis, few studies have assessed the specificity of the two kinds of stir bars for larger screening.

Furthermore, bacterial strains have a distinct metabolism, leading to a production of specific volatile compounds [24–26]. The detection of these molecules represents a high interest in clinical diagnostics and in the monitoring of infections, but only a limited amount of research is available at this moment [27] and there is a need for new and innovative detection methods.

In this paper, we propose for the first time to assess an approach coupling the two bars (in SBSE or HSSE format) for the analysis of volatile or semi-volatile metabolites produced by bacteria.

In this first approach, the method was developed from a reference *Staphylococcus aureus* strain. Interestingly, *S. aureus* has already been successfully detected in cases of cystic fibrosis or pulmonary infections by the volatile biomarkers it produces [28,29]. Moreover, the volatile profile of this strain has been described in the literature [27]. Thus, it is a bacterial model particularly adapted to the development of new methods to detect volatile or semi-volatile metabolites.

2. Material and methods

2.1. Materials

SBSE and HSSE extractions were performed with 10 mm twister® magnetic stir bars coated with PDMS or EGS (both 0.5 mm film thickness). Stir bars were obtained from Gerstel and were conditioned prior to use according to the manufacturer's instructions. In order to control the identification of some compounds, standards were used: ethanol (99.8%) and acetic acid (99.8%) were obtained from VWR (Fontenay-sous-Bois, France). Acetaldehyde (99%), propan-2-one (99.8%), benzaldehyde (99%) and 3-methylbutanoic acid (99%) were purchased from Sigma-Aldrich (Saint-Louis, USA). A standard mixture containing linear alkanes from C7 to C40 from Sigma-Aldrich (Saint-Louis, USA) was used to evaluate the retention index (RI).

2.2. Culture conditions

S. aureus ATCC 25923 [30] was the reference strain used in this study. The strain was maintained as frozen stock on Microbank™ bead (Pro-Lab Diagnostic, Richmond Hill, Canada) at −80 °C, and cultivated overnight onto Luria Bertani (LB) medium (tryptone (Fluka, Saint-Louis, USA), yeast extract (Amresco, Solon, USA) and sodium salt (Panreac, Barcelona, Spain)) at 37 °C with shaking at 210 rpm. From this culture, sterile 20 mL glass vials sealed with PTFE/Silicone screw caps (Agilent, Santa-Clara, USA) containing a magnetic stirrer and 10 mL of LB medium were inoculated at 0.1 uDO mL^{-1} and placed at 37 °C under shaking at 600 rpm.

Growth was monitored by spectrophotometry at 600 nm every hour for 24 h. Each experiment was performed in triplicate.

2.3. SBSE/HSSE extraction procedure

Several extraction conditions were tested to determine the contribution of each stir bar in order to determine the performance of a simultaneous extraction (PDMS and EGS in the same vial) compared to a series of separate extractions (one twister per vial). In all cases, PDMS and EGS twisters were inserted into liquid cultures of LB medium and in headspace, respectively, at the beginning of the experiment (t = 0 h), and remained for 3, 6 or 9 h of culture at 37 °C and shaking at 600 rpm. When EGS twister was used alone, an inert and sterile magnetic bar was added into the culture to facilitate the volatilisation of organic compounds and shake the culture.

After exposure, the stir bars were removed from the bacterial culture. The PDMS twister was rinsed with ultrapure water and blotted with a lint-free tissue before being placed in a glass thermal desorption tube. The EGS twister was directly placed into a glass thermal desorption tube.

To eliminate the compounds present in the media, the same experiments were performed on LB medium alone in three replicates in the same conditions as the sample. These conditions are defined as control.

2.4. GC-MS analysis

The analytical system was composed of an Agilent 7890 B gas chromatograph coupled to an Agilent 5977 A mass spectrometer with an MPS autosampler, a thermal desorption unit (TDU) and a cooled injection system (CIS) (Gerstel). The data acquisition software MSD Chemstation F.01.00 (Agilent) was used to program the GC-MS. The gas chromatograph was fitted with a VF-WAXms fused silica capillary column (30 m × 0.25 mm × 0.25 µm, Agilent) and was used with helium as carrier gas at 0.8 mL min^{-1}. The tube was thermally desorbed in the TDU at 220 °C (suitable temperature for both bars, recommended by the supplier) for 5 min.

Two desorption conditions were tested: (i) a separate desorption, with one stir bar per tube, (ii) or a simultaneous desorption, with PDMS and EGS in the same tube. After desorption, volatile or semi-volatile compounds were focalised on the CIS at −10 °C for 2 min, ramped to 250 °C at a heating rate of 12 °C per second, and finally held for 2.5 min in splitless mode to ensure complete desorption of analytes. The column temperature was initially kept at 40 °C for 7 min and then increased from 40° to 240°C at 8 °C min^{-1}, the temperature was maintained for 3 min. Then the sample was introduced into the ion source of the Agilent 5977 A mass spectrometer. The transfer line temperature was set at 250 °C and ion source temperature at 230 °C. Ions were generated by a 70 eV electron beam. Masses were acquired from *m/z* 33–500 amu.

2.5. Data processing and statistical analysis

All peaks were integrated using MassHunter Qualitative Analysis software B.06.00 (Agilent Technologies). This software deconvoluates the chromatograms, separating the co-eluted compounds. Compounds were then identified by their mass spectra by using the National Institute of Standard and Technologies (NIST) and the Wiley7 reference libraries.

Compounds identified present in at least two out of three replicates were conserved for the analysis. Compounds found in the controls were not considered.

For each compound available in the databases, the retention index was determined using the one previously obtained on a polar column, as described by Van Den Dool and Kratz [31]. A maximum relative deviation of 2.5% from literature values was accepted to identify bacterial compounds (Supplement material, Table S1).

3. Results and discussion

3.1. Assessment of SBSE for the concentration and extraction of volatile and semi-volatile bacterial metabolites

The first objective was to determine the efficiency of the two types of stir bars (PDMS and EGS) for a tentative identification of *S. aureus* metabolites. Each bar was exposed individually to a culture of *S. aureus*

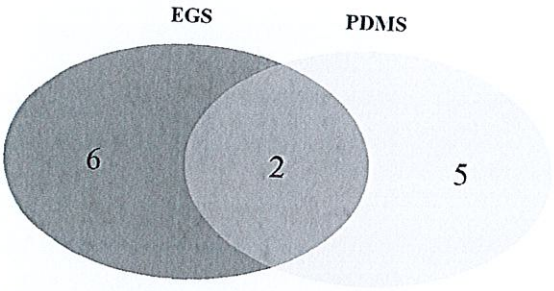

Fig. 1. Distribution of the number of volatile and semi-volatile compounds produced by *S. aureus* and extracted with each stir bar. Venn diagram illustrating the degree of overlap of extracted and identified volatile and semi-volatile compounds between the two twisters. The central section in grey represents the compounds that are found by both stir bars. Specific compounds extracted by EGS twister are in dark grey while those extracted with PDMS are in light grey.

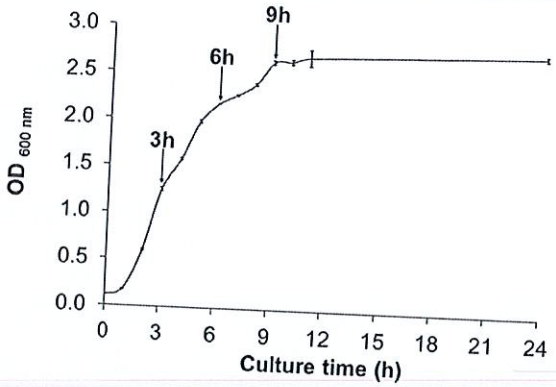

Fig. 2. Selected extraction times of *S. aureus*. Growth curve of *S. aureus* in GC vials was followed by optical density measurement at 600 nm. The data shown average obtained from three independent experiments with error bars representing standard deviation.

for 6 h. Then, the bars were desorbed separately in the TDU and the identified compounds were compared using a Venn diagram (Fig. 1). A total of 13 compounds were detected (Table 1): eight were extracted with the EGS twister and seven with the PDMS twister. Only two compounds were retained by both EGS and PDMS stir bars.

These results are in agreement with the literature. Baltussen et al. explained that the octanol-water partition coefficient (K_{ow}) and the amount of sorptive phase coated on the bar are the main parameters influencing the extraction [14]. The PDMS phase has also been described to preferentially adsorb compounds with K_{ow} between 2 and 3 [14], while the EGS phase was developed for compounds with moderate polar profiles. Due to its silicone-based nature, the EGS twister can retain analytes with a wider range of polarity and with a significant increase in the recovery of molecules with K_{ow} smaller than 2 while maintaining satisfactory capabilities for extraction of apolar/slightly polar compounds [32].

Interestingly, two compounds were extracted by the two phases: acetic acid and 4-methylquinoline, although the extraction yields between methods showed a better affinity for one of the two phases: 80% of acetic acid ($K_{ow} = -0.17$) was extracted by the EGS twister and 87% of 4-methylquinoline ($K_{ow} = 2.61$) by the PDMS twister.

The results showed that the SBSE method allowed extraction of a large range of bacterial metabolites. The two kinds of stir bars were complementary; EGS extracting the polar compounds and PDMS the apolar ones.

3.2. Optimization of extraction method

Bacterial growth comprises three phases: a lag phase, an exponential phase and a stationary phase (Fig. 2). Three extraction times corresponding (i) to the middle of exponential phase (3 h), (ii) to the end of exponential phase and the beginning of the stationary phase (6 h) and (iii) to the stationary phase (9 h) were selected to determine the best conditions between the exposure time and the number of extracted metabolites.

The experiment was carried out in triplicate for each point with simultaneous extraction and desorption (Chromatograms available in Supplement material, Fig. S1). Only the compounds found in at least two of the three replicates were considered (Fig. 3).

After 3 h of growth, 16 volatile and/or semi-volatile compounds found in *S. aureus* cultures could be identified, while 22 and 23 compounds were found after 6 and 9 h, respectively. The difference between the number of compounds found at 3 h and 6 h of extraction was statistically significant ($p = 0.039$). No significant difference was observed between 6 h and 9 h ($p = 0.882$).

Among the compounds extracted after 6 h, two were not detected after 9 h, probably due to either a total or partial consumption during growth, or a modification of their production during the bacterial growth (with a decrease during the stationary phase) as previously shown for formic acid [33]. Similarly, of the 23 compounds extracted after 9 h, three were not detected after 6 h. However, two of them were identified in one of the three replicates and were thus not considered. One explanation could be the low production of these metabolites, making it difficult to detect the signal against the background noise.

Therefore, the final protocol requires some handling (positioning and removal of the stir bars in the vial, followed by their rinsing, drying and positioning in the desorption tube) for a total extraction time of 6 h. Compared to other more common extraction techniques focussed on a specific bacterial growth time, the proposed protocol is longer but lead

Table 1
Identification and main characteristics of the volatile and semi-volatile compounds extracted by each twister. K_{ow} values were obtained by the software EPI SUITE. Compounds recovered on the EGS twister have low range K_{ow} from −4.15 to 2.61 while those extracted with the PDMS have higher range K_{ow} from 0.4 to 4.09.

Compound	Formula	Retention time (min)	Molecular weight ($g.mol^{-1}$)	Log K_{ow}	EGS peak area	PDMS peak area
Acetaldehyde	C_2H_4O	5.932	44.0	−0.34	5,087,208	/
Propan-2-one	C_3H_6O	6.886	58.0	−0.24	243,238	/
Ethanol	C_2H_6O	9.031	46.0	−0.31	647,891	/
1-methyl-1-propylhydrazine	$C_4H_{12}N_2$	12.097	88.1	−0.21	326,360	/
3-methylbutan-1-ol	$C_5H_{12}O$	15.331	88.1	1.16	/	499,654
Acetic acid	$C_2H_4O_2$	20.237	60.0	−0.17	3,249,288	822,073
Formic acid	C_2H_6O	21.412	46.0	−0.54	3,694,860	/
Undecan-2-one	$C_{11}H_{22}O$	22.585	170.2	4.09	/	117,805
1,3,5,7-Tetraazatricyclo[3.3.1.1^{3,7}]decane	$C_6H_{12}N_4$	27.478	140.1	−4.15	1,483,838	/
4-methylquinoline	$C_{10}H_9N$	30.033	143.1	2.61	138,612	901,885
3-phenylpyridine	$C_{11}H_9N$	31.555	155.1	2.57	/	129,777
Isoquinoline-1-carbonitrile	$C_{10}H_6N_2$	32.811	154.1	1.69	/	1,363,594
Quinoline-4-carbaldehyde	$C_{10}H_7NO$	33.968	157.1	0.4	/	2,442,258

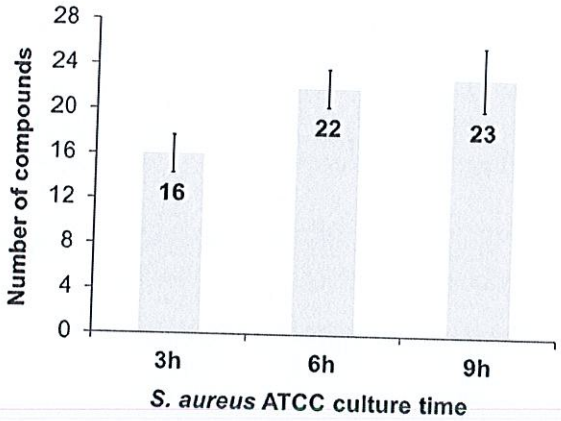

Fig. 3. Number of *S. aureus* metabolites identified by GC-MS after simultaneous PDMS/EGS extraction at different points during bacterial growth. Errors bars indicate the standard deviation of the identification of the compounds in the three independent replicates.

to an overall metabolic profile over the full growth period of the bacteria. Such process avoids small biological variations and significantly increases the number of compounds detected.

3.3. Impact of simultaneous or separated extraction and desorption

Finally, we assessed the impact of separate (one bar per vial) versus simultaneous (two bars in the same vial, with the EGS in headspace and the PDMS in liquid culture for 6 h) extraction. The results are presented in Fig. 4.

In separate extraction (test 1 A), 13 bacterial volatile and/or semi-volatile compounds were identified by GC-MS: six extracted from the EGS twister, four from the PDMS and three from the two bars.

In simultaneous extraction (test 1B), 20 compounds were extracted (10 from the EGS, six from the PDMS and four from the two bars). Simultaneous extraction allowed extraction and tentative identification of significantly more compounds than separate extraction ($p = 0.007$) (Fig. 4A and B). This difference could be explained by the fact that the PDMS twister, at the bottom of the vial, captures the heavier and more apolar molecules, thus increasing the available surface of coating phase of the EGS stir bar thus promoting the adhesion of a large number of volatile and light compounds (Table 2).

The simultaneous extraction and desorption was tested (test 2, Fig. 4). Simultaneous desorption (220 °C) detected 22 molecules, but compared to test 1B, no statistical significant difference ($p = 0.768$) was observed (Chromatograms available in Supplement material, Fig. S2).

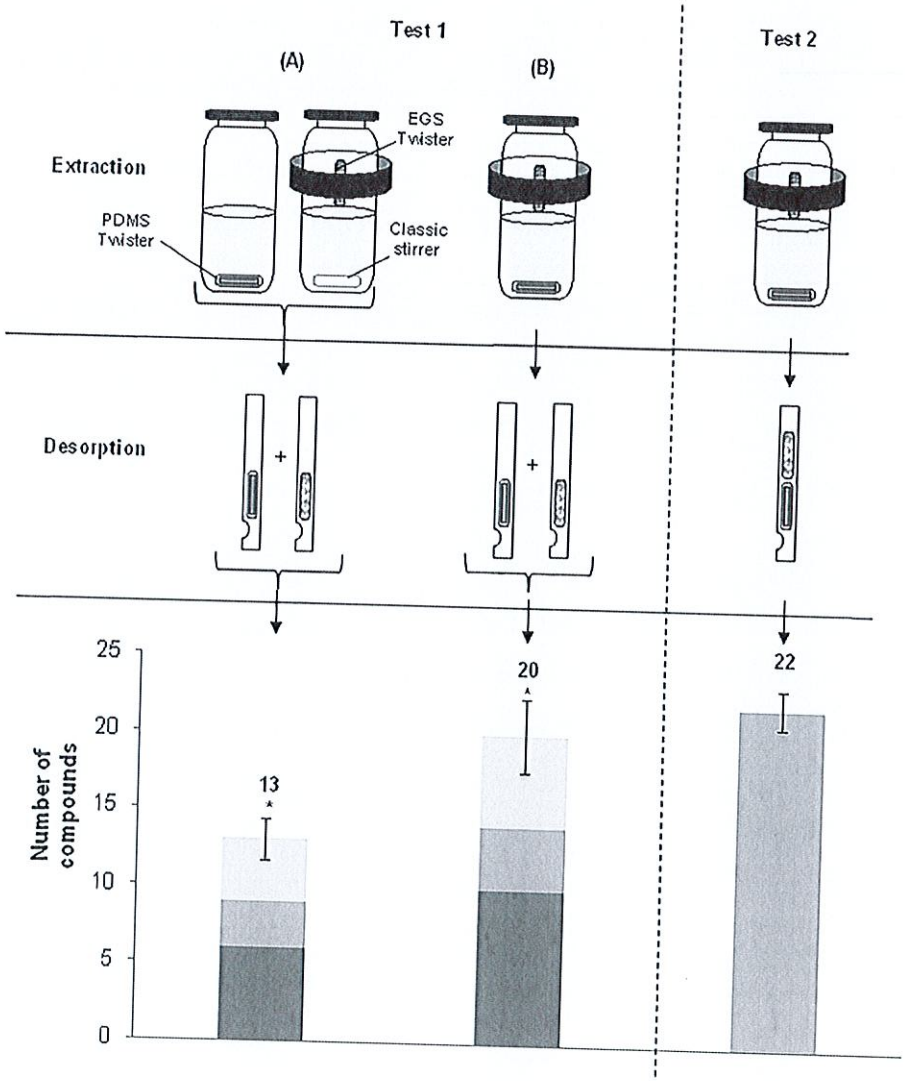

Fig. 4. Schema and results of separate or simultaneous extraction and desorption. The histograms are stacked with the compounds found with EGS twister in dark grey, while those found with PDMS twister are in light grey; in grey, molecules extracted with these two stir bars are visible. The significant difference between tests is indicated by a star. Errors bars indicate the standard deviation of the identification of the compounds in the three independent replicates. "Test 1" compares the efficiency of separate extraction (two vials/two bars - A) and a simultaneous one (one vial/two bars - B) with a separate desorption. "Test2" implements a simultaneous extraction (one vial/two bars) followed by a simultaneous desorption.

Table 2
Identified compounds of each extracted and desorbed condition.

Compound	Retention Time (min)	Molecular weight (g.mol^{-1})	Henry LC (Pa.m^3.mol^{-1})	Test 1 A	Test 1 B	Test 2
Acetaldehyde[2]	5.930	44.0	6.76	EGS	EGS	Both
2-methylpropanal[1]	6.814	72.1	1.82×10^{1}			Both
Propan-2-one[1]	6.876	58.0	3.55	EGS	EGS	Both
Methyl acetate[1]	6.998	74.0	1.17×10^{1}		EGS	Both
Prop-2-enal	7.250	56.0	1.24×10^{1}		EGS	
Ethanol[1]	9.044	46.0	5.07×10^{-1}	EGS	EGS	Both
(methyldisulfanyl)methane	10.806	94.0	1.23×10^{2}		PDMS	
1-methyl-1-propylhydrazine[1]	12.094	88.1	1.24×10^{-2}	EGS	EGS	Both
4-methylheptan-2-one[1]	15.320	128.2	2.07×10^{1}		PDMS	Both
3-methylbutan-1-ol	15.331	88.1	1.43	PDMS		
(methyltrisulfanyl)methane[1]	18.775	126.0	1.89×10^{2}		EGS	Both
3-ethyl-2,5-dimethylpyrazine[1]	20.123	136.1	5.28×10^{-1}			Both
Acetic acid[1]	20.306	60.0	1.45×10^{-2}	Both	Both	Both
Formic acid[1]	21.527	46.0	1.69×10^{-2}	EGS	Both	Both
Benzaldehyde[2]	21.697	106.0	2.71		EGS	Both
Undecan-2-one	22.585	170.2	6.44	PDMS		
2-hydroxybenzaldehyde[1]	23.456	122.0	5.68×10^{-1}			Both
3-methylbutanoic acid[1]	23.719	102.1	8.44×10^{-2}		EGS	Both
Acetamide[1]	25.051	59.0	1.13×10^{-3}			Both
1,3,5,7-Tetraazatricyclo[3.3.1.1^{3,7}]decane[1]	27.463	140.1	1.66×10^{-4}	EGS	EGS	Both
4-methylquinazoline	29.714	144.1	3.18×10^{-2}		PDMS	
4-methylquinoline[2]	30.039	143.1	1.67×10^{-1}	Both	Both	Both
Decanoic acid[2]	31.546	172.1	1.36×10^{-1}			Both
3-phenylpyridine[1]	31.565	155.1	4.83×10^{-2}	PDMS	PDMS	Both
Isoquinoline-1-carbonitrile[1]	32.788	154.1	6.74×10^{-4}	Both	Both	Both
Quinoline-4-carbaldehyde[2]	33.961	157.1	8.21×10^{-4}	PDMS	PDMS	Both
1H-indole[1]	33.974	117.0	5.35×10^{-2}		PDMS	Both

The tests shown here correspond to those described in Fig. 3 with the different extraction and desorption conditions. [1]produced compounds and [2]consumed compounds.

3.4. Analysis of the volatiles and semi-volatiles identified

Table 2 shows all the compounds detected in the different tests. A total of 22 volatiles and/or semi-volatiles were obtained and identified under optimal SBSE/HSSE extraction conditions (test 2).

In order to identify products consumed or produced by *S. aureus*, the areas of the peaks of interest were compared with the controls (data not shown). Seventeen volatile or semi-volatile compounds were produced by *S. aureus*, while five were consumed (acetaldehyde, benzaldehyde, 4-methylquinoline, decanoic acid and quinoline-4-carbaldehyde). Among the 17 produced compounds, nine were also present in the controls but their area was markedly increased during the test (from 22% to 630%). Similarly, four out of the five consumed compounds showed a decreased area varying from 30% (for acetaldehyde) to 86% (for benzaldehyde). Only quinoline-4-carbaldehyde showed a small variation, with an area decreasing by 2%.

Interestingly, five compounds (prop-2-enal, (methyldisulfanyl)methane, 3-methylbutan-1-ol, undecan-2-one and 4-methylquinazoline) were not found in test 2. This could be explained by the fact that (i) these compounds have very small areas in separate desorption and (ii) when twisters were desorbed simultaneously, the background noise increased. The area of these compounds was also below the detection limit. In contrast, two compounds were not detected in test 1B but only in simultaneous desorption: 2-hydroxybenzaldehyde and 3-ethyl-2,5-dimethyl-pyrazine. These two compounds were detected with the PDMS and EGS stir bars in test 1B, but their concentrations were too low and these compounds were detected only once out of three replicates. The desorption of the bars simultaneously in test 2, increased the concentration retained by the two twisters enough to overcome the background noise.

Finally, 22 compounds were detected by SBSE/HSSE method, of which 17 were produced and five were consumed. Regarding the 17 compounds produced, seven have already been described as produced by *S. aureus*, although one of them, the indole, is controversially described as either produced or consumed by the bacterium [25,34–38]. The major advance of the method described is the potential identification, for the first time to our knowledge, of 10 new metabolites produced by *S. aureus*: methyl acetate, 1-methyl-1-propylhydrazine, 4-methylheptan-2-one, 3-ethyl-2,5-dimethylpyrazine, formic acid, 2-hydroxybenzaldehyde, acetamide, 1,3,5,7-tetraazatricyclo [3.3.1.1^{3,7}]decane, 3-phenylpyridine and isoquinoline-1-carbonitrile. Their role and interest in clinical practice must be evaluated in the future.

4. Conclusion

This paper presents an original method for the tentative identification of bacterial volatile and/or semi-volatile metabolites. It is based on the use of a SBSE/HSSE multi-extraction procedure with two complementary sorptive PDMS and EGS stir bars. To our knowledge, this is the first time this technique has been applied to extract bacterial metabolites. The main objective of this study was to evaluate the potential of this technique by using a screening of a single bacterial strain. Development of the method was obtained by (1) inserting both twisters in the same vial: the PDMS stir bar at the bottom (liquid phase) and the EGS in headspace of the vial, and (2) a simultaneously desorption in the TDU. Exposure time of the bars with the culture medium was observed to be more efficient (detection of the highest number of bacterial metabolites) towards the end of exponential growth phase. These experiments demonstrated that simultaneous extraction with EGS twister in headspace and PDMS in liquid phase, combined with simultaneous desorption, was the most suitable conditions for the extraction and identification of bacterial metabolites by GC-MS of strains grown in vials. By applying all these parameters, we define a first tentative SBSE/HSSE metabolite profile of *S. aureus*, which contains 17 molecules probably produced by this bacterium.

Therefore, this method will be applied to other bacterial strains in order to identify and to establish their volatile and semi-volatile metabolic profiles. Once identified, specific metabolites of each strain could be quantified and be used as biomarker to monitor the infections.

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Conflicts of interest

There are no conflicts to declare.

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