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1 **Early release of 1-pyrroline by Pseudomonas aeruginosa cultures discovered using ambient**
2 **corona discharge ionization mass spectrometry**

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24 ## Abstract

25 *Pseudomonas aeruginosa* (PA) is a leading cause of nosocomial infections in humans with
26 increasing number of health-threatening implications, which urges faster clinical detection of
27 this pathogen. In present study, we discovered for the first time the early release of 1-pyrroline
28 vapor by PA cultures using direct ambient mass spectrometry (AMS) analysis of bacterial culture
29 headspace based on corona discharge ionization. Importantly, the concentration of 1-pyrroline
30 in PA cultures was found to greatly increase during the lag phase and early log phase of
31 bacterial growth (3 – 6 h, 200-800 ppb), enabling early detection. Typically, 1-pyrroline
32 produced by PA cultures could be detected in our experiments already after 0.5 – 4 h of
33 incubation at the clinically relevant initial bacterial counts. Reference AMS screen of common
34 infectious microbes from other genera, including *Staphylococcus aureus*, *Staphylococcus*
35 *epidermidis*, *Staphylococcus haemolyticus*, *Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter*
36 *baumannii, Enterococcus faecalis, Klebsiella oxytoca, Candida albicans, Candida tropicalis,*
37 *Candida parapsilosis, Enterococcus faecalis, Enterobacter cloacae,* did not reveal notable
38 release of 1-pyrroline. Our results indicate the high suitability of volatile 1-pyrroline for the
39 early and reliable diagnosis of *Pseudomonas* infections using commonly available MS
40 instrumentation.

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44 **1. Introduction**

45 *Pseudomonas aeruginosa* (PA) is an opportunistic, nosocomial pathogen which frequently
46 causes pneumonia, urinary tract infection, bacteremia, surgical wound infection, osteomyelitis,
47 and various other diseases.[1] PA exhibits multi-resistance to commonly used antimicrobials, and
48 therefore the effective eradication of PA infections requires early identification and targeted
49 medical treatment.[2] Clinical diagnosis of PA infection usually relies on microorganism
50 identification in a clinical laboratory, which is laborious, time-consuming (2-3 days) and carries a
51 risk of misidentification.[3] Hence is the increasing attention to analytical tools with higher
52 molecular specificity and speed of the analysis.[3-5]

53 Among a variety of strategies, identification of bacteria via the direct analysis of emitted
54 volatile organic compounds (VOCs) is particularly attractive, owing to the non-invasiveness,
55 practical simplicity, cost-efficiency, and toxicological safety.[6, 7] A number of workflows have
56 been developed for the rapid identification of bacteria, mostly based on VOC analysis using
57 chemical sensing or mass spectrometry detection.[8-11] Although the reported methods have
58 many evident analytical merits, their integration into clinical practice is hindered due to the
59 requirement of dedicated instrumentation. For example, the identification of most specific PA
60 volatiles such as hydrogen cyanide[4] and methyl thiocyanate[12] requires selected ion flow tube
61 mass spectrometry (SIFT-MS),[13] which is yet not widely available in analytical laboratories.
62 Other VOCs identified in PA cultures have lower pathogen specificity and typically occur at trace
63 amounts, which necessitate sample collection.[14, 15] Currently, there is no reliable biomarker for
64 the early recognition of PA using commonly available analytical instrumentation.

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65 In ambient mass spectrometry (AMS), ions are formed outside the mass spectrometer
66 without sample preparation or separation.[16-18] AMS can be implemented on any type of a mass
67 spectrometer with atmospheric interface (e.g., linear ion trap, time-of-flight, triple-quadrupole,
68 Orbitrap) commonly available in bioanalytical laboratories and core facilities. A large variety of
69 AMS methods for the direct molecular analysis of complex samples have been developed over
70 past decade, including desorption electrospray ionization (DESI-MS),[19] direct analysis in real
71 time (DART-MS),[20] laser ablation electrospray ionization (LAESI-MS),[21] low-temperature plasma
72 probe (LTP-MS),[22] paper spray ionization,[23, 24] leaf spray ionization,[25] rapid evaporative
73 ionization (REIMS),[26] desorption atmospheric pressure chemical ionization (DAPCI-MS)[27] and
74 many others. Dedicated AMS approaches for bacterial differentiation have been reported
75 based on the ambient desorption/ionization of nonvolatile metabolites (most commonly, lipids)
76 from intact bacterial cells.[28-34] Further, differentiation of bacteria has also been demonstrated
77 based on the AMS of released VOCs using ambient ionization by electrospray droplets[35, 36] and
78 atmospheric corona discharge.[37, 38] Compared to SIFT-MS, ionization of volatile molecules in
79 AMS is usually less selective, but the possibility of tandem mass analysis in AMS greatly
80 facilitates identification of signals.

81 In present study, the VOCs emitted by common infectious microbes were analyzed using
82 tandem AMS analysis of bacterial culture headspace based on corona discharge ionization. The
83 method is a variation of the classical atmospheric pressure chemical ionization (APCI)[39] in which
84 bacterial VOCs are transported to the tip of the discharge needle using room-temperature
85 nitrogen gas without carrier solvent and accessory heating. Using this simple approach for the
86 direct VOC analysis, we discovered the release of 1-pyrroline produced by PA cultures during

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87 the lag and early log growth stage, after 0.5 – 4 h incubation. 1-Pyrroline signal could be
88 detected in PA cultures at the initial counts < 100 colony-forming units (CFU) per mL, which is
89 far below clinically relevant concentrations. Reference AMS screen of common infectious
90 microbes from other genera did not reveal notable release of 1-pyrroline. Our results strongly
91 indicate the excellent potential of targeted 1-pyrroline detection for the much earlier
92 recognition of PA compared to common clinical diagnostic routines (2-4 h vs. 2-3 days).
93 Beneficially, 1-pyrroline detection is achieved using widely available type of MS instrumentation
94 with high simplicity, speed and cost efficiency of operation.

95

96 2. Materials and methods

97 2.1. Bacterial culture

98 PA isolates were incubated in 10 mL centrifuge tubes (Solarbio, Beijing, China) containing 5 mL
99 aqueous medium at 35 °C and 150 rpm. Two types of liquid media were used: tryptic soy broth
100 (20 g/L tryptone and 5 g/L NaCl) and Luria-Bertani broth. PA cultures were grown at four
101 different original PA concentrations (5×10^2 CFU/mL, 5×10^3 CFU/mL, 5×10^4 CFU/mL and 3×10^6
102 CFU/mL) and at different incubation times (10-15 time points within total 25 h). For each
103 incubation time and initial PA concentration, at least four replicate samples were
104 independently prepared and analyzed. Growth media without bacteria were cultured under
105 identical conditions for reference analysis.

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106 Sputum and urine from seven healthy volunteers were divided into four equal batches.
107 Simulated sputum cultures were prepared at the initial PA concentration 2×10^5 CFU/mL by
108 mixing 1 mL of sputum inoculated by 1×10^6 CFU/mL PA with 4 mL medium solution. Simulated
109 urine cultures were prepared at the initial PA concentration 2×10^4 CFU/mL by mixing 1 mL of
110 urine inoculated by 1×10^5 CFU/mL PA with 4 mL medium solution. The four batches from each
111 volunteer were incubated for four different time periods (0 h, 2 h, 4 h, 6 h). In total, 28 sputum
112 and 28 urine cultures were analyzed. Informed consent was obtained for any experimentation
113 with urine and sputum samples.

114

115 **2.2. AMS analysis of bacterial VOCs**

116 AMS analysis of bacterial VOCs directly from the headspace of incubated tubes was done on
117 commercial ion trap mass spectrometers (LTQ-XL and Orbitrap-XL, Thermo Scientific, San Jose,
118 CA, USA) interfaced with a home-made corona discharge ionization source as detailed in our
119 earlier studies.[37, 38] Briefly, +4 kV was applied to a stainless steel needle (OD 150 μm) with a
120 sharp tip (curvature radius ~7.5 μm) to create corona discharge at ambient pressure. The
121 headspace VOCs of bacterial cultures were continuously transferred into ionization region via
122 plastic tubing (ID 1.0 mm) assisted by nitrogen gas flow (0.1 MPa, 1 L/min). The angle between
123 the discharge needle and the sample tubing was 30°. The distance from the tip of the needle to
124 the end of the outlet tubing was 2 mm. The distance from the tip of the needle to the inlet of
125 the LTQ capillary was 6 mm. Mass spectra for each sample were accumulated for at least 10 s.
126 The spectra of pure culturing medium incubated over the same time period without PA were

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127 collected as background spectra. Reference standard compounds were analyzed from aqueous
128 solutions under identical experimental conditions. Collision-induced dissociation (CID) analysis
129 of 1-pyrroline and reference authentic isomeric compounds was done on LTQ-XL mass
130 spectrometer (Thermo Scientific, San Jose, CA, USA).

131

132 **2.3. LC-MS analysis of bacterial VOCs**

133 PA cells were filtered out by centrifugation, and supernatant solution was heated to 90 °C.
134 VOCs were collected under ambient conditions for 10 min onto the bottom surface of a glass
135 slide placed 1 cm above the solution. The glass slide was passively cooled by dry ice powder
136 placed on its top surface. The collected condensate was scraped from the slide into an
137 Eppendorf tube using a clean spatula, melted at room temperature, centrifuged to remove
138 possible contaminant particles and then directly analyzed by LC-LTQ-MS (Thermo Scientific, San
139 Jose, CA, USA; C18: length 150 mm; bead diameter 4.6 mm). LC solvent (water/methanol:
140 20%/80%) was run without gradient at a flow rate of 200 μL/min. VOCs of reference standard
141 compounds dissolved in water were collected and analyzed under identical experimental
142 conditions.

143

144 **2.4. Chemicals**

145 Authentic 3-pyrroline (95% purity), *n*-butyronitrile (99% purity), isobutyronitrile (99% purity), *n*-
146 propyl isocyanide and isopropyl isocyanide (97% purity) for reference experiments were

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147 purchased from Sigma-Aldrich. N-propyl isocyanide (99% purity) was purchased from AR, J&K
148 Bailingwei (Beijing, China). 1-Pyrroline is not commercially available due to its poor chemical
149 stability. In present study 1-pyrroline was obtained at 54% purity as a kind gift from Prof. Gao
150 Chen and Prof. Chengfeng Xia (Kunming Institute of Botany). 1-Pyrroline synthesis has been
151 described in a recent publication.[40] Milli-Q water (18.2 MΩ cm) was prepared in house.
152 Methanol was purchased at HPLC purity from TEDIA Co Inc, (Fairfield, Ohiao, USA).

153

154 3. Results and Discussion

155 3.1. High-resolution AMS analysis of PA volatiles

156 Fig. 1 shows a typical mass spectrum of volatiles emitted by PA cultured in 5 mL aqueous
157 medium (20 g/L tryptone and 5 g/L NaCl) for 1.5 h recorded using commonly available linear ion
158 trap MS instrument (LTQ, Thermo Scientific, San Jose, CA, USA). The spectrum was dominated
159 by an unknown signal at *m/z* 70. Based on high-resolution mass measurements (Orbitrap XL,
160 Thermo Scientific, San Jose, CA, USA), the exact *m/z* value of this signal was determined as *m/z*
161 70.066. $C_4H_8N^+$ is the only possible chemical formula that fits this value within the mass
162 accuracy of Orbitrap detection ($\Delta \approx 10$ ppm). All other possible chemical formulas with the
163 nominal mass 70 differ from *m/z* 70.066 by > 150 ppm.

164

165 3.2. CID analysis

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166 The structural CID analysis of $C_4H_8N^+$ ion displayed the major characteristic fragments at *m/z* 43
167 and *m/z* 28 (Fig. 2a). Using the same experimental conditions, we performed reference analysis
168 of model volatile metabolites discovered in bacteria, humans and other living organisms that
169 could form $C_3H_8CN^+$ ions upon protonation.[7, 41] Out of the studied reference compounds, only
170 protonated 1-pyrroline cations and 3-pyrroline cations produced CID pattern with exactly the
171 same intensity ratios for product ions as in the CID spectrum of *m/z* 70 signal from PA culture
172 (Fig. 2 a-c). The protonated cations of other model compounds, including *n*-butyronitrile,
173 isobutyronitrile, *n*-propyl isocyanide and isopropyl isocyanide, were also found to yield the CID
174 fragments at *m/z* 28 and *m/z* 43 but with notably and reproducibly different intensity ratios (Fig.
175 2 d-g). This data strongly suggest that the $C_3H_8CN^+$ ion detected in the volatile headspace of PA
176 cultures was formed by the protonation of pyrroline.

177

178 **3.3. LC-MS analysis**

179 To further validate the structural assignment of $C_3H_8CN^+$ ion to protonated pyrroline cation as
180 well as to identify the specific pyrroline isomer produced by PA cultures, we also performed LC-
181 ESI-MS analysis of VOCs from the headspace of PA culture as well as LC-ESI-MS analysis of VOCs
182 from the headspace of reference 1-pyrroline and 3-pyrroline aqueous solutions. Note that the
183 observation of pyrroline signal by LC-ESI-MS directly from the supernatant solution of a PA
184 culture was suppressed due to a high salt content in the culturing medium. Nonvolatile salt
185 interferences were efficiently removed by collecting VOCs on a cold glass surface and then
186 analyzing the condensate as described in Experimental section. Fig. 3 shows single ion

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187 chromatograms for the *m/z* 70 signal, which clearly indicate the selective generation of 1-
188 pyrroline isomer by PA cultures. The chromatographic separation of 1-pyrroline and 3-pyrroline
189 was easily achieved due to the notable difference of their PK_a values (≈6.7 for 1-pyrroline;
190 ≈10.5 for 3-pyrrolnie).[42] To our knowledge this is the first evidence for the release of 1-
191 pyrroline by PA.

192

193 **3.4. Time profile of 1-pyrroline release by PA**

194 We found that the release of 1-pyrroline by PA cultures followed highly characteristic transient
195 time profile. Fig. 4 shows the time profiles of 1-pyrroline signal from the headspace of cultures
196 with different initial counts of PA (5×10^2 – 3×10^6 CFU/mL). Each time point for different initial
197 PA concentrations was analyzed by AMS using at least four independently grown cultures.
198 Standard deviation of signal response was mostly in the range of 3-8%. In total, ca. 400 PA
199 cultures were analyzed (four different original PA concentrations in TSB medium; two different
200 original PA concentrations in LB medium × 10-15 time points × 4-5 replicate samples + 28 urine
201 cultures + 28 sputum cultures ≈ 400 cultures). In all the cultures, 1-pyrroline production was
202 substantially enhanced during the lag phase and early log phase of bacterial growth (3 – 6 h, Fig.
203 S-1). We also observed that the higher initial counts of PA resulted in the earlier onset of 1-
204 pyrroline release (Fig. 4). For example, at the initial PA count of 3×10^6 CFU/mL, notable release
205 of 1-pyrroline was observed after 30 min of incubation, early in the lag phase preceding
206 bacterial growth (Fig. S-1). The initial increase of 1-pyrroline level in PA cultures was followed

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207 by a steady decrease lasting for > 20 h. The enhancement of 1-pyrroline emission occurring in
208 the early phase of bacterial culture is particularly beneficial for the rapid identification of PA.

209

210 **3.5. Quantification of 1-pyrroline release by PA**

211 Quantification of 1-pyrroline in PA cultures was done based on the comparison of 1-pyrroline
212 signal intensity with reference AMS measurements of standard 1-pyrroline dilutions in pure
213 culture medium (Fig. S-2). The data indicated that the peak solution concentration of 1-
214 pyrroline in PA cultures was in the range from 200 ppb to 800 ppb depending on the original
215 number of PA cells (Fig. 4). The quantitative evaluation of 1-pyrroline concentration derived
216 from AMS analysis was consistent with more rough estimations based on analogous LC-MS
217 analysis. The limit of detection for 1-pyrroline in aqueous solution using AMS analysis of vapor
218 phase was ca. 10 ppb.

219

220 **3.6. Specificity of pyrroline release**

221 Besides PA, the early release of 1-pyrroline was also discovered in two taxonomically close
222 bacterial strains, *P. putida* and *Burkholderia cepacia* (Fig. S-3). In fact, *B. cepacia* was originally
223 classified as *Pseudomonas* and has only recently been re-classified to *Burkholderia*. The time
224 profile of 1-pyrroline release was very similar in different culturing media (tryptic soy broth,
225 Luria-Bertani broth). Importantly, the reference AMS screen of common infectious microbes,
226 including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*,

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227 *Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter baumannii, Enterococcus faecalis,*
228 *Klebsiella oxytoca, Candida albicans, Candida tropicalis, Candida parapsilosis,*
229 *Enterococcus faecalis, Enterobacter cloacae*, did not reveal notable release of 1-pyrroline (Fig.
230 S-3). These results indicated the high specificity of 1-pyrroline to *Pseudomonas* and to PA,
231 which is the most common *Pseudomonas* infection in humans.

232

233 **3.7. Ionization mechanism of 1-pyrroline**

234 The prevalence of 1-pyrroline over the rest of VOC signals in PA culture is very remarkable. The
235 ionization of bacterial volatiles by corona discharge in our experiments most likely occurs via a
236 similar mechanism to that in atmospheric pressure chemical ionization (APCI).[39] In APCI, analyte
237 solution is pneumatically nebulized when passed through a heated capillary (> 400 °C), and the
238 aerosol cloud is subjected to atmospheric corona discharge. The majority of ion-molecule
239 reactions in corona discharge area are gas-phase acid-base type reactions in which protons are
240 gradually transferred along the ladder from chemicals with lower proton affinity – primarily
241 from protonated water cations – to chemicals with higher proton affinity value.[39] This ladder
242 proton transfer is very fast owing to the high rate of ion-molecule collisions at atmospheric
243 pressure. As a result, thermodynamic equilibrium between reacting species is typically reached
244 during ionization. 1-Pyrroline has a very high proton affinity value of 926 kJ/mol.[43] For
245 comparison, proton affinity of water is 691 kJ/mol, proton affinity of ammonia is 854 kJ/mol,
246 and proton affinity of pyridine is 930 kJ/mol.[43] Owing to its high proton affinity and high vapor
247 pressure, 1-pyrroline acts as an efficient proton scavenger to suppress ionization of VOCs with

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248 lower proton affinity values. Similarly, the addition of highly basic pyridine or trimethylamine
249 into sample solution is known to heavily suppress ionization of other analyte molecules. Apart
250 from protonated 1-pyrroline, the only other signals observed at high intensity in AMS of PA
251 corresponded to radical water cations (m/z 36 and m/z 54) formed via electron abstraction
252 rather than protonation. Other signals were observed at a much weaker abundance. Therefore,
253 ambient ionization by corona discharge can be considered as a highly sensitive and selective
254 approach to detect 1-pyrroline in PA cultures.

255 Interestingly, earlier studies revealed the ubiquitous presence of protonated 1-pyrroline
256 cations in the Earth troposphere.[43] The process of tropospheric ion formation proceeds via
257 essentially the same charge-transfer ladder as described above. Initially, cosmic rays and
258 radioactive decay in the troposphere initiate the production of protonated water cations. These
259 primary water cations donate protons to the successively less abundant neutral species with
260 higher proton affinity to form progressively more stable protonated cations. As a result, trace
261 neutral compounds, such as pyridine and 1-pyrroline, can serve as precursors for relatively
262 abundant tropospheric ions.[43] The ubiquitous observation of 1-pyrroline cations in the air
263 illustrated the high chemical selectivity of corona discharge ionization toward 1-pyrroline and
264 the high stability of protonated 1-pyrroline cations in the gas phase.

265

266 **3.8. Analytical challenges of 1-pyrroline detection by other methods**

267 Although the detection of 1-pyrroline in the volatile headspace of PA cultures is readily
268 achieved using the ambient corona discharge ionization, it appears to be more challenging by

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269 many other methods. To our knowledge neither offline approaches (e.g., gas chromatography
270 mass spectrometry (GC-MS))[14, 44, 45] nor online methods (e.g., selected ion flow tube mass
271 spectrometry (SIFT-MS)[46] or secondary electrospray ionization mass spectrometry (SESI-MS)[36])
272 reported until today could reveal the presence of 1-pyrroline in PA or in other bacterial species.
273 The detection of 1-pyrroline is complicated by its low chemical stability. In room-temperature
274 aqueous solutions 1-pyrroline usually degrades on the time scale of several days,[47] and the
275 degradation process is further accelerated at higher temperatures.[47, 48] Therefore, significant
276 degradation of 1-pyrroline could be expected if high temperatures are used for vapor collection
277 and/or analysis, such as in GC-MS. Another problem associated with the poor chemical stability
278 of 1-pyrroline is the lack of commercially available reference standard, which greatly
279 complicates the unambiguous chemical assignment of 1-pyrroline signal. Finally, because 1-
280 pyrroline is released transiently within the early period of incubation, the detection of 1-
281 pyrroline is best achieved at relatively short incubation times. Therefore, too long incubation
282 times in some studies could be another possible explanation for the lack of 1-pyrroline
283 observation.[36] Our data demonstrate the APCI type of ionization is highly suitable for the
284 sensitive MS detection of 1-pyrroline vapor. Importantly, vapors from the bacterial cultures are
285 sampled to ionization at room temperature without additional heating. The mild sampling
286 conditions protect the intact molecular structure of 1-pyrroline and may contribute to the high
287 visibility of 1-pyrroline in our experiments. We expect that the detection of 1-pyrroline in the
288 headspace of PA cultures should also be easily achieved using other types of ambient plasma
289 ionization, e.g., DART-MS[20] or LTP-MS.[22]

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291 **3.9. 1-Pyrroline metabolism in PA**

292 1-Pyrroline is known to be released by some plants presumably for odor mimicking purposes[40,
293 49] and by some animals presumably as pheromones.[50-52] However, the exact mechanism of 1-
294 pyrroline formation by living organisms remains unknown. Below we mention a possible
295 explanation for the high specificity of 1-pyrroline release by PA, even though experimental
296 proof is lacking. 1-Pyrroline is a possible oxidation product, directly or indirectly, from at least 7
297 common metabolites, including pyrrolidine, proline, ornithine, 4-aminobutanol, putrescine,
298 spermidine and spermine.[42] The generation of 1-pyrroline from putrescine by spontaneous
299 atmospheric oxidation[42] or from proline by the Strecker degradation[53] was demonstrated in
300 laboratory conditions. In living organisms, both proline and putrescine can mediate cellular
301 response to abiotic stresses, such as cold and dehydration.[54, 55] Therefore, the enhanced
302 emission of 1-pyrroline during the lag phase of PA culture could possibly be related to the
303 enhanced turnover of proline, putrescine and other structurally relevant molecules over the
304 process of bacterial adaptation to growing conditions. The specific observation of 1-pyrroline
305 emitted by PA cultures but not by the other strains might thereby reflect the mechanistic
306 distinction of stress adaptation in *Pseudomonas* relative to other genera. The gradual decrease
307 of 1-pyrroline level in PA cultures after the initial increase (Fig. 4) indicated that the produced 1-
308 pyrroline was readily metabolized by bacteria into other products or degraded due to its low
309 chemical stability. The short metabolic life-time of 1-pyrroline has also been suggested by
310 earlier studies[52, 56] and might be another factor complicating the detection of 1-pyrroline in PA
311 cultures using offline methods.[14]

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312

313 **3.10. Identification of PA infection in clinical samples via targeted 1-pyrroline detection**

314 PA infections are most frequently acquired by immunocompromised patients in hospitals and
315 nursing homes, accounting for ca. 10% of hospital-acquired infections, causing pneumonia,
316 urinary tract infection, bacteremia, surgical wound infection, osteomyelitis, etc.[1] Predisposing
317 conditions for acquiring PA infection include hematologic malignancies, immunodeficiency
318 relating to AIDS, neutropenia, diabetes mellitus, severe burns, and particularly cystic fibrosis.
319 The lungs of cystic fibrosis patients can be chronically infected by PA for several years.[57] Clinical
320 diagnosis of PA infection usually relies on microorganism identification in a clinical laboratory,
321 which is laborious, time-consuming and carries a risk of misidentification.[3] Hence is the
322 increasing attention to identification of microorganisms using MS approaches, which allow
323 much higher molecular specificity of the analysis.[3-5]

324 Based on our findings, 1-pyrroline can be proposed as a volatile differential metabolite
325 for the rapid identification of PA infections in humans using commonly available MS
326 instrumentation. In a recent study, we analyzed blood cultures of 61 adult volunteers at
327 suspicion of bacteremia and 39 patients confirmed with bacteremia.[38] Out of the studied
328 cultures, 3 cases of bacteremia were assigned to PA infection through conventional
329 microorganism identification in clinical laboratory for 2–3 days, and the AMS of the same
330 cultures showed a notable signal at *m/z* 70 after few hours of incubation (0.5–6 h). Further, 30
331 blood cultures of healthy individuals inoculated with PA (10^4 CFU/mL) were tested by AMS, and

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332 each of them produced the *m/z* 70 signal upon incubation.[38] However, no conclusive structural
333 assignment for *m/z* 70 was made in our preliminary work.[38]

334 In current study, using tandem analysis we confirmed that the *m/z* 70 biomarker signal
335 in the blood cultures of PA patients indeed corresponded to protonated 1-pyrroline. The same
336 approach was directly extended for the rapid identification of PA infections in urinary and
337 respiratory tracts using the AMS analysis of urine and sputum cultures. 1-Pyrroline was clearly
338 visible by AMS of the volatile headspace from seven sputum cultures and seven urine cultures
339 incubated for 2 h at the initial PA count of 2×10^5 CFU/mL and 2×10^4 CFU/mL accordingly (Fig. 5a
340 and 5b). The important feature of urine and sputum spectra was a relatively low inter-individual
341 variability in MS signal intensity of 1-pyrroline, with RSD within 5-10% (Fig. 5c and 5d). In
342 contrast, our earlier study showed pronounced inter-individual variability of 1-proline signal in
343 simulated blood cultures.[38] Some blood cultures of PA did not reveal any bacterial growth even
344 after 16 h of incubation. The considerably slower bacterial growth in the blood cultures of
345 certain human individuals was attributed to possibly higher antibacterial blood immunity of
346 those individuals.[38] Apparently, both urine and sputum have weaker bacterial resistance than
347 blood, which could account for the much lower inter-individual signal variability. The low inter-
348 individual signal variability is important for the quantitative evaluation of PA concentration in
349 culture solution.

350 In this study we did not pursue quantitative measurements of PA concentration in urine
351 and sputum cultures after incubation. For the accurate quantitative analysis of PA
352 concentration based on 1-pyrroline AMS signal intensity one should definitely take into account

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353 matrix effects, most importantly those associated with solution pH. The pKa value of 1-
354 pyrroline is ≈ 6.7, which means that 1-pyrroline is half ionized at pH 6.7.[42] At higher pH values
355 the relative share of nonionic 1-pyrroline will increase, resulting in the increase of molecular
356 volatility. In contrast, at lower pH values the relative share of ionic 1-pyrroline will increase,
357 resulting in the decrease of molecular volatility. The sensitivity of 1-pyrroline vapor pressure to
358 solution pH should be particularly high around pH 6.7. Therefore, calibration curve for 1-
359 pyrroline should be built in the same matrix as the clinical sample. To account for possible inter-
360 individual differences of matrix composition, the method of standard additions can be
361 recommended for the most accurate and reliable quantification of 1-pyrroline in patient
362 cultures based on the AMS analysis of volatiles.

363

364 **3.11. Analytical merits and limitations of VOC screening by AMS**

365 The principal limitation of MS analysis compared to the traditional biochemical approaches is
366 that different culture tubes are analyzed sequentially rather than simultaneously. Therefore,
367 the speed of MS sampling is particularly important, because it limits the number of samples
368 that can be measured during the available instrument time. VOC analysis by AMS is featured by
369 the high speed and throughput of sampling. At present we can routinely achieve scanning rate
370 of ca. 5 samples per min. This high throughput of sampling is achieved owing to the technical
371 simplicity of experimental procedure and the efficient obviation of sample carry-over effects in
372 VOC mode of analysis. We estimate that the sampling throughput can be further increased
373 using robotized sampling procedure, similar to that in commercial GC instruments.

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374 An important analytical merit of ambient corona discharge ionization is the total
375 obviation of solvents during sampling and ionization of volatile chemicals. This allows for the
376 low cost of operation and for the high stability of approach. When operated on a daily basis,
377 the signal intensity of reference 1-pyrroline standard usually displayed relative deviations
378 below 10%. Abrupt changes of signal intensity (> 50%) were sometimes noticed upon
379 instrument maintenance, in which case the calibration curve for 1-pyrroline (Fig. S-2) was
380 rebuilt to account for the change in instrument response.

381

382 ## 4. Conclusion

383 **I**n this study we discovered 1-pyrroline as a differential volatile metabolite of *Pseudomonas*
384 *aeruginosa*. 1-Pyrroline emitted by PA features early release, high chemical specificity and
385 excellent visibility by widely available MS instrumentation with atmospheric ion interface. The
386 diagnostic value of 1-pyrroline was demonstrated for several clinical samples, including blood,
387 urine and sputum. While common clinical diagnostic routines usually require ca. 2-3 days of
388 analysis, our approach offers the same day diagnosis of PA, which allows earlier and more
389 efficient disease treatment.

390

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398

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479

480 ## Figure captions

481

482 **Fig. 1** AMS spectrum of volatiles emitted by a PA culture after 1.5 h incubation, featuring an
483 abundant signal at *m/z* 70.

484

485 **Fig. 2** Collision-induced dissociation of VOC signal at *m/z* 70 produced by AMS from the
486 headspace of a) PA culture; b) 1-pyrroline; c) 3-pyrroline; d) *n*-butyronitrile; e) isobutyronitrile;
487 f) *n*-propyl isocyande; g) isopropyl isocyanade. CID was performed at the collision energy of 25
488 instrument units (left column) and 30 instrument units (right column).

489

490 **Fig. 3** Single ion chromatogram of *m/z* 70 signal in LC-ESI-MS of vapor condensate collected
491 above the aqueous solution containing a) pure culture medium; b) PA cluture (after 12 h
492 incubation); c) 1-pyrroline in pure culture medium; d) 3-pyrroline in pure culture medium. The
493 CID pattern of *m/z* 70 signal produced by LC-ESI-MS fully matched the CID pattern of *m/z* 70
494 signal produced by AMS.

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496 **Fig. 4** Time profiles of 1-pyrroline signal (*m/z* 70) detected by AMS from the headspace of

497 cultures with different initial concentration of PA.

498

499 **Fig. 5** AMS spectra of VOCs from a PA sputum culture (a) and PA urine culture (b) incubated for

500 2 h at the initial PA counts of 2×10^5 CFU/mL and 2×10^4 CFU/mL accordingly. Signal intensity of

501 1-pyrroline signal (*m/z* 70) is shown at four different incubation times (0 h; 2 h; 4 h; 6 h) for

502 seven biological replicates (c, d).

503

504 **Graphical abstract entry:** 1-Pyrroline detected by ambient mass spectrometry is suggested as a

505 potential volatile biomarker for early identification of Pseudomonas aeruginosa infections.

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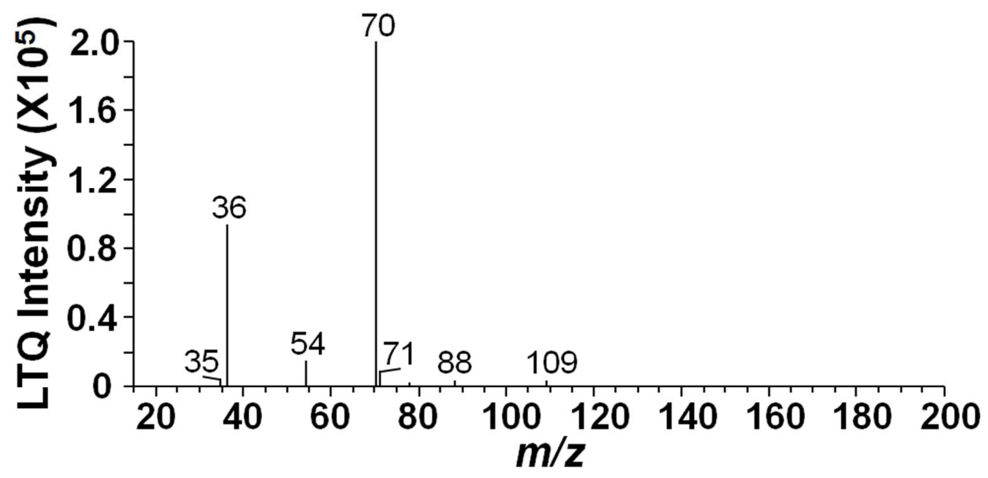

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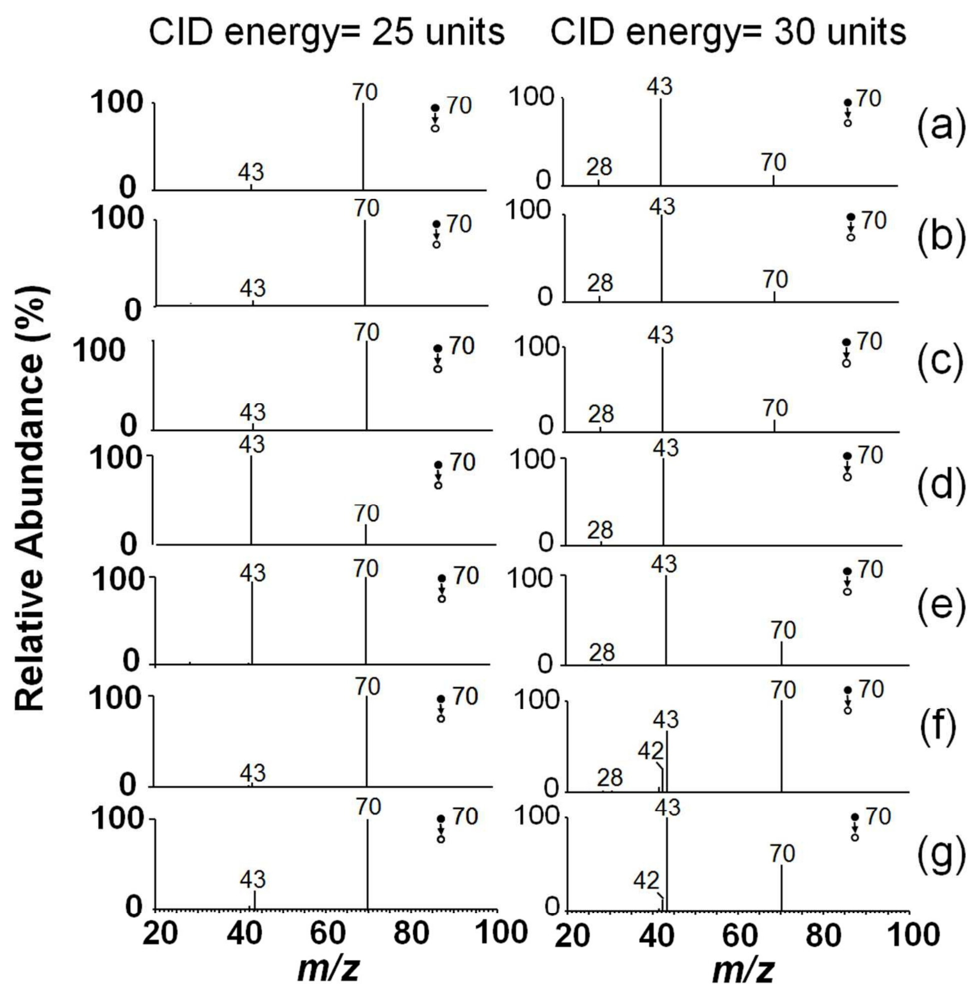

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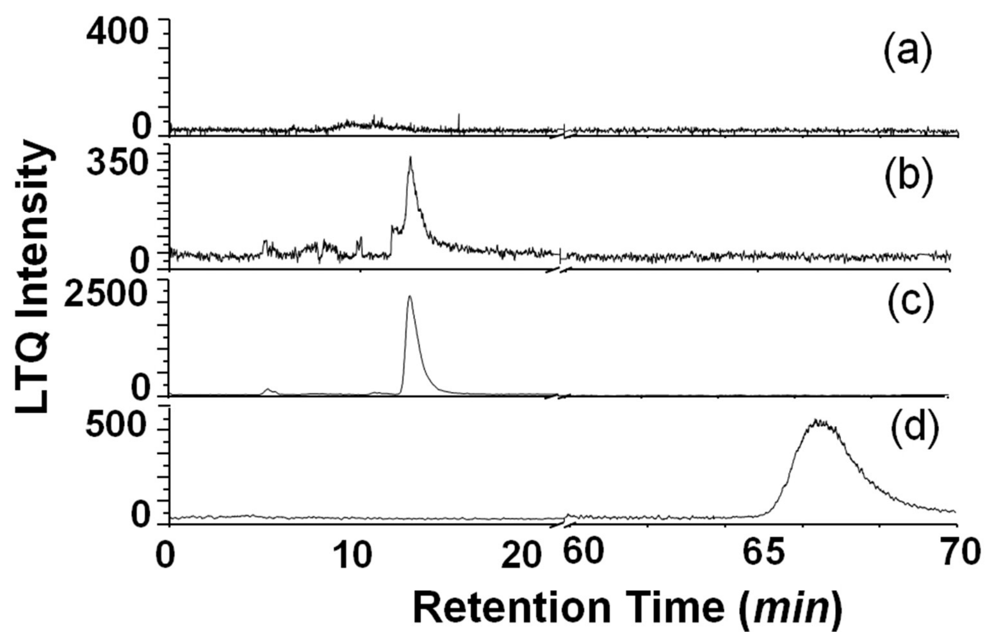

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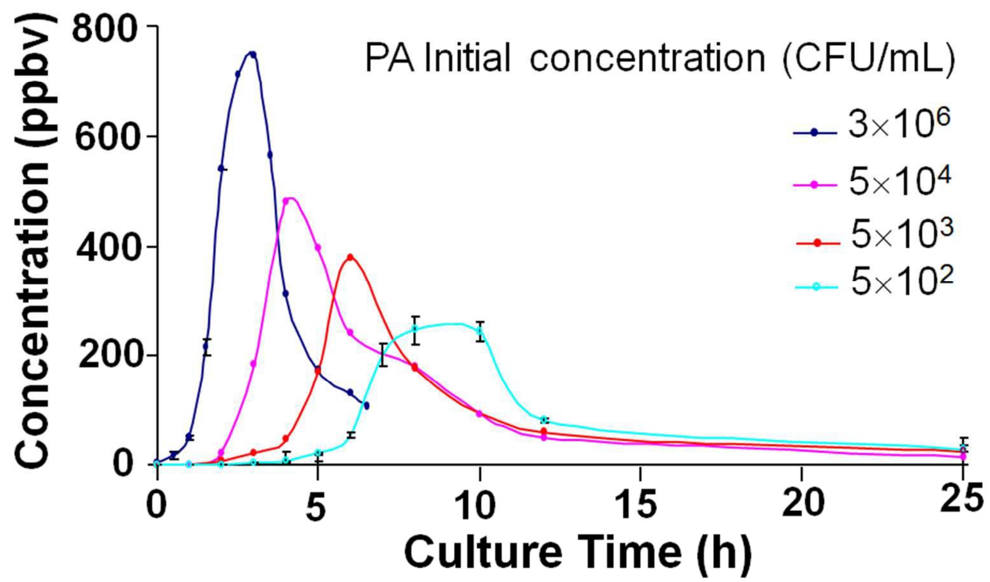

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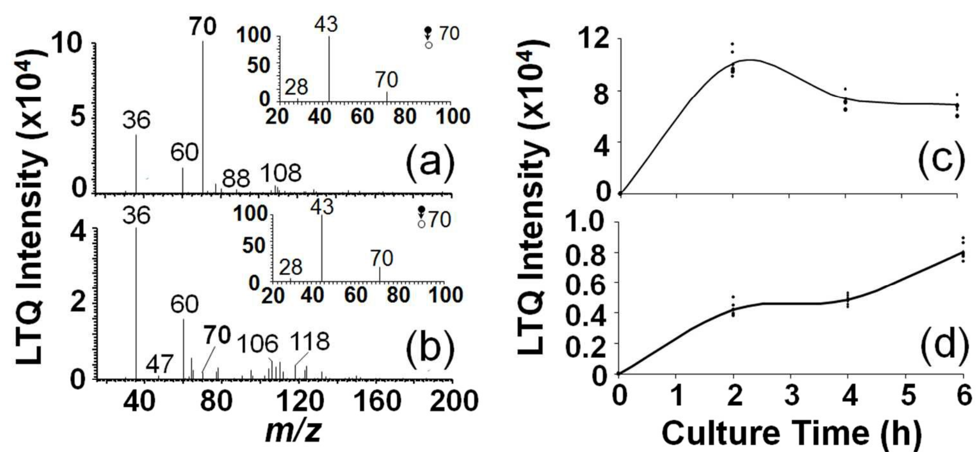

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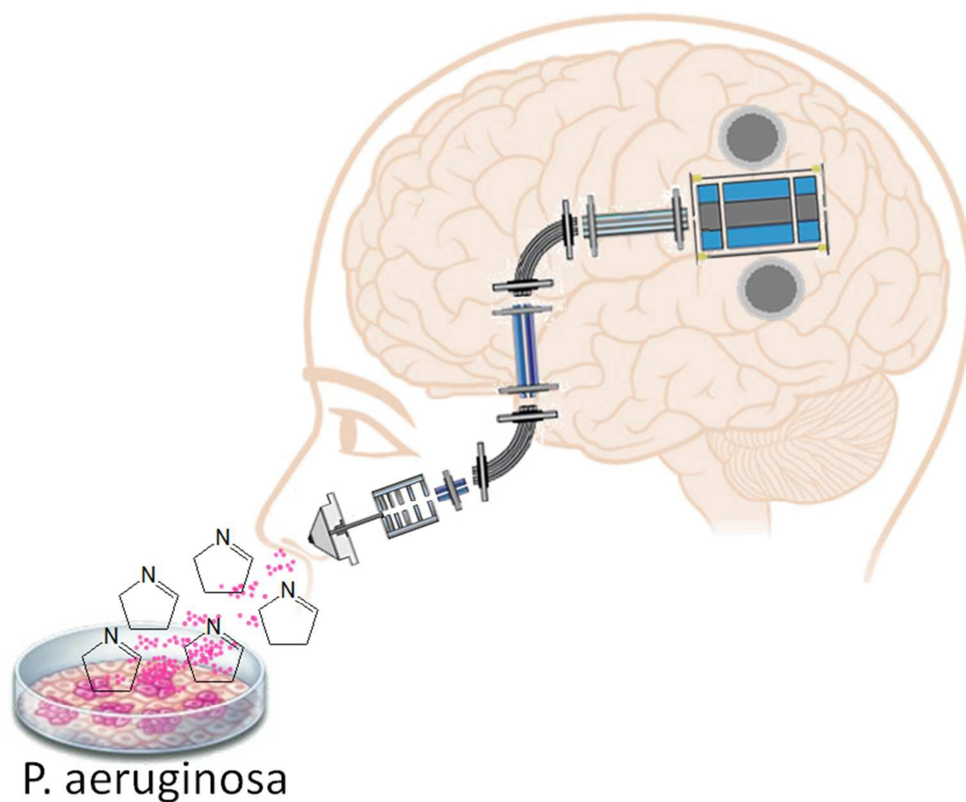

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