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Rapid differentiation of microbial cultures based on the analysis of headspace volatiles by atmospheric pressure chemical ionization mass spectrometry

Juchao Liang,[1] Yaping Hang,[2] Konstantin Chingin,*[1] Longhua Hu,[2] and Huanwen Chen*[1]

[1]Jiangxi Key Laboratory for Mass Spectrometry and Instrumentation, East China Institute of Technology, Nanchang 330013 P.R. China

[2]The Second Affiliated Hospital of Nanchang University, Nanchang 330006 P.R China

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Corresponding authors:
Dr. Huanwen Chen (chw8868@gmail.com), Dr. Konstantin Chingin (chingin.k@gmail.com)
Room 804, Sci. & Tech. Building, 418 Guanglan Road, Nanchang City, Jiangxi Province 330013, P. R. China
Tel: (+86)791-83879275; Fax: (+86)791-83896370

Keywords: microbial cultures; mass spectrometry; non-invasive sampling; volatile organic compounds (VOC); molecular fingerprinting; biomarker discovery; corona discharge; atmospheric pressure chemical ionization (APCI); secondary electrospray ionization (SESI); selected-ion flow-tube mass spectrometry (SIFT-MS); proton-transfer reaction mass spectrometry (PTR-MS).

Abstract

We report the direct analysis of volatile compounds emitted by microorganisms using atmospheric pressure chemical ionization mass spectrometry (APCI-MS) with atmospheric corona discharge as a source of primary ions. APCI-MS fingerprinting of headspace volatiles was used to differentiate nine microbial cultures without any sample pretreatment, chromatographic separation and wet chemistry using ion trap detection.

Identification of bacteria and other microbes based on the characteristic composition of volatile organic compounds (VOCs) released as part of their metabolism is of sustained interest in clinical diagnosis and other biomedical applications.[1-3] The major figures of merit associated with sampling volatile metabolites include non-invasiveness, ease of practical implementation and toxicological safety. Microbial VOCs can be examined by a wide selection of analytical methods, including chemical sensing, optical spectroscopy, "electronic noses", ion mobility spectroscopy, etc.[4-6] Mass spectrometry (MS) has higher chemical specificity of detection than the aforementioned methods, but the traditional MS approaches involving gas chromatography (GC) separation are slow, require sample collection and cannot be implemented in real time.[7,8] The invention of selected-ion flow-tube mass spectrometry (SIFT-MS),[9] proton-transfer reaction mass spectrometry (PTR-MS)[10] and ion-molecule reaction mass spectrometry (IMR-MS)[11] enabled direct profiling of VOC metabolites without the need for chromatographic separation and sample collection.[12,13] However, the user base of these methods because they require dedicated MS instrumentation.

With the introduction of ambient ionization,[14,15] a variety of techniques became available for the direct molecular analysis of microorganisms on mass spectrometers with atmospheric pressure ionization (API) interface.[16,17] However, up to now the research in this field has mostly been done on nonvolatile metabolites, only few reports on VOC detection being available in the literature.[16] Secondary extractive electrospray ionization (SESI) is perhaps the most successful ambient ionization method for the analysis of microbial VOCs by far.[18-21] In present study, the investigation of microbial VOCs by ambient MS is extended toward atmospheric pressure chemical ionization (APCI). APCI-MS was primarily used for the analysis of

volatile airborne substances in various environmental applications.[22] In recent years, the interest in APCI and direct analysis has re-emerged with a focus on the rapid analysis of chemicals thermally desorbed from solid surfaces.[23] For our application we chose APCI with the direct current corona discharge as a source of primary ions because of the simplicity, robustness, and high efficiency of this approach.[23] Headspace VOCs from growing cultures were continuously transferred for APCI with nitrogen carrier gas without any pre-concentration, as detailed in Figure S1. Volatile metabolites were ionized by the ambient air plasma in the vicinity of a corona discharge needle, and the secondary ions were monitored with an LTQ linear ion trap mass spectrometer (ThermoFischer, San Jose, CA, USA).

Figure 1 shows the ion chromatograms of two selected signals in the APCI+ screen of eight microbial cultures corresponding to five bacterial species (*Klebsiella pneumonia* (KP)*, Acinetobacter baumannii* (AB)*, Escherichia coli* (EC)*, Staphylococcus aureus* (SA) *and Pseudomonas aeruginosa* (PA)) and three fungal species (*Candida albicans* (CA)*, Candida tropicalis* (CT)*, Candida parapsilosis* (CP)) grown aerobically in Mueller-Hinton (MH) medium at 35°C for 16 h. Three successive samplings of each strain in Figure 1 correspond to three biological replicates. The baseline level in ion chromatograms corresponds to the sampling of an empty centrifuge tube. The samplings of bacterial cultures and pure non-inoculated growth medium are indicated accordingly. The details about APCI-MS experiment are summarized in Supplementary Information. Compared to the pure growth medium, the signal intensity for *m/z* 140 was dramatically increased in all the three studied *Candida* strains but not in the rest of the microorganisms (Fig. 1a). The signal at *m/z* 70 was observed in the headspace of a pure medium and was notably increased in PA cultures. Interestingly, the signal at *m/z* 70 was significantly

depleted in the rest of the samples except for SA (Fig. 1b). Similar phenomenon was also observed for some other signals. This reflects that the same VOCs can be both consumed and released by microorganisms depending on the particular strain and growth environment.[24] The spike in signal intensity at the beginning of each sampling in Figure 1 is most likely related to the volatility of ionized chemicals. While the culture tube is kept closed, volatile metabolites gradually saturate the headspace. When the sampling is started, the accumulated headspace VOCs are rapidly transferred out of the tube to APCI-MS. As a result, the concentration of headspace metabolites gradually decreases until it reaches the steady state at which further decrease is compensated by the emission of new metabolites from the culture.

Mass spectra of microbial cultures are averaged in the *m/z* 60-200 range from the 20 scans obtained over the initial sampling period of 10 s, when the headspace VOC concentration is maximal (Fig. 1). Each mass spectrum is converted into a microbe-specific fingerprint by the subtraction of signals from non-inoculated growth medium incubated under the same conditions (Fig. 2). Out of the studied strains, the largest total number of signals (25) was detected in SA cultures (5 in APCI+ and 20 in APCI-), and the smallest number of signals (6) was detected in AB (5 in APCI+ and 1 in APCI-). Notable variation in total ion current was also observed for different strains (up to ca. 2 orders of magnitude). VOC patterns are well reproduced in biological replicates (Fig. 2a-c), and different strains can be readily distinguished by visual inspection (Fig. 2c-e). We also compared VOC fingerprints for the two sets of KP cultures grown on two different media and found pronounced difference in the spectral pattern and signal intensities (Fig. 2e and 2f). Because media-specific signals are subtracted from the fingerprints, the observed difference can be directly linked to the altered metabolism of KP. The

effect of growth media on the types and abundance of VOCs emitted by bacterial cultures has been recognized in earlier studies.[18,25]

The degree of difference/similarity between the microbial VOC fingerprints is qualified using PCA plot (Fig. 3). Mass spectra were exported for PCA as a list of nominal *m/z* values and signal intensities. For higher specificity of analysis, APCI+ and APCI- data for each sample were combined in one fingerprint. Ten points for each culture in PCA plot corresponds to ten biological replicates. Overall, all the strains are well separated in PCA. Fungi are clustered separately from bacteria. The replicates of SA are clustered at the largest distance from the rest of bacterial strains. This observation is likely related to the gram-positivity of SA, whereas the other four bacterial species in this study were gram-negative. Interestingly, the same KP strains cultured on two different media are clustered in PCA at a greater distance from each other compared to different gram-negative bacteria cultured on the same medium. This observation shows that bacterial metabolism is strongly dependent on microenvironment. The strong variability of bacterial VOC profiles with growth conditions is a frequent source of inconsistency for the results reported by different research groups.[3] This strong variability also raises concerns whether in vitro bacterial VOC fingerprints can be directly applied as a reference to identify corresponding species in vivo, e.g., by the analysis of expired breath.[20]

Identification of microorganisms by MS fingerprinting does not require chemical assignment of the observed signals. However, the knowledge of chemical identity can be useful in related research on microbial VOCs. Of particular interest are the signals that are only observed in just one or few strains, because these signals hold the highest biomarker capacity.

Thus, the signal at *m/z* 101 dominated the APCI- fingerprint of SA (Fig. 2a-c) but was totally missing in the rest bacterial species. Based on the literature search and tandem MS analysis, *m/z* 101 was tentatively assigned to deprotonated isovaleric acid. Earlier MS studies established isovaleric acid as a highly specific SA biomarker.[3,8,24,26,27] Interestingly, in this study we also observed the signal at *m/z* 101 in fungal *Candida* strains (Table S1). Out of the eight analyzed microorganisms, the signal at *m/z* 116 was only found in the APCI- fingerprint of EC (Fig. 2d) and was tentatively assigned to deprotonated indole. Indole has been recognized as a characteristic EC biomarker by several earlier studies.[3,28-32] Table S1 gives a summary of VOC signals observed in this study specific to one or more of the microbial strains. For some of the observed signals tentative chemical assignment is provided based on the earlier MS reports of bacterial volatiles (Table S1).

The developed experimental setup obviates wet chemistry for sampling and ionization of VOCs and therefore allows long-term stability and high day-to-day reproducibility of microbial fingerprints (Fig. 2a-c). Long-term stability is crucial in fingerprinting approaches, where identification of an unknown sample relies on comparing its spectrum with a bank of reference fingerprints collected earlier. The obviation of wet chemistry distinguishes APCI from the ambient methods for VOC analysis based on electrospray ionization (ESI). ESI is a very sensitive process and is known to be vulnerable to instabilities, particularly on the long time scale.[33] In our hands, APCI ionization allowed much better day-to-day reproducibility of VOC fingerprints than extractive electrospray ionization (EESI). In both settings, the headspace of microbial cultures was analyzed using the same LTQ-MS instrument with the same sampling interface (Fig. S1). Other benefits associated with the lack of water, methanol and other volatile

solvents in APCI include the lower level of chemical background, as well as the improved cost efficiency and speed of analysis.

In conclusion, our results indicate that APCI-MS allows direct and simple molecular analysis of volatile metabolites emitted by microorganisms. Nine different cultures corresponding to eight microbial species and two growth media were readily distinguished based on characteristic fingerprints. Although APCI with atmospheric corona discharge has generally lower sensitivity than the techniques in which ionization is done in vacuum or at a reduced pressure, the use of API mass spectrometers in this approach is a step toward broader user base.

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Figures

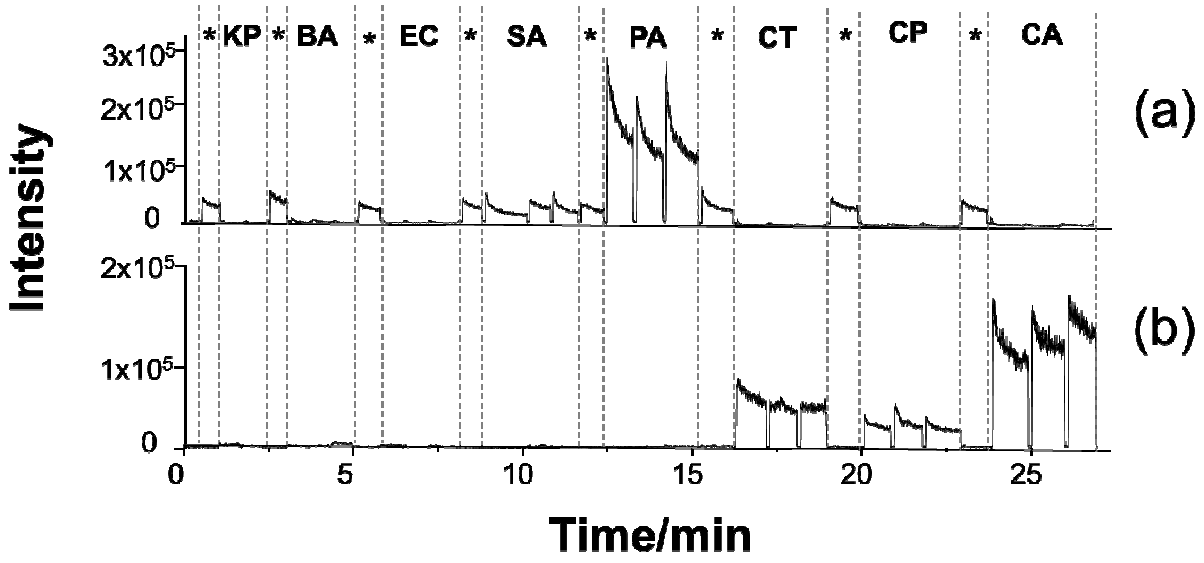
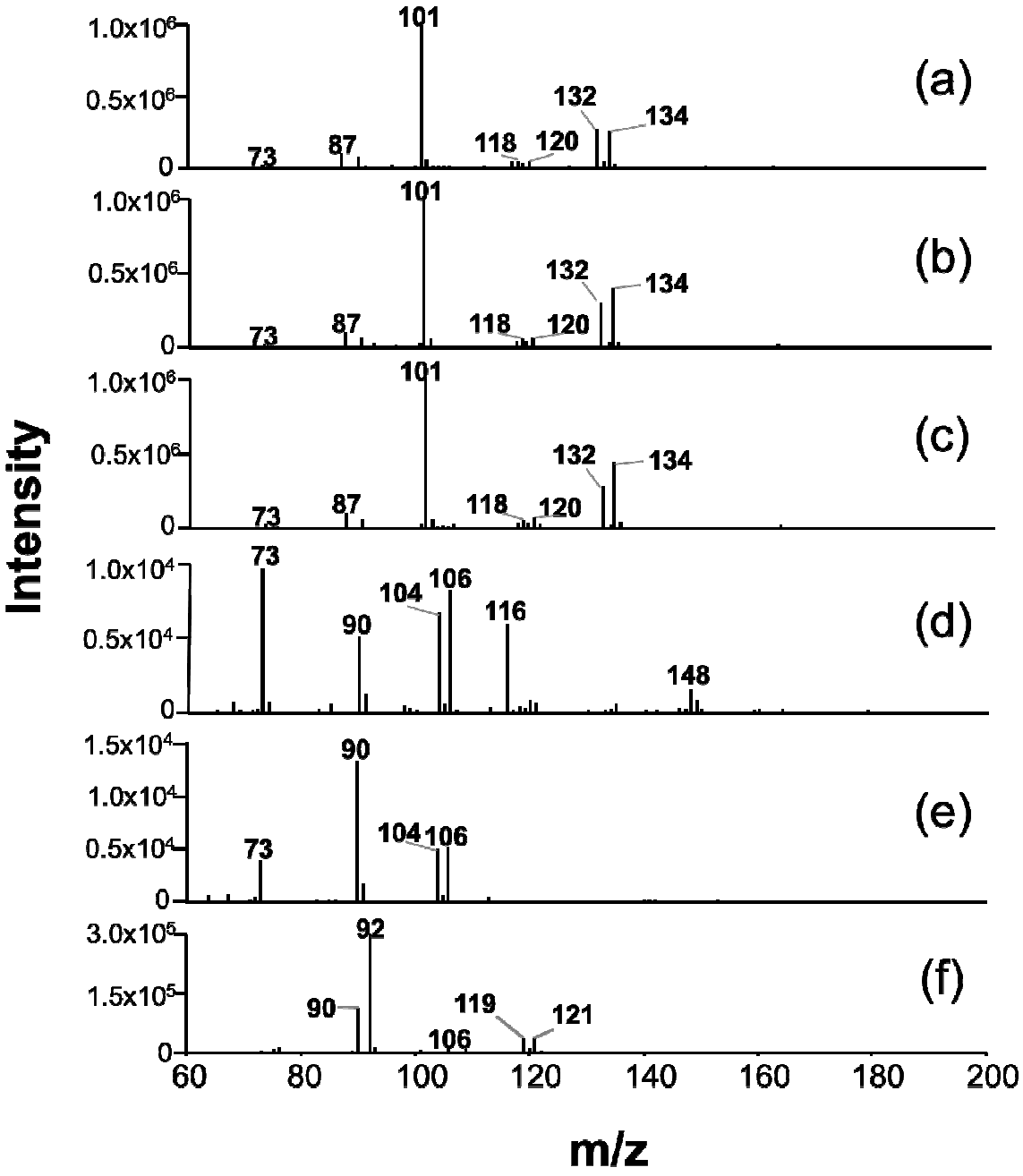


Figure 1: Single ion chromatograms for *m/z* 140 (a) and *m/z* 70 (b) signals in the APCI+ screen of eight microbial cultures corresponding to five bacterial and three fungal strains. Three successive samplings of each strain correspond to three biological replicates. The reference samplings of pure growth medium incubated under the same conditions are indicated with asterisk.



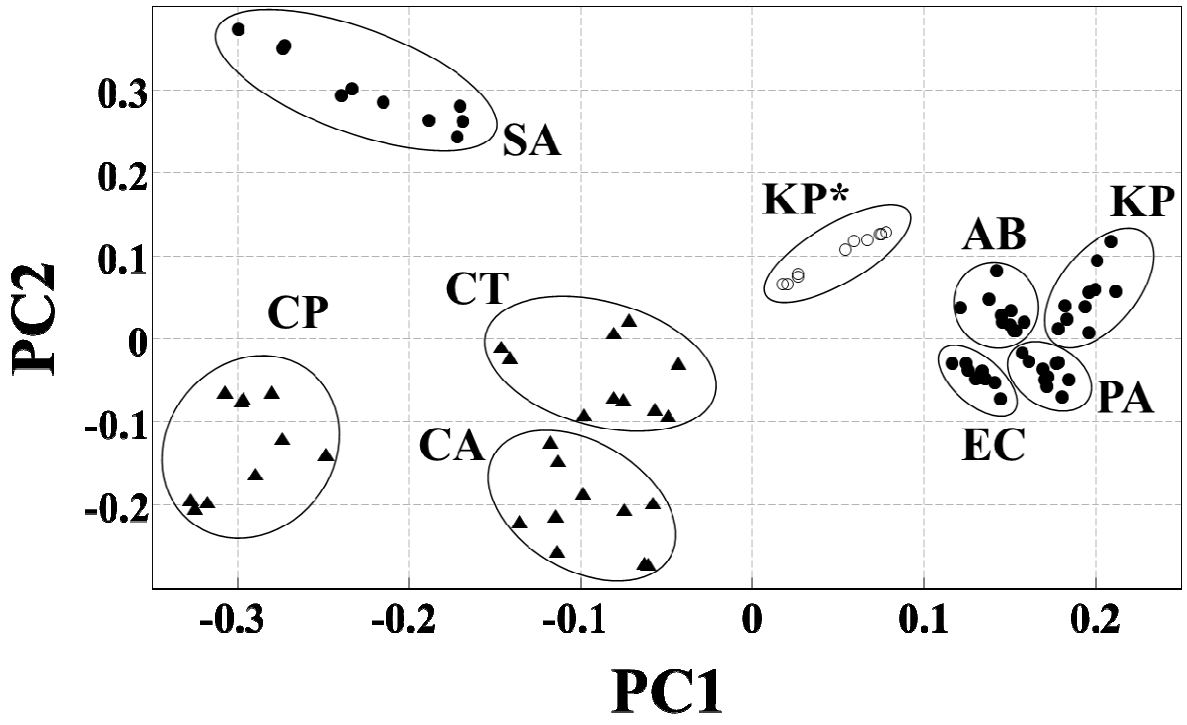
185

186 **Figure 2**: VOC fingerprinting by APCI-MS. (a-c): APCI- fingerprints for three biological replicates of SA in

187 Mueller-Hinton (MH) medium sampled on three different days; (d, e): APCI- fingerprints of EC and KP in

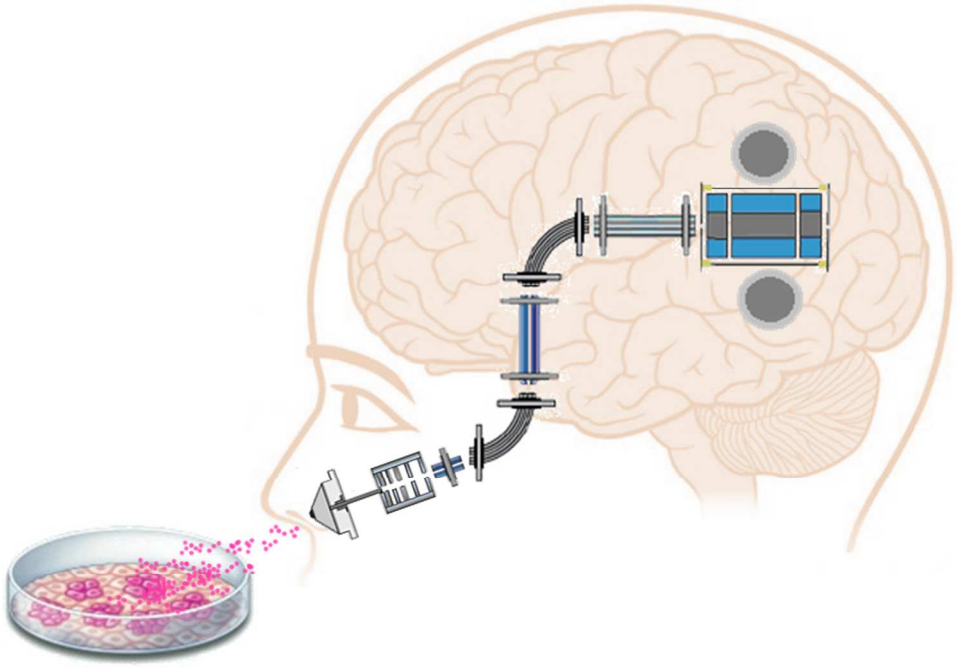
188 MH medium; (f): APCI- fingerprint of KP in China Blue Agar (CBA) medium grown under the same

189 conditions.



190

191 **Figure 3**: PCA score plot of VOC fingerprints from nine different microbial cultures. Ten points in the PCA
192 plot for the same species correspond to ten biological replicates analyzed on three different days. Circles
193 indicate bacterial cultures, and triangles indicate fungal cultures. Asterisk and open circles are used to
194 indicate KP cultures grown in CBA medium. The rest of the cultures were grown in MH medium.



APCI-MS fingerprinting of headspace volatiles was used to differentiate microbial cultures without any sample pretreatment, chromatographic separation and wet chemistry.
236x159mm (96 x 96 DPI)