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Rapid recognition of bacteremia in humans using atmospheric pressure chemical ionization mass spectrometry of volatiles emitted by blood cultures

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Abstract

Rapid recognition of pathogenic bacteria in humans is a serious problem in clinical research. In this six-month study, molecular volatiles of blood cultures from 61 patients at the suspicion of bacteremia and 39 patients positively diagnosed with bacteremia were fingerprinted by atmospheric pressure chemical ionization mass spectrometry (APCI-MS) for the presence of five common pathogens (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*) which statistically account for ca. 50% cases of bacteremia in humans. All the infected blood cultures revealed characteristic and clearly distinct MS patterns specific to the presence of one of the five pathogens after the incubation time of 3-16 h. Technical replicates were incubated over 2-3 days for the reference diagnosis using traditional blood culture detection. Overall, the results of our six-month hospital screening show that APCI-MS of blood culture volatiles allows rapid, reliable and cost-efficient diagnosis of bacteremia in humans. The integration of this approach in clinical practice will be further promoted by the growing availability of atmospheric pressure ionization mass spectrometers in bioanalytical laboratories and core facilities.

1. Introduction

Bloodstream bacterial infections pose severe health risks to humans. For example, one of the most frequent cause of bacteremia worldwide, *Staphylococcus aureus*, is associated with a 12-week mortality rate of 22%.[1] The efficiency of medical treatment largely depends on the speed of bacteremia recognition, but the lack of specific symptoms may delay the diagnosis. This urges the development of more rapid and affordable diagnostic tools to replace the traditional biochemical routines employed in clinics.

Among a variety of strategies, identification of bacteria via the direct analysis of emitted volatile organic compounds (VOCs) is particularly attractive, owing to the non-invasiveness, practical simplicity, cost-efficiency, high speed of analysis and toxicological safety.[2-5] Over recent years, a number of workflows have been developed for the rapid identification of bacteria based on VOC analysis, mostly using chemical sensing or mass spectrometry (MS) detection.[6-26] Particularly notable progress in VOC analysis has been achieved by selected ion flow tube mass spectrometry (SIFT-MS).[15-26] Although the reported methods have many evident analytical merits for rapid bacterial identification, their integration into clinical practice is hindered due to the requirement of dedicated instrumentation which is not widely available in analytical laboratories.

Ionization of volatiles in the ambient atmosphere enables molecular analysis of bacteria to be done on atmospheric pressure ionization (API) mass spectrometers.[12, 27] API mass spectrometers (e.g., ion trap, time-of-flight, triple-quadrupole, Orbitrap) have by now become common in bioanalytical laboratories and core facilities, and their application is further

increasing. Compared to SIFT-MS, ionization in API-MS is usually less efficient and less selective, but the possibility of tandem mass analysis in API-MS greatly facilitates identification of biomarker signals and differentiation of structural isomers. The power of API-MS for the direct molecular analysis of complex samples has dramatically increased over the past decade with the introduction of ambient ionization techniques, such as desorption electrospray ionization (DESI-MS),[28] direct analysis in real time (DART-MS),[29] laser ablation electrospray ionization (LAESI-MS),[30] low-temperature plasma probe (LTP-MS),[31] paper spray ionization,[32, 33] leaf spray ionization,[34] rapid evaporative ionization (REIMS),[35] desorption atmospheric pressure chemical ionization (DAPCI-MS)[36] and many others. A number of API-MS approaches for bacterial differentiation have been reported based on the ambient desorption/ionization of nonvolatile metabolites (most commonly, lipids) from intact bacterial cells.[37-43] Analysis of bacterial volatiles on API-MS instruments has been demonstrated by secondary electrospray ionization (SESI-MS)[44-47] and by atmospheric pressure chemical ionization (APCI-MS),[48] but the potential of these approaches in real clinical practice remains largely unexplored.

In present study we extend the investigation of VOC analysis by APCI-MS toward clinical diagnosis of bacteremia in humans. Over the time period of six months, we analyzed blood cultures of 61 suspects, 39 positively diagnosed patients and 180 blood cultures of healthy individuals inoculated with five common bloodstream pathogens, including *Staphylococcus aureus* (SA), *Escherichia coli* (EC), *Klebsiella pneumonia* (KP), *Acinetobacter baumannii* (AB) and *Pseudomonas aeruginosa* (PA). These species account for ca. 50% of microorganisms derived from 9613 consecutive blood isolates studied in the framework of SENTRY antimicrobial surveillance program.[49] Headspace VOCs from growing cultures were continuously sampled to

solvent-assisted APCI-MS with nitrogen carrier gas without any pre-concentration and chromatographic separation, as detailed in our recent publication.[48] Secondary ions were monitored with a LTQ linear ion trap mass spectrometer (ThermoFischer, San Jose, CA, USA). Overall, our results indicate that APCI-MS analysis of VOCs emitted by blood cultures can shorten the diagnostic time and improve the cost-efficiency of currently accepted clinical protocols. Because VOC analysis is a nondestructive method, the analyzed blood cultures can be further interrogated by conventional biochemical assays for complementary diagnosis.

2. Materials and methods

2.1. Blood culture

Over the time period of six months, we analyzed blood cultures of 61 non-diagnosed individuals at suspicion of bacteremia and 39 clinically diagnosed patients. 30 healthy volunteers for reference measurements were selected based on the lack of bacteremia symptoms. All the volunteers were instructed about the goal of the study. Informed consent was obtained for any experimentation with blood samples. Each volunteer donated 10 mL of blood. The collected 10 mL blood was mixed with 38 mL liquid culture medium (tryptic soy broth, TSB). For healthy volunteers, the resulting 48 mL was equally split into six 8 mL fractions. Five out of the six fractions were inoculated with five KP, AB, EC, SA and PA at the concentration of 10^4 colony-forming units (CFU) per mL. The remaining sixth fraction was not inoculated. All the fractions were incubated in 10 mL centrifuge tubes (Solarbio, Beijing, China) at 35 °C for 16 h unless

specified otherwise. After incubation, ca. 2 mL of each fraction was withdrawn for biochemical analysis, and the remaining volumes (ca. 6 mL) were analyzed by APCI-MS. Patient samples were incubated without splitting (48 mL) in glass bottles (Hemoline, BioMerieux, Shanghai, China) under the same conditions for 16 h. After incubation, each sample was split into six replicates (6 mL per centrifuge tube). The remaining volume was deposited for biochemical identification.

2.2. APCI-MS analysis of VOCs emitted by blood cultures

The majority of MS experiments was done on a commercial linear ion trap mass spectrometer (LTQ-XL, ThermoFischer, San Jose, CA, USA) interfaced with a home-made corona discharge ionization source introduced earlier[50] (schematically shown in Fig. S1). Briefly, a high voltage (+4 kV in positive and -3.5 kV in negative ion detection mode) was applied to a stainless steel needle (OD 150 μm) with a sharp tip (curvature radius ~7.5 μm) to create corona discharge at ambient pressure. Nitrogen with a purity of 99.9999% was used as the nebulizing gas at a flow rate of 3.2 L/s (1.6 MPa). Sheath solvent solution (methanol/water = 50/50) was introduced through a fused silica capillary (ID 0.15 mm, OD 0.17 mm) of channel I at 5 μL/min for higher discharge stability. The headspace VOCs of blood cultures were continuously transferred into ionization region with nitrogen gas (0.1 MPa) via plastic tubing (ID 1.0 mm; OD 1.6 mm) (channel II). Earlier introduced sampling interface was used.[48] The cap of a blank centrifuge tube was detached from the rest of the tube, and the inlet and the outlet gas lines were sealed onto the top of the cap. The assembly was firmly fixed in front of the LTQ. For MS analysis,

103 open centrifuge tube with a microbial culture was connected to the mounted cap. To change
104 sample simply required disconnecting the tube and connecting a tube with the next sample to
105 the same cap. This operational workflow allowed the high throughput and reproducibility of
106 analysis. No notable sample carry-over effects were observed, as verified by reference
107 headspace measurements of a pure centrifuge tube sampled right after blood cultures. The
108 angle between the discharge needle and the outlet tubing was 30°. The distance from the tip of
109 the needle to the end of the outlet tubing was 2 mm. The distance from the tip of the needle to
110 the inlet of the LTQ capillary was 6 mm. In total, each sample was analyzed in five replicates
111 grown independently. Mass spectrum of each replicate was collected for 10 s in the *m/z* range
112 15-200. The headspace of pure growth medium without bacteria was analyzed for background
113 correction.

114

115 **2.3. Biochemical identification of bacteremia**

116 After the initial incubation period (usually 16 h), 12 mL of blood culture was transferred into an
117 automated BACTEC-9240 detection system (Becton, Dickinson and Company, New Jersey, USA).
118 If the culture was positively alarmed within five days (typically after 1-3 days), 0.5 mL of blood
119 culture was withdrawn for gram stain analysis (ca. 2 h), and another 0.5 mL was inoculated into
120 blood agar medium, Chinese blue medium and chocolate medium. Biochemical identification
121 was done after the overnight growth at 35 °C. Optionally, the results of biochemical
122 identification were confirmed (ca. 24 h) using an automated VITEK-2 microbiology analyzer
123 (bioMerieux, Durham, USA).

3. Results and discussion

3.1. Ionization mechanism

APCI of ambient air in positive ion polarity generated a series of H_2O cluster ions (Fig. 1a), including radical cations (*m/z* 36, $[H_2O]_2^{\cdot+}$), as well as protonated clusters (*m/z* 37, $[H_2O]_2H^+$; *m/z* 55, $[H_2O]_3H^+$; *m/z* 73, $[H_2O]_4H^+$; *m/z* 91, $[H_2O]_5H^+$). However, during the sampling of a blood culture, most of the primary $[H_2O]_nH^+$ clusters were readily neutralized via H^+ transfer to the VOCs with higher H^+ affinities (Fig. 1b). Some of these VOCs, particularly NH_3,[24] are released by bacteria at high concentrations but remain invisible in LTQ-MS due to their low molecular weight. Here we 'visualized' the release of NH_3 by PA blood cultures using gas-phase Ag^+ as primary ions for ionization (Fig. S2). The high abundance of NH_3 and other low-molecular H^+ scavengers in the discharge area suppresses protonation of many common compounds released by blood cultures, such as alcohols, aldehydes, ketones, etc. As a result, lower number of bacterial VOCs is detected in APCI-MS compared to the techniques in which ionization is done at reduced pressure, e.g. SIFT-MS and GC-MS.[14, 22, 24] The detected VOCs mostly include volatile amines with higher proton affinity than NH_3, e.g. trimethylamine and indole (Table S1). Unlike the $[H_2O]_nH^+$ ions, primary radical cations (e.g. $[H_2O]_2^{\cdot+}$) usually remained visible in APCI-MS in the presence of bacterial VOCs, although the intensity of $[H_2O]_2^{\cdot+}$ signal was found to be notably decreased in some cultures. The reactivity of H_2O radicals towards specific volatiles can be responsible for some of the non-identified signals listed in Table S1. In contrast to the positive ion polarity, background APCI-MS signals from ambient air in negative ion polarity

remained nearly unchanged in the presence of bacterial VOCs (Fig. S3). Organic acids, e.g. acetic acid, butyric acid and isovaleric acid, were preferentially ionized from the headspace of blood cultures via proton abstraction (Table S1).

3.2. APCI-MS fingerprinting of simulated blood cultures

In order to create bacterial MS fingerprint with high molecular specificity, the signals related to bacterial metabolism need to be separated from the signals related to indigenous blood metabolism. However, such separation can be difficult to achieve just by screening patients population, because indigenous blood metabolome exhibits great variability between individuals as well as at different environmental factors.[51, 52] Accordingly, in this study we observed that VOC fingerprints of non-inoculated blood cultures undergo inter-individual variations (Fig. S4). To create strain-specific MS fingerprint, we analyzed simulated blood cultures, i.e. blood cultures of healthy individuals inoculated with a particular bacterial strain in vitro. The analysis of simulated cultures obviates the problem of inter-individual variability because reference analysis is done on non-inoculated cultures from the same individuals grown under the same conditions. For the simulated culture analysis we selected five common bloodstream pathogens, including *Staphylococcus aureus* (SA), *Escherichia coli* (EC), *Klebsiella pneumonia* (KP), *Acinetobacter baumannii* (AB) and *Pseudomonas aeruginosa* (PA). In total, these bacterial species account for ca. 50% of microorganisms derived from 9613 consecutive blood isolates studied in the framework of SENTRY antimicrobial surveillance program, the two most frequent causes of bacteremia being EC (20%) and SA (17.6%).[49] Fig. 2 shows typical VOC

APCI-MS fingerprints of simulated blood cultures inoculated with one of the five bacterial species (KP, AB, EC, SA and PA) at the concentration of 10^4 CFU/mL and grown aerobically at 35°C for 16 h. Each mass spectrum is shown after the subtraction of signals from non-inoculated blood culture originated from the same individual and incubated under the same conditions. Whereas a number of signals, e.g., *m/z* 59 and *m/z* 92, are shared between different bacteria, the fingerprints can be readily distinguished by visual inspection. It is worth noting that the peaks observed in background-subtracted spectra do not necessarily belong to bacterial VOCs. Some peaks in the spectra can instead belong to blood metabolites released in response to inoculation. Even though the identification of bacteria by MS fingerprinting does not require the knowledge of chemical identity for the observed signals, such information can be useful, particularly to help delineate the molecular specificity of detection. Based on MS/MS experiments involving the measurements of standard compounds and on the results of earlier published reports, we assigned some of the most specific biomarker signals in bacterial fingerprints to indole (EC, negative ion mode, *m/z* 116; positive ion mode, *m/z* 118),[4, 17, 19, 20, 53, 54] 1-vinyl aziridine (PA, positive mode, *m/z* 70),[14] trimethylamine (AB and PA, positive mode, *m/z* 60),[15, 55] butyric acid (SA, negative ion mode, *m/z* 87) and isovaleric acid (SA, negative ion mode, *m/z* 101).[4, 14, 48, 56-58] Unlike EC, PA, SA and AB, the most specific biomarker signals for KP (particularly, *m/z* 106 in negative ion mode) were observed at a low intensity, which prevented their unambiguous chemical identification. The entire list of biomarker signals observed in this study is summarized in Table S1. Signals that did not yield notable fragments (e.g., *m/z* 59 in negative ion mode), either due to the lack of charged fragments within the detection window or due to the insufficient abundance of parent ion, were left unidentified. It is important to

note that the relative signal intensity for a particular VOC in APCI-MS does not necessarily reflect its relative concentration in the culture headspace. The ionization efficiency of VOCs by APCI-MS is affected by the abundance of other volatiles. Reliable estimation of VOC concentration is achieved when ionization is done in vacuum such as in SIFT-MS and GC-MS.[14, 15, 18, 22, 23]

3.3. Features of bacterial growth in blood cultures

To exclude possible isobaric interferences, biomarker signals are usually monitored in selected-transition mode (signal intensity of the most abundant fragment in MS/MS). Fig. 3 shows time profiles of indole (EC biomarker) and isovaleric acid (SA biomarker) in VOC screen of simulated blood cultures from four individuals recorded in negative ion detection mode. The baseline level in ion chromatograms corresponds to the sampling of an empty centrifuge tube. The samplings of non-inoculated blood cultures are indicated as blank, and the samplings of simulated culture are marked accordingly (Fig. 3). Interestingly, despite the same initial bacterial concentration and culturing conditions, we observed notable inter-individual variability in MS signal intensities. Thus, all the simulated blood cultures of the second volunteer were reported negative after 16 h. Out of the total 30 tested volunteers, two did not reveal bacterial growth after 16 h. Bacterial growth in those individuals only became notable after ca. 40 h. The results were confirmed by the clinical cell culture approach. The considerably slower bacterial growth in the blood cultures of certain people likely reflects the higher antibacterial immunity of their blood. We found that the same bacterial isolates grew faster

when incubated in pure TSB rather than in blood/TSB mixture (Fig. S5). Also, the isolates grown in pure TSB exhibited much better reproducibility of MS signals for the same initial number of inoculated bacteria (Fig. S6). These observations further validate that the deviations in MS signal intensities from the blood bacterial cultures (Fig. 3) are individual-specific.

3.4. Stability of blood culture APCI-MS fingerprints

In infected people, the number of bacteria in blood is not only dependent on the host resistivity to bacteria but also on the duration of a disease, i.e. the time passed after inoculation. The duration of a disease varies from patient to patient and affects the blood composition. We tested VOC fingerprints of the same simulated blood culture obtained at different incubation times (Fig. 4). For the five bacterial species studied we found that the biomarker signals are preserved in the fingerprint during the incubation of at least 48 h, although their relative intensities did undergo significant alterations. The signal intensity of different bacterial metabolites during the incubation grew at a different rate, and the intensity of some signals remained nearly constant. Moreover, the signal at *m/z* 70 in the positive ion mode fingerprint of PA cultures revealed transient intensity profile peaked at a certain incubation time followed by a gradual decrease. In the earlier GC-MS study Filipiak et al. also found several nitrogen-containing VOCs released by PA in a transient manner, indicating the intermediary role of these VOCs in bacterial metabolism.[14]

3.5. Patient screening

The constructed VOC fingerprints (Fig. 2) were used to screen 61 adult volunteers at suspicion of bacteremia and 39 patients diagnosed with bacteremia over the time period of six months (Fig. S3). Blood cultures were grown for 16 h in the hospital and transported to MS laboratory in sealed tubes without freezing. MS analysis was done directly without any sample pretreatment. Bacteremia was reported if the VOC fingerprint of a blood culture contained the entire characteristic set of biomarker signals for one of the five bacterial genera (Table S1). Relative signal intensities were not taken into account for the reason of inter-individual fluctuations discussed above. Among the selected group of people, we identified 14 cases of SA, 5 cases of EC, 7 cases of KP, 4 cases of AB and 3 cases of PA. Every positive and negative bacteremia assignment was successfully confirmed by biochemical identification of technical replicates incubated over 2-3 days (see Experimental).

3.6. Analytical performance

The full agreement between the results by ambient MS and the results by the conventional biochemical analysis demonstrated on a group of patients indicates a good perspective of ambient MS for clinical applications. Bacteremia screening by ambient MS has several potential advantages. The speed of diagnosis (< 1 day) is higher than that by culture methods (total 2-3 days). The sensitivity of detection is particularly high for certain species, such as PA and EC, which allows the recognition of bacteremia already after few hours from the beginning of incubation. However, it should be remembered that bacterial growth reveals strong individual

specificity (Fig. 3). As a very rough estimate, biomarker signals usually become visible in cultures of all studied pathogens after 16 h growth at the initial bacterial concentration of 10^5 CFU/mL. Another advantage of VOC analysis is its high throughput (ca. 10-20 s per sample), because absolutely no sample pretreatment is necessary. We estimate that the cost per sample by ambient MS is approximately two times lower than that by traditional multi-step blood culture (~ 10 USD per sample). The cost reduction in MS based workflow is mainly allowed by the economy of chemicals due to the shorter culturing time. Finally, because VOC analysis is rapid and noninvasive, the same cultures can be later interrogated by biochemical assays for the verification of results and/or for more specific diagnosis.

3.7. Outlook

The incubation time of 16 h mostly used in this study is substantially shorter than in traditional clinical routines and allows next-morning diagnosis of patients. However, some species, e.g., SA or PA, stably reveal biomarker signals much faster, within 3-10 h, and can be diagnosed same day. There also remains space for further analytical improvement. For example, over the course of this study we found that the bacterial growth can be accelerated by more than two times if gentle agitation of incubated cultures is applied. The growth of certain isolates can be significantly accelerated by selecting a proper medium, e.g., lysogeny broth (LB) medium is known to promote rapid EC growth.[59] Also, the signal response of particular biomarkers can be selectively enhanced for targeted analysis. For example, the characteristic release of isovaleric

acid by SA cultures (*m/z* 101 in negative ion mode) can be regulated by the content of leucine precursor.[60]

4. Conclusion

The results of our six-month hospital study strongly suggest that VOC fingerprinting of blood cultures by APCI-MS can be successfully employed for the clinical diagnosis of bacterial infections in humans. Particular advantages of APCI-MS include the high speed, throughput, low operational cost and simplicity of diagnosis. Depending on pathogen, the method allows the same-day or the next-morning diagnosis of bacteremia. It can be expected that the continuing improvement in the sensitivity of commercial MS instruments as well as the development of more targeted approaches will further shorten the time as well as the cost of diagnosis. The samples processed by the rapid and nondestructive MS analysis can be further interrogated by more expensive and time-consuming biochemical assays for complementary analysis.

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Figure captions

Fig. 1 Positive ion polarity APCI-MS of ambient air (a) and the headspace of a blood culture (b) recorded in low mass range.

Fig. 2 Positive (top) and negative (bottom) ion mode VOC fingerprints of five bacterial species grown in blood cultures of healthy volunteers. SA = *Staphylococcus aureus*, EC = *Escherichia coli*, KP = *Klebsiella pneumonia*, AB=*Acinetobacter baumannii,* PA = *Pseudomonas aeruginosa.*

Fig. 3 Single ion chromatograms for indole (a) and isovaleric acid (b) signals in the MS screen of simulated bacterial cultures from four individuals. The reference samplings of non-inoculated blood culture incubated under the same conditions are indicated as “blank”.

Fig. 4 VOC fingerprints of a simulated SA blood culture at a different incubation time.

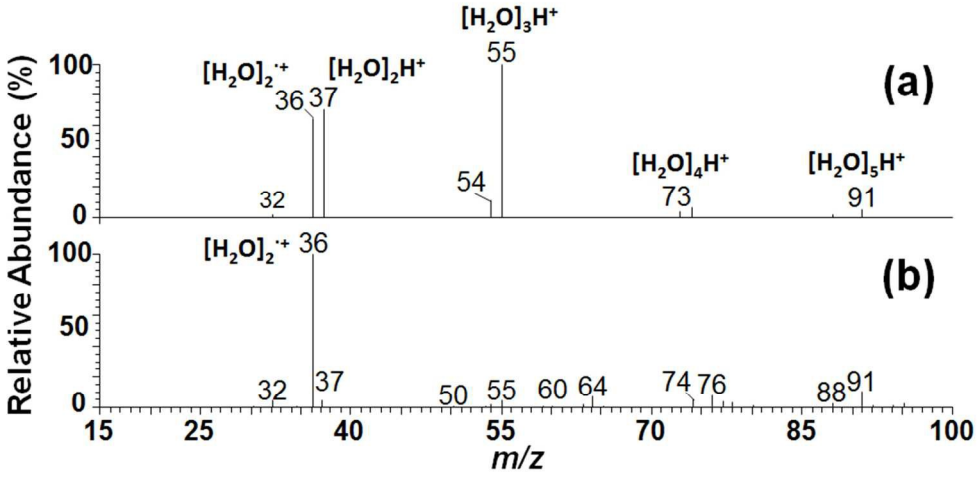

Fig. 1 Positive ion polarity APCI-MS of ambient air (a) and the headspace of a blood culture (b) recorded in low mass range.
262x132mm (96 x 96 DPI)

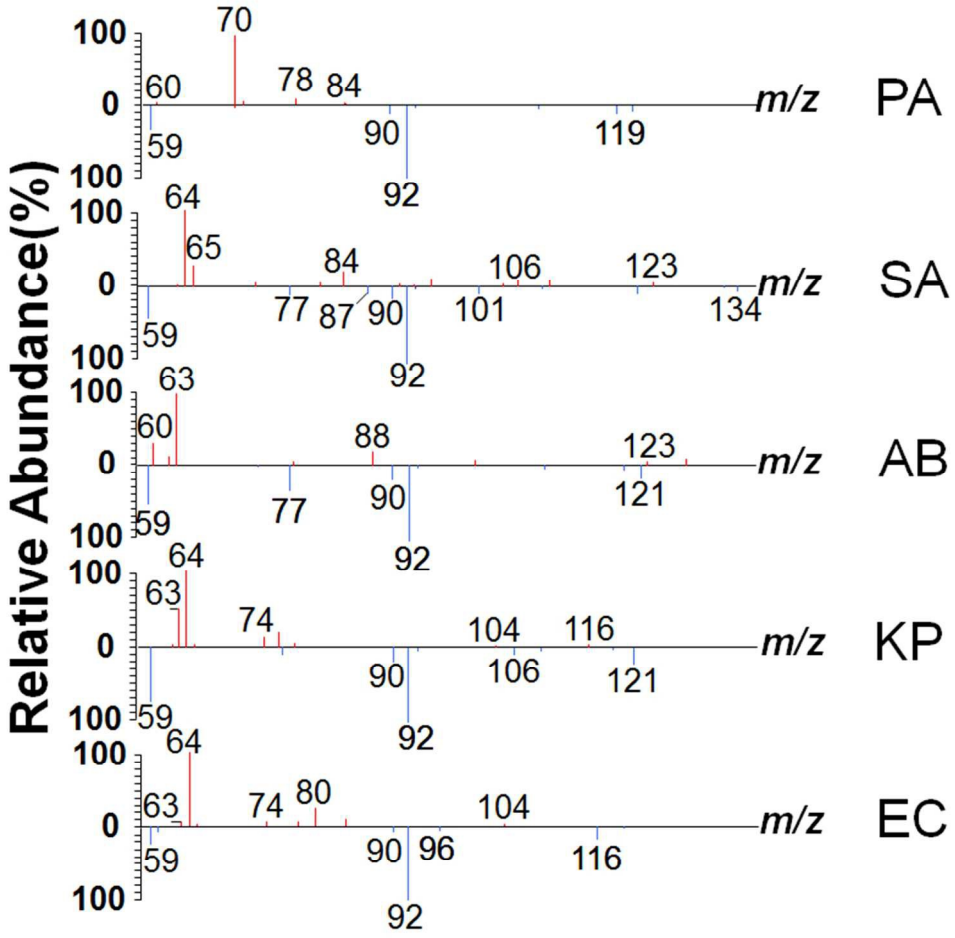

Fig. 2 Positive (top) and negative (bottom) ion mode VOC fingerprints of five bacterial species grown in blood cultures of healthy volunteers. SA = Staphylococcus aureus, EC = Escherichia coli, KP = Klebsiella pneumonia, AB=Acinetobacter baumannii, PA = Pseudomonas aeruginosa.
217x210mm (96 x 96 DPI)

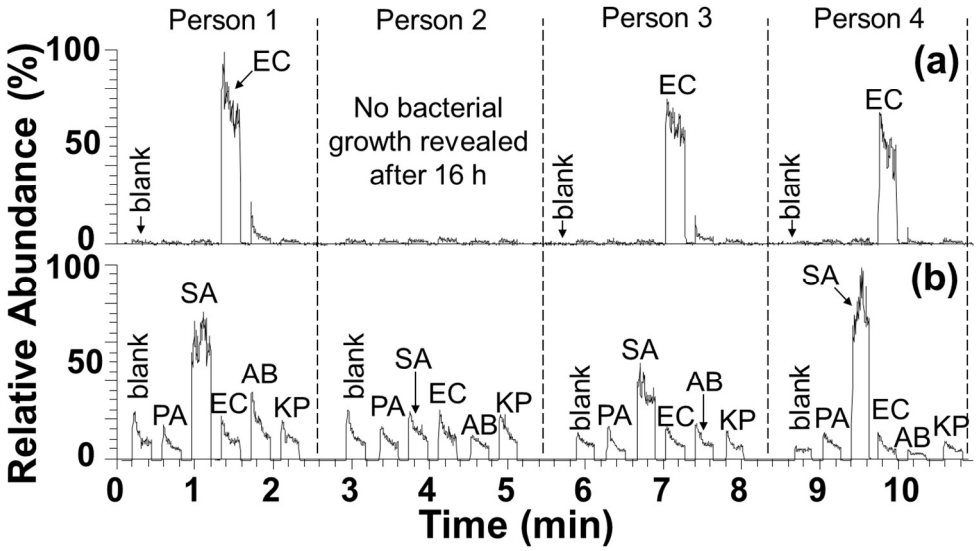

Fig. 3 Single ion chromatograms for indole (a) and isovaleric acid (b) signals in the MS screen of simulated bacterial cultures from four individuals. The reference samplings of non-inoculated blood culture incubated under the same conditions are indicated as "blank".
394x228mm (96 x 96 DPI)

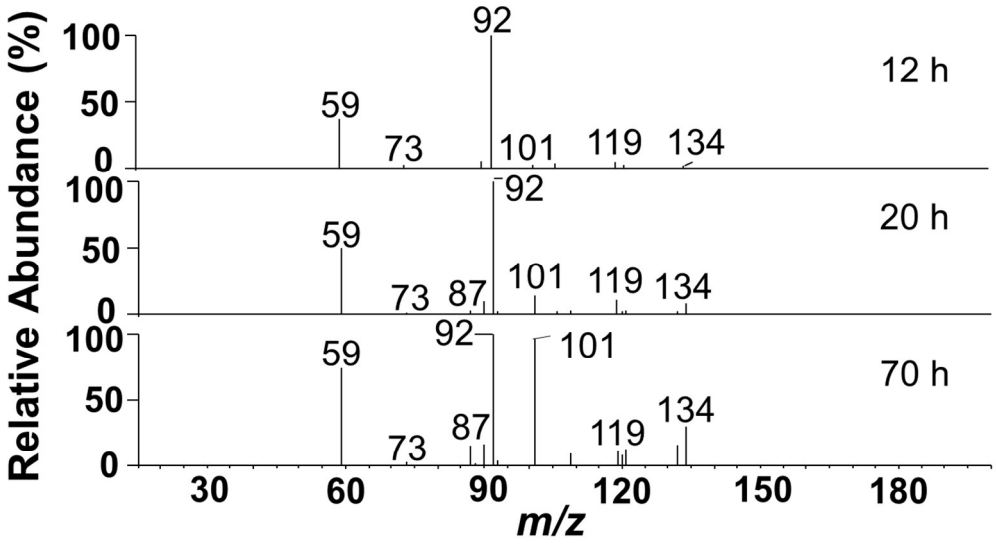

Fig. 4 VOC fingerprints of a simulated SA blood culture at a different incubation time.
369x207mm (96 x 96 DPI)