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## Detection of Volatile Organic Compounds from Preclinical Lung Cancer Mouse Models

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1 DETECTION OF VOLATILE ORGANIC COMPOUNDS FROM PRECLINICAL  
2 LUNG CANCER MOUSE MODELS

3  
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## 39 **Abstract**

40 Volatile organic compounds (VOCs) may help detect cancer tumour. This study addressed this question, as well  
41 as two methodological issues: 1) repeatability, comparing VOCs profiles obtained with two Solid Phase Micro  
42 Extraction (SPME) fibers used simultaneously; 2) detectability of cancer VOCs biomarkers, comparing profiles  
43 obtained following 1h versus 24h exposure of SPME fibers. We analyzed VOCs composition of soiled bedding  
44 obtained from a lung adenocarcinoma mouse model in which cancer was induced by doxycycline ingestion. We  
45 compared the VOCs profile of soiled bedding of cancerous (CC) and non-cancerous (NC) mice, before (T0),  
46 after two-weeks (T2) and after twelve weeks (T12) doxycycline ingestion. The results indicate : 1) qualitative  
47 and quantitative consistency in VOCs detection by two distinct SPME fibers ; 2) although more VOCs were  
48 detected following a 24h compared to 1h SPME exposure, none of the former molecules were related to cancer;  
49 3) doxycycline impacted VOCs emissions in both CC and NC mice; 4) cancer impacted four VOCs at T12 only  
50 : the benzaldehyde which showed higher levels in CC mice and the hexan-1-ol and two mice pheromones, the 2-  
51 *sec*-butyl-4,5-dihydrothiazole and the 3,4-dehydro-*exo*-brevicomine, which showed lower levels in CC mice.  
52 Our study points out that the use of two SPME fibers and an extraction duration of 1h may be considered a good  
53 compromise allowing detection of cancer biomarkers while easing bench constraints.

54

55 **Key Worlds-** SPME, repeatability , odour signature, cancer biomarkers, EFGR cancer, *Mus musculus*.

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## 60 1. Introduction

61 Volatile organic compounds (VOCs) are carbon-based small molecules, which are characterized  
62 by their volatility at ambient temperature. During the last decades, increasing attention has been devoted  
63 to exploring the relevance of VOCs emitted by the body as potential biomarkers of pathologies. VOCs  
64 are emitted and can be analysed from breath, skin, saliva, sweat, blood, urine and faeces. Nowadays,  
65 VOCs are commonly used as biomarkers of various diseases [1,2] such as fibrosis [3], asthma [4],  
66 Alzheimer disease [5], diabetes and tuberculosis [6,7], and an increasing attention has been given at  
67 using them for cancer detection [8].

68 VOCs extraction involves the use of effective trapping methods, of which Solid-Phase Micro-  
69 Extraction (SPME) is the most commonly used [9]. For instance, this technique has been successfully  
70 used to detect VOC biomarkers in the context of diseases such as gastrointestinal disorders [10], cholera  
71 [11], tuberculosis [12] and cancers [13–15]. Accuracy and repeatability of VOC extraction strongly rely  
72 on the characteristics of the SPME fiber used [16] and on extraction conditions such as temperature and  
73 duration [17–19]. Some journals recommend using a single SPME in comparative studies, arguing that  
74 the use of more than one fiber in such studies may result in biased interpretations. However, when a  
75 study includes a large number of samples, it may be unpractical to use a single fiber for all experiment,  
76 and multiple successive use of the same fiber may also induce measurements biases due, for example,  
77 to wear. Another methodological issue is sampling duration. Emission of VOCs from biological  
78 substrate can be dynamic [20] and, in order to account for emission variations, comparative studies need  
79 to homogenize sampling duration. A short sampling duration may not allow capturing the diversity of  
80 VOCs emitted by the substrate nor detecting components present in very low concentrations, partly  
81 because competition between molecules present in the headspace may disadvantage the adsorption of  
82 components present in relatively small proportions [21–23].

83 In the context of investigations of variation in VOCs composition induced by lung cancer in mice, the  
84 present study addressed repeatability of results obtained when sampling involved two different SPME  
85 fibers, and the impact of sampling duration on the detection of changes in the VOCs composition of  
86 mice soiled bedding during the course of tumour development. We also assessed whether VOCs induced  
87 by lung cancer were better detected after a 24h as compared to a 1h SPME exposure, and sought to  
88 identify murine lung adenocarcinoma VOC biomarkers. The odour sources were soiled beddings  
89 obtained from a genetically modified mouse model suffering from lung cancer, and considered an early  
90 and a late stage of tumour development.

## 91 2. Material and methods

92

### 93 2.1 Ethical clearance

94

95 All the precautions for animal welfare were followed during our experiments, and our study received  
96 ethic clearance from the Ethical Committee for Animal Experimentation (French Ministry of Higher  
97 Education, Research and Innovation) number 1645-22123.

## 98 2.2 Animals

99

100 Two genotype lines were involved in this study: WT and CCSP-rtTA / EGFR<sup>T790M/L858R</sup> [24,25]. The  
101 WT mice, lacking both CCSP-rtTA and EGFR<sup>T790M/L858R</sup> transgenes, do not develop any tumour upon  
102 doxycycline (antibiotic) induction, hereafter non-cancerous (NC) mice, and the CCSP-rtTA /  
103 EGFR<sup>T790M/L858R</sup> mice, carrying both transgenes, develop lung adenocarcinoma upon doxycycline  
104 induction, hereafter cancerous (CC) mice. To induce the EGFR<sup>T790M/L858R</sup> in a lung specific manner,  
105 the mice received a doxycycline supplemented food (1mg/kg). The treatment lasted 12 weeks starting  
106 when the mice were 13 weeks old.

107 NC and CC male mice were obtained from the IRCM (Montpellier Cancer Research Institute) at the  
108 age of 6 weeks and maintained at the breeding facilities of the IRD (Institute of Research and  
109 Development) in Montpellier. Before the start of the experiment they were maintained in groups of 2  
110 to 4 mice in transparent plastic cages (26.8cmW\*21.5cmL\*14.1cmH). Each cage contained sawdust, a  
111 cellulose square, hay and a cardboard tunnel. The mice were observed and weighted once a week  
112 during the entire experimental period to monitor their health. All mice were euthanized at the end of  
113 the experiment at the IRCM at 25 weeks-of age. The non-cancerous status of all NC and the cancerous  
114 status of all CC mice were confirmed by histopathological analysis of haematoxylin & eosin stain of  
115 whole lung sections obtained after necropsy.

## 116 2.3 Odour source sampling

117 Odour sources were two-week old soiled bedding of CC and NC mice. Each mouse was isolated at  
118 the age of 10 weeks in a cage containing 130g sawdust and a cellulose square. The housing conditions  
119 of all donors were homogenized as much as possible, and all mice were given the same quantity of  
120 food. Every two weeks, all the soiled bedding of every cage was collected in a plastic bag and kept at -  
121 20 ° C and replaced by cleaned bedding (i.e. 130g sawdust). Soiled bedding analyzed in this study  
122 were pools of bedding obtained from 3 to 4 different mice. A sample of clean sawdust was also  
123 collected in a plastic bag and frozen to serve as a control for the VOCs analyses.

124

## 125 2.4 VOCs extraction

126  
127 PMDS-DVD (polydimethylsiloxane and divinylbenzene) SPME fibers, recently purchased, and which  
128 had never been used before, were used in this study (thickness 65µm, Stable Flex 24Ga, Sigma-  
129 Aldrich, Bellefonte, USA). The odour samples were processed as follows. A frozen soiled bedding  
130 was thawed while being maintained on ice during the entire procedure. Then a 3g aliquot was

131 transferred into a 125 ml glass vial, which was then sealed with a rubber septum. The VOCs sampling  
132 phase took place in an oven maintained at 22°C. The SPME fiber was introduced into the vial after  
133 piercing the septum with a needle after a 3min equilibration time, its position was always the same,  
134 ~2cm above the stimulus (**Fig. 1**).

135

### 136 2.5 VOCs characterisation by Gas Chromatography- Mass Spectrometry

137

138 The SPME extract was injected into a Gas Chromatography - Mass Spectrometry (GC-MS), quadrupole  
139 mass spectrometer Shimadzu QP2010-SE (Shimadzu, Kyoto, 282 Japan) for further identification and  
140 relative quantification of its chemical content. Desorption was achieved by inserting the SPME fiber  
141 into the Ultra Inlet liner (for SPME/Purge and trap, 0.75mmID, Agilent CrossLab, Santa-Clara, USA)  
142 placed in Split/Splitless GC-MS injector heated at 250°C. The injection was made using a split ratio of  
143 1:4 to allow a Gaussian form of low boiling point compounds and so, a better separation and further  
144 integration. The GC was equipped with an Optima 5-MS fused silica capillary column (30 m x 0.25 mm  
145 x 0.25 µm film thickness, Macherey-Nagel, Düren, Germany). Helium was used as the carrier gas (1  
146 ml.min<sup>-1</sup>). The GC temperature was maintained at 40 °C for 2 min, after which the temperature increased  
147 by 5°C every minute till it reached 175 °C, and then by 12°C/min. till it reached 220°C.

### 148 2.6 Identification of the VOCs present in the extracts

149 Spectra were analysed with the resident software (GCMS Solution, Shimadzu, Kyoto, Japan).  
150 Identification of compounds was based on chromatograms analysis of peak retention time (RT) and  
151 examination of their mass spectra. Retention indices (RI) were calculated using as reference the RI of a  
152 series of n-alkanes injected in the same apparatus (Alkanes standard solution, 04070, Sigma-Aldrich).  
153 Identification of compounds was made by both spectral and RI comparison with reference databases  
154 (NIST 2011, Wiley 292 Registry Ninth; e.g. Adams, 2007, Pubchem, 293  
155 <https://pubchem.ncbi.nlm.nih.gov/>). Synthetic compounds were also used as a reference to confirm the  
156 identity of some VOCs. The peak surface area of each compound was based on the Total Ion Current  
157 Chromatogram (TICC). To be conservative, we excluded from the analyses compounds that were  
158 present in both our controls (clean bedding) and our biological samples, even when they were in higher  
159 proportion in the biological samples.

160 Compounds identified with their mass spectra but present in the form of traces could not be quantified  
161 with the above described method and were given an arbitrary value corresponding to 10% of the surface  
162 area calculated for the smallest peak identified in the entire dataset. Peak areas for all compounds were  
163 then summed per chromatogram, and their relative proportion calculated as the ratio of the compound  
164 surface area divided by the sum of the surface areas of all other compounds identified in that  
165 chromatogram.

## 166 2.7 Testing the repeatability of odour sampling

167

168 We tested the repeatability of odour sampling among two different SPME fibers (F1 and F2)  
169 pertaining to the same new pack of 3 fibers (Sigma-Aldrich). The fibers were conditioned following  
170 the manufacturer's recommendations, i.e. 30 minutes at 250°C. They were simultaneously introduced  
171 into a glass bottle containing the odour sample (3g of soiled bedding of 13-weeks old NC mice, Fig.1)  
172 and were exposed at the same time to the bottle headspace for 1h. The distance from the odour sample  
173 was controlled to be the same for the two fibers (~2cm), and the distance between the two fibers was  
174 ~1.5 cm. The same procedure was repeated at seven occasions with the same fibers and the aliquots  
175 extracted were obtained from the same odour source, maintained at -20° between samplings. The  
176 seven occasions corresponded to the first, 8<sup>th</sup>, 15<sup>th</sup>, 22<sup>th</sup>, 30<sup>th</sup>, 39<sup>th</sup> and 45<sup>th</sup> use of each fiber.

177

## 178 2.8 Testing the influence of sampling duration

179 We analyzed the qualitative and quantitative variations of VOCs obtained from 18 samples of soiled  
180 bedding sampled with two fibers simultaneously, one exposed to the headspace for 1h the other for  
181 24h. The odour sources were obtained from CC and NC mice at three experimental conditions: T0,  
182 i.e. before ingestion of doxycycline, T2, i.e. after two weeks' doxycycline treatment, and T12, i.e. after  
183 12 weeks' doxycycline treatment. (Fig. 1). We proceeded as follows, two SPME fibers were inserted  
184 into the same glass bottle containing soiled bedding and were exposed simultaneously to an odour  
185 source. The two fibers were placed at the same distance from this source and at a 1cm-distance from  
186 each other. After 1h exposure, one of the fibers was removed, while the other was left for another 23h.  
187 The procedure for the 18 samples lasted three days, and each day an additional glass bottle containing  
188 clean bedding was also sampled following the same protocol and served as a control.

## 189 2.9 Identifying VOCs biomarkers of murine lung cancer

190

191 The same 18 odour sources as the above were processed. However, here each of the sources was  
192 represented by three replicates to control for sampling bias (i.e. collection of 3g aliquots for VOCs  
193 extraction). All together we analyzed 54 biological samples, In addition, a sample of clean bedding  
194 was extracted every day to serve as a control. Two SPME fibers were used alternatively for the  
195 purpose of this analysis, randomizing their use among NC and CC.

196

## 197 2.10 Statistical analyses

198 All statistical analyses were performed with Rstudio version 3.4.4 [26]. We used the following packages:  
199 ade4 [27], vegan [28], mixOmics [29], ggplot2 [30], RVAideMemoire [31], Hotelling [32]. Variation  
200 of the relative proportions of VOCs was assessed with a multivariate approach using redundancy  
201 analyses (RDAs) followed by permutation F tests [33] and the R package vegan [28]. Relative  
202 proportions were CLR-transformed prior to RDAs, and as our data included zeroes, a small constant of  
203 an order of magnitude smaller than the smallest non-zero value was added to all values prior to the  
204 transformation (i.e. 0.01 if the smallest non-zero value is 0.1). Factors included in RDAs depended on  
205 the questions tested. The first model questioned the consistency of extraction by the two SPME fibers  
206 used simultaneously at 7 sampling occasions, and included “fiber identity” and “sampling occasions” as  
207 factors. The second RDA model addressed the impact of sampling/extraction duration on detection of  
208 cancer biomarker VOCs. Two models were considered. Because the number of VOCs detected after 24h  
209 extraction was higher than that after 1h extraction, the first model analysed only the data obtained after  
210 24h and sought to identify presence of VOCs induced by cancer tumour, not detected after 1h extraction.  
211 The model included experimental conditions (T0, T2, T12), health status (CC, NC) and their interactions  
212 as factors. The second model considered the VOCs identified both after 1h and 24h sampling and  
213 addressed whether quantitative differences existed. This model included duration (1h/24h),  
214 experimental conditions (T0, T2, T12), and health status (CC, NC) and their interactions as factors.

215 Finally, the entire data set (56 samples corresponding to 3 replicates of each of 18 odour sources)  
216 obtained after 1h extraction was analysed using four RDA models to identify candidate cancer VOCs.  
217 In the two first models we addressed the impact of experimental condition (T0, T2 and T12) on VOCs  
218 composition for each health status separately. This model included experimental conditions as a fixed  
219 factor and replicates (3 per sample) and fiber identity (2 fibers used) as random factors (condition). The  
220 third model asked whether the VOCs composition of NC and CC at T0 was similar and included health  
221 status (NC/CC) as a fixed factor and replicates (3 per sample), and fiber identity (2 fibers used) as  
222 random factors (condition). The last model excluded the VOCs identified as different between NC and  
223 CC before the doxycycline treatment (at T0) and addressed whether the rest of the VOCs differed  
224 between health status, experimental conditions and their interactions (with replicate and fiber identity  
225 as random factors).

226 When relevant, we identified and listed the VOCs showing an absolute correlation coefficient  $> 0.8$  with  
227 the RDA constrained axis associated with significant effects in a given model.

228

## 229 3. Results

230

### 231 3.1 Consistency of VOCs sampling across fibers and impact of multiple samplings



232

233 We detected 89 compounds (**Table.S1**) in the technical controls (clean bedding). When compounds  
234 from this list were also detected in the biological samples at similar amounts (soiled bedding), they  
235 were not considered in the final list of compounds. The composition of the volatile profiles specific to  
236 the biological samples is given in **Table.1**. We identified the same 17 compounds with the two fibers  
237 (**Table.1**). Quantitative variation was assessed with a RDA analysis including the 17 compounds with  
238 “fiber identity” (2 modalities) and “sampling occasions” (7 modalities) as factors (model 1: Table 3).  
239 VOCs composition did not vary significantly with fiber identity ( $F= 0.957$ ,  $P = 0.395$ ), while it did  
240 between sampling occasions ( $F= 34.185$ ,  $P < 0.001$ ) (**model 1 : Table.2**). Almost all compounds,  
241 except two, showed variations in proportions over time (Figure S1). However, these variations were  
242 not linear and did not seem to relate to fiber wear. Although the same quantity of soiled bedding from  
243 the same odour source was analysed each time, variation in the quantity and quality of urine or faeces  
244 present in each aliquot might be the source of VOCs variation. These results further suggested that it  
245 might be important to use multiple replicates of every sample when comparing relatively small  
246 quantities (here 3g) of a complex substrate such as soiled bedding.

247

248 3.2 Influence of sampling duration on VOCs’ detection: 1h versus 24h

249

250 The same 17 compounds as in the first experiment were detected after 1h extraction and the 17 VOCs  
251 plus 11 others after 24h extraction (**Table.S2, Fig. 3**). A RDA was first performed to address if among  
252 the 11 VOCs only found after 24h extraction any or all were related to cancer tumour development. We  
253 designed a model considering as fixed factors experimental conditions (T0, T2, T12), health status (CC,  
254 NC) and their interaction (**model 2: Table.2**). VOCs detected only after a 24-h extraction did not differ  
255 between NC and CC nor interactively with experimental conditions. Variation in VOCs composition  
256 differed significantly only between experimental conditions ( $F=3.910$ ,  $P < 0.001$ ), pointing out  
257 significant differences in the VOCs profiles at T0 compared to T2 and T12 (**model 2 : Table.2**). We  
258 then analysed the 17 VOCs common to the 1h and 24h extractions, and tested the same model with an  
259 additional fixed factor, sampling duration, and its interactions with the other two factors mentioned  
260 above (**model 3 : Table 2**). The results indicated a significant influence of the three main factors, but  
261 no interaction between sampling duration and health status (**Fig.4**) further suggesting that 24h extraction  
262 was not necessary to address the impact of cancer on the mice VOCs profile.

263 3.3 Cancer influence on VOCs production

264

265 We did not detect qualitative differences in VOCs composition between CC and NC mice (**Table.3**). To  
266 address possible quantitative differences, we proceeded in several steps. First we tested the impact of  
267 experimental conditions within the CC and within the NC samples. The two RDA analyses comprising  
268 replicates (3 per sample) and fiber identity (2 fibers used) as random factors, and experimental  
269 conditions (T0, T2 and T12) as fixed factor, indicated that the random factors explained a relatively  
270 negligible part of the total variation (9.60 % and 2.60% respectively for NC and for CC). The  
271 experimental conditions explained 24.87 % (NC) and 57.85% (CC) of the total variance. The VOCs  
272 composition varied significantly between the three experimental conditions, both for NC and CC mice  
273 (NC:  $F = 4.174$  ; CC :  $F = 16.085$  ;  $P < 0.001$ ; post hoc tests all  $P < 0.001$ ; **model 4 & 5 : Table.2**),  
274 further indicating the influence of experimental conditions on the VOCs profiles of both NC and CC  
275 mice.

276 The second step consisted in evaluating the impact of cancer. To address this question, we first verified  
277 if the VOCs composition of CC and NC mice differed significantly before the start of the doxycycline  
278 diet, at T0, which was the case (**model 6 : Table.2**) indicating that the two genotypes had a different  
279 VOCs profile at the start of the experiment. Seven compounds had an absolute correlation  $> 0.8$  with  
280 the RDA main axes, and hence were identified to be involved in the differences between CC and NC at  
281 T0 (**Table. 3**). To further address the impact of cancer we removed these molecules from the data set  
282 and performed a RDA analysis including only the 10 VOCs that did not differ between CC and NC mice  
283 at T0 (**model 7 : Table.2 and Table.4**). The two random factors, replicates and fiber identity, accounted  
284 for a negligible part of the total variance, respectively, 3.32% and 1.05%. Both experimental conditions  
285 (T0, T2 and T12), health status (NC and CC) and their interaction were found to be significant (all  $P <$   
286  $0.005$ ; **Table.2**). As expected, we did not detect a significant difference in VOCs' composition between  
287 CC and NC mice at T0 ( $P=0.622$ )(**Table.4**). At T2 the two mice types (CC, NC) were also characterised  
288 by the same VOCs profile ( $P=0.495$ ) but different from T0 ( $P<0.001$ ), suggesting that at T2 only the  
289 doxycycline diet had altered the VOCs composition and that it influenced similarly CC and NC mice.  
290 The VOCs profiles of NC and CC were significantly different at T12 ( $P < 0.001$ ; **Table.4**), and the CC  
291 profile at T12 was significantly different from that of CC at T2 and from that of NC both at T2 and T12,  
292 indicating that lung adenocarcinoma development influenced the VOCs' composition of CC mice (**Fig.**  
293 **5**). The doxycycline treatment did not seem to have induced a change between T2 and T12 as far as NC  
294 mice were concerned (T2-T12 NC,  $P = 0.259$ ). However, our analyses pointed out 4 compounds  
295 differing between the VOCs profiles of CC at T2 and at T12. Three of these compounds, 3,4-dehydro-  
296 *exo*-brevicomine, 2-sec-butyl-4,5-dihydrothiazole and hexan-1-ol, were present in lower relative  
297 proportions in CC mice at T12 as compared to both NC and CC individuals at T2. The fourth compound,  
298 benzaldehyde, was present in higher relative proportions in CC at T12 (**Table.3**). Our analyses pointed  
299 out 4 other compounds impacted by the doxycycline treatment: the hexan-1-ol, 3-methyl-butan-1-ol,  
300 (*x*)-2,4,4-trimethyl-pent-2-enal and the fenchone. The hexan-1-ol and the 3-methyl-butan-1-ol were

301 either absent or present as a track before the start of the doxycycline diet (**Table.3**), while (x)-2,4,4-  
302 trimethyl-pent-2-enal disappeared from the VOCs profiles after tumour induction by doxycycline.  
303 Concerning the fenchone, it decreased over time in NC mice, while in CC mice it decreased at T2 and  
304 then increased again at T12 (**Table.3**).

305

#### 306 4. Discussion

307 Characterization of VOCs composition can quickly become tedious when one needs to analyze  
308 a large number of samples with a single SPME fiber, and when fiber exposure duration for each  
309 sample is long. However, when several fibers are used and extraction duration per sample is reduced,  
310 the repeatability of the results and the detectability of the molecules may be questioned. This paper  
311 addressed these two methodological issues and sought to identify VOCs present in mice soiled  
312 bedding that could be considered as cancer biomarkers.

313

##### 314 *Repeatability when using multiple SPME fibers.*

315 The use of several SPME fibers (with the same adsorption phase) to analyse a large number of  
316 samples might induce a bias due to inherent heterogeneity between fibers [34]. However, our results  
317 showed that, when all other conditions were controlled (duration, temperature etc.), we did not detect  
318 significant qualitative and/or quantitative difference in the VOCs profiles obtained by two distinct  
319 SPME fibers exposed simultaneously to the same source of odour. Our results also indicated that when  
320 two different fibers were used in a comparative study and fiber identity was included in the model as a  
321 random factor, the percentage of variation caused by this factor was marginal (**models 4 and 5 :**  
322 **Table.2**).

323 Unexpectedly, our results also indicated strong variation in the VOCs profile of the same source of  
324 odour sampled at several occasions. The variation in the relative proportions of the different VOCs  
325 was not linear and did not suggest an effect of fiber wear (between the first and the 45th use). A  
326 possible explanation of our results might be sample heterogeneity. Soiled bedding is a complex  
327 substrate, containing saw dust, food leftover, urine, faeces and other body secretions, difficult to  
328 homogenise. For the purpose of our study, we used pools of two-weeks old soiled bedding that we  
329 mixed as much as possible before sampling our aliquots (3g). Our results suggest that some of the  
330 aliquots used in this part of our study were not similar, further suggesting that to avoid such bias larger  
331 volumes than 3g should be used, and if not possible several replicates should be used to control for  
332 sampling heterogeneity, which we did to address cancer impact on VOCs (see below).

333

334 *Influence of sampling duration on detection of VOCs related to cancer.*

335 The release and adsorption of volatile compounds is dynamic [35–37]. Some molecules will be  
336 released immediately after exposure while others will not, this may be due to their chemical property  
337 and/or their state in the odour source (e.g. bound to urinary proteins [38,39] Low molecular weight  
338 VOCs will be quickly adsorbed by the SPME fiber while other molecules will take longer to be  
339 emitted and hence their detection could be delayed [20]. Increasing sampling duration can increase the  
340 number of molecules likely to be adsorbed. By increasing the duration of VOCs extraction to 24h, we  
341 were able to detect 11 additional molecules compared to the 1h sampling. Compounds detected only  
342 after a 24h sampling were mostly relatively heavy compounds that came out at the end of the  
343 chromatogram profile. The lower volatility of such molecules may explain why they were only  
344 observed in the 24h samples [40,41]. However, the increase in sampling duration did not necessarily  
345 result in an increase of the amount of compound trapped on the fiber for all compounds. For example,  
346 the relative proportions of some compounds such as 3,4 -dehydro-*exo*-brevicomine did not change  
347 between the 1h and 24h profiles while the proportion of thiazoline was higher in the 24h profiles  
348 compared to the 1h ones. This could be explained by the fact that while 3,4 -dehydro-*exo*-brevicomine  
349 is released during the first 20 minutes of sample exposure, thiazoline is emitted continuously for 24h  
350 [20]. Among the 11 molecules exclusively detected after 24h extraction hexanoic acid and (*E*)-  
351 nerolidol were described in mice urine that were heated at different temperatures between 45°C and  
352 65°C, enhancing their volatility [42–45]. Some of the other compounds such as heptanoic acid and p-  
353 cymen -8 -ol are derived from microbial communities [46] and their emission was facilitated by  
354 heating or longer exposure to oxidative degradation as it is the case when extraction time lasts 24h.

355 Nonetheless, among the 11 compounds detected exclusively after a 24h extraction, none differed  
356 between CC and NC mice, further indicating that 1h sampling should suffice to address cancer impact  
357 on mice VOC's profile (at least for such mouse model and such cancer type). Further, considering the  
358 cancer literature, none of the 11 molecules detected only in the 24h profiles was referenced as cancer  
359 biomarkers in mice. One compound was mentioned as a possible cancer biomarker in humans, the  
360 hexanoic acid, in gastric [47,48] and gastroesophageal cancer [49].

361

362 *Cancer influence on VOCs production.*

363 The use of soiled bedding allows to sample VOCs emitted by mice in their everyday activities in their  
364 cages, making the sampling non-invasive and non-stressful for the mice. It also allows collection of  
365 VOCs from a variety of sources simultaneously such as faeces, urine, saliva, preputial gland etc. Among  
366 the 17 compounds identified in this study, seven had not been reported in another publication on mice  
367 to our knowledge (**Table.3**). The other compounds, such as propanoic acid, hexan-1-ol or butanoic acid,  
368 were described before [44]. The propanoic acid was reported to be emitted from the body or from faeces,

369 while other compounds such as benzaldehyde was reported to be present in mice urine [43,44,50]. Some  
370 compounds that we found both in the control and in the biological samples, that we decided not to  
371 consider here, were reported as mouse compounds in the literature, such as acetic acid, pentan-1-ol,  
372 heptanal [44], nonanal [44,51] or again p-cymene [51].

373 Our results revealed significant quantitative differences in the VOCs profiles of the two mice lines (NC,  
374 CC) before doxycycline treatment (at T0). As these mice were kept in exactly the same conditions, and  
375 were all same age males and litter mates, the only difference that we see that could explain their different  
376 VOCs profile is their relatively slight different genetic background: presence versus lack of CCSP-rtTA  
377 and EGFR<sup>T790M/L858R</sup>. Small differences in genetic background were already shown to impact the VOCs  
378 profile of mice, [52–54]. Seven compounds differed between NC and CC at T0. Except for the 3-  
379 methylbutan-1-ol, these compounds were present in greater relative proportion in NC than in CC mice.  
380 Among the molecules described in the mouse VOCs literature, only the propanoic acid and the 3-  
381 methylbutan-1-ol were reported [55], the other four compounds, nopinone, camphor, camphene hydrate  
382 and (*E*)-3-pinocamphone are terpenes also found in plants.

383 Our study revealed the effect of both doxycycline and cancer tumour development on the mice VOCs  
384 profile. Doxycycline diet seems to influence VOCs emissions both in NC and CC mice. One compounds  
385 disappeared from the profile (e.g. (x)-2,4,4-trimethyl-pent-2-enal) while two other compounds were  
386 present in higher proportions after the addition of doxycycline, i.e. hexan-1-ol and 3-methylbutanol,  
387 which are compounds notably emitted by bacteria [56], indicating that the doxycycline treatment, by  
388 eliminating some bacteria, might have favoured others, impacting the mice gut microbiome composition  
389 and thus inducing a change in the VOCs emitted by the mice [57].

390 Our results indicated that the impact of cancer on VOCs composition was only detectable at T12, i.e.  
391 after 12 weeks of treatment when all mice harboured lung adenocarcinoma [25], and that this impact  
392 involved four compounds. Benzaldehyde was found in higher proportions in CC than NC mice at T12.  
393 It is a compound commonly found in mice urine [44,58–60], and was already reported in studies using  
394 mice to study cancer [61–63]. Benzaldehyde was also found in human cancers and might be considered  
395 as a relatively general biomarker of lung [64–67], colorectal [68] or breast cancer [69]. Most of the time  
396 this compound was studied in cell cultures and was found to increase in cancerous cells, here we show  
397 that cancer impact the whole body emission. Benzaldehyde was also found to be discriminating in  
398 studies on Alzheimer's disease [70]. The three other candidate VOCs identified in this study to be  
399 impacted by cancer development were found in smaller proportions in CC as compared to NC mice,  
400 namely, hexan-1-ol, 2-*sec*-butyl-4,5-dihydrothiazole (Thiazoline) and 3,4-dehydro-*exo*-brevicomine  
401 (Brevicomine). Of these three compounds, two are known mice pheromones described in male mice,  
402 brevicomine and thiazoline. These pheromones were shown to be involved in male dominance and male  
403 attractiveness to female mice [71]. Thiazoline was pointed as a candidate in another mouse lung cancer

404 study [72]. The onset of cancer therefore seems to impact pheromone emissions in sick mice, and hence  
405 is expected to impact their social life. The third candidate molecule is the hexan-1-ol also found in mice  
406 urine [44]. Like in our study, hexan-1-ol was found in smaller proportions in cancerous as compared to  
407 non-cancerous individuals in investigations of human lung cancer [63], melanoma [73] and head and  
408 neck cancer [74] Finally, among the compounds identified in similar proportions in both NC and CC  
409 sources in our study, the camphor was found to discriminate against colorectal cancer in mice [75], and  
410 the 3 methylbutanoic acid was found to discriminate melanoma [73].

## 411 5. Conclusion

412 We were able to show that when used under strict controlled conditions, the use of different SPME  
413 fibers may have a marginal impact on the variance observed between samples. The use of two fibers  
414 allowed us to perform a larger number of samples in a relatively short time. In addition, our study  
415 validated the use of mouse soiled bedding to extract VOCs from mice without being invasive. A  
416 longer sampling period may make it easier to identify compounds present in small amounts during a  
417 shorter sampling period. However, in our study, 1h extraction was sufficient to isolate the compounds  
418 that differentiated cancerous from non-cancerous mice. We did not detect cancer induced VOCs  
419 variation at an early stage of tumour development (T2), although behavioural investigations showed  
420 that mice discriminated between CC and NC soiled bedding at T2 [76]. The latter results suggest  
421 either, that our study method was not sensitive enough (compared to a mouse nose) to detect  
422 informative VOCs at T2, or most probably that other molecules, e.g. proteins, might be impacted by  
423 tumour development [77]. We identified four candidate compounds that discriminated CC from NC  
424 soiled bedding at T12: the benzaldehyde and the hexan-1-ol confirming earlier studies on mice and  
425 humans cancer, and two mice pheromones the Thiazoline and the Brevicomine, indicating that cancer  
426 could potentially impact social and sexual interactions in mice natural populations and, as such, might  
427 be selected against.

428

429

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728 COMPETING INTERESTS

729 The authors declare no competing financial interests.

730

731 AUTHOR CONTRIBUTIONS

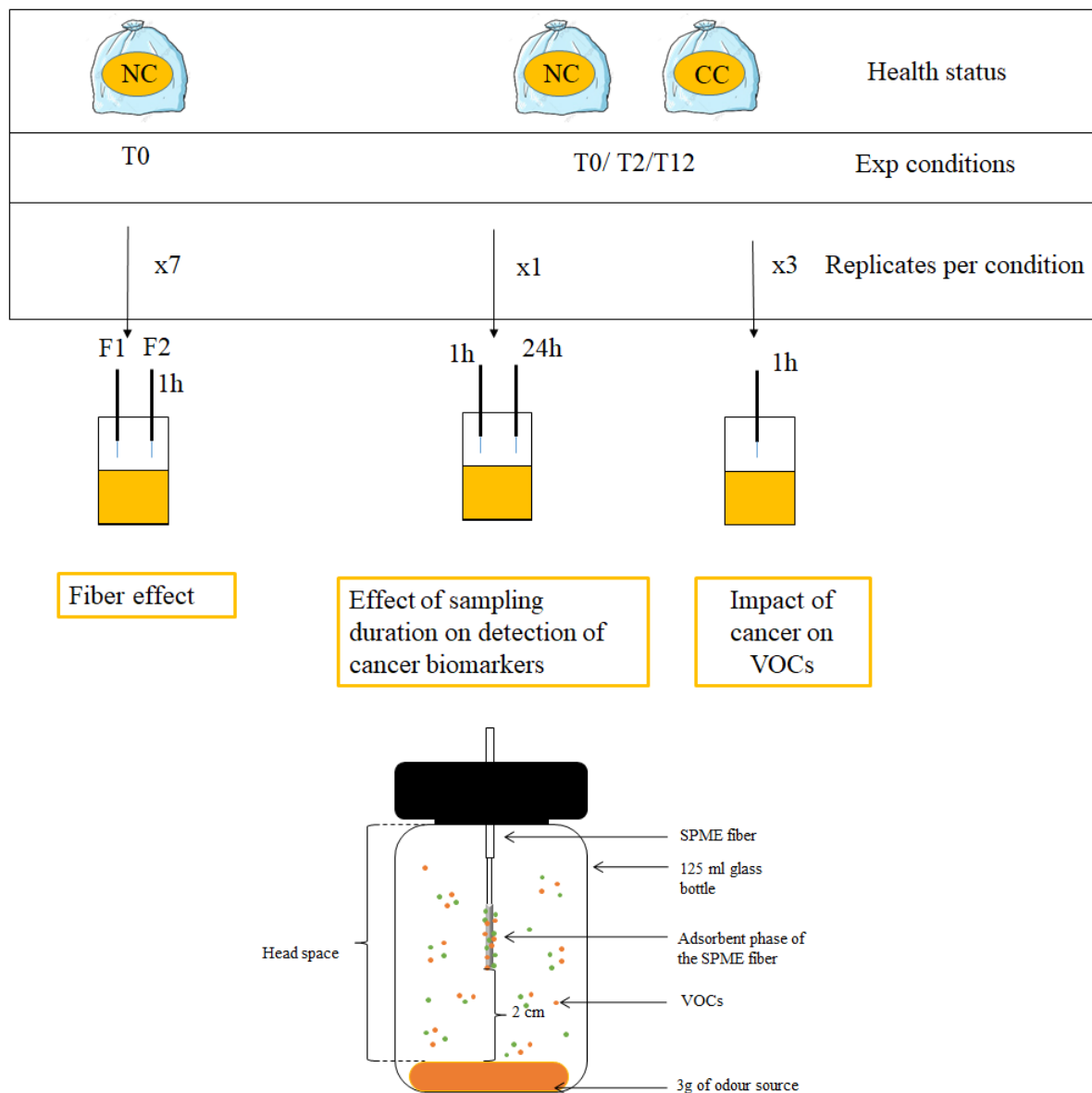
732 FG, GG, FT, LD developed the concept, FG realized the experiments, FG and GG performed the  
733 analytical measurements and analyzed the data with MH. BB supervised the chemical ecology aspects.  
734 MM and AM performed euthanasia of EGFR mice and verification of their cancer status. FG and GG  
735 prepared and edited the manuscript and all authors have reviewed the manuscript.

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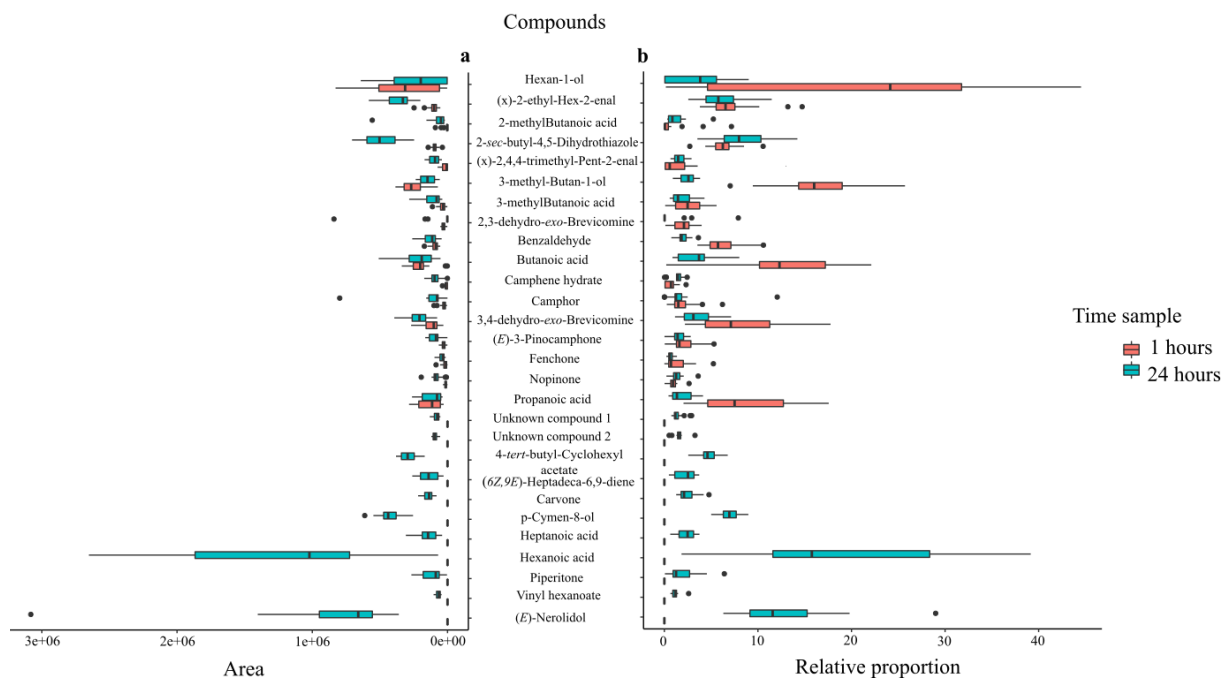
738 FIGURE

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740

741 **Fig. 1 : A schematic of the experimental protocol.** Each bag contained a mixture of bedding obtained  
742 from 3 to 4 mice, at different experimental conditions (T0: before treatment, T2: after 2-weeks  
743 doxycycline diet; and T12: after 12 weeks doxycycline diet). Three different types of tests were  
744 performed: - 1h VOCs sampling using two different SPME fibers exposed simultaneously to the head  
745 space and involving T0 non-cancerous (NC) soiled bedding (testing “Fiber effect”); - Two fibers  
746 exposed simultaneously, one for 1-h the other for 24h and involving soiled bedding of NC and CC  
747 (cancerous) soiled bedding sampled at T0, T2 and T12 (testing “Effect of sampling time”); and – A  
748 single fiber exposed for 1h and involving 3 replicates of the same source of NC and CC soiled bedding  
749 at T0, T2 and T12 (“Cancer influence”).

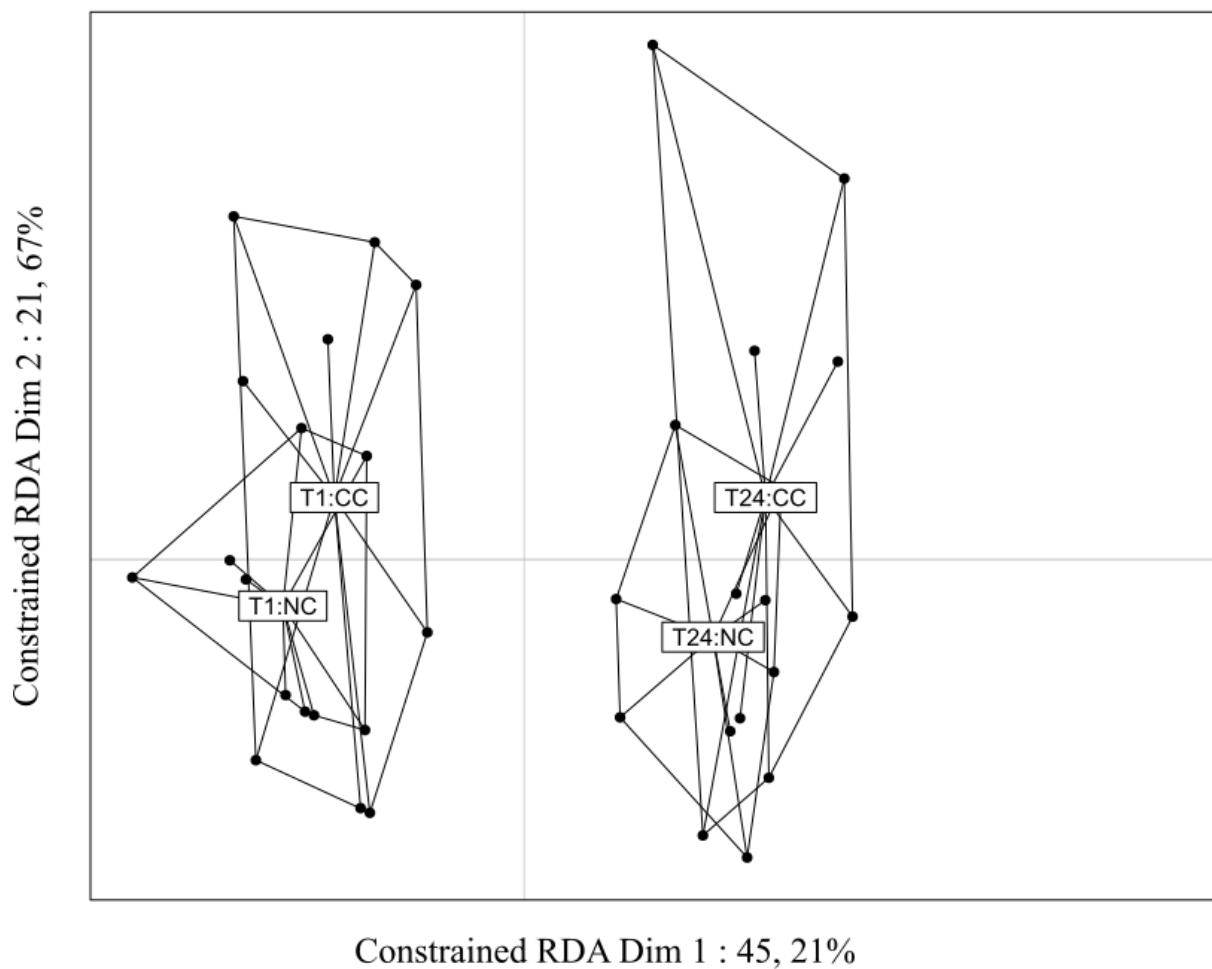


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752 **Fig. 2 :** Pic surface areas (a), and relative proportions (b) of compounds identified in mice soiled  
 753 bedding samples after 1h versus 24h SPME extractions.

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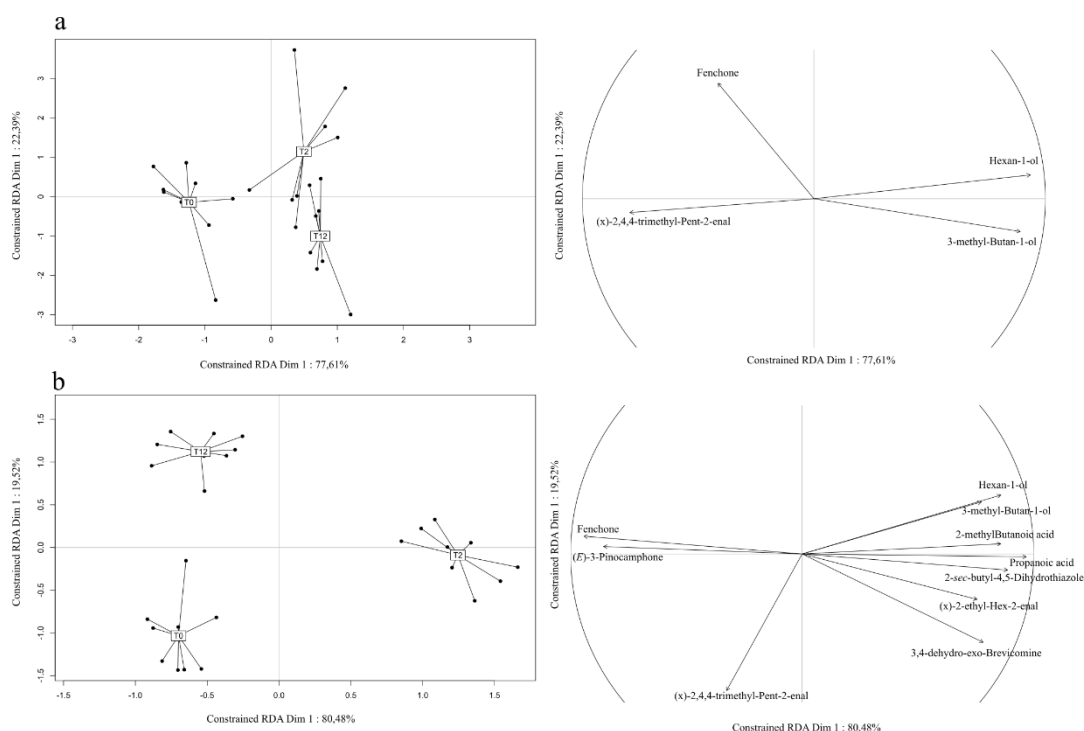


755

756 **Fig. 3** : Score plots of the redundancy analysis (RDA) comparing variation of 17 VOCs identified in  
 757 the 1h extraction profiles (T1) versus the 24h extraction profiles (T24) of non-cancerous (NC) and  
 758 cancerous (CC) mice soiled bedding.

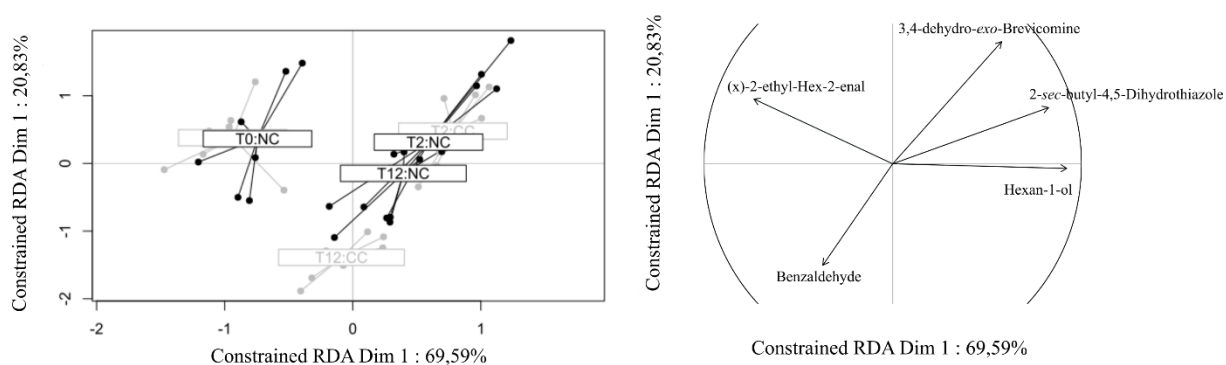
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 762 **Fig. 4** : Score plots of the redundancy analyses (RDAs) comparing variation of 17 VOCs identified  
 763 following 1h SPME extraction of non-cancerous soiled bedding (A) and cancerous soiled bedding (B)  
 764 at three experimental condition (T0: before treatment, T2: after 2 weeks doxycycline diet; and T12:  
 765 after 12 weeks doxycycline diet). Correlation circles represent the position of VOCs showing an  
 766 absolute correlation coefficient > 0.8.

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 771 **Fig. 5** : Score plots of the redundancy analysis (RDA) addressing variation of 10 VOCs present in the  
 772 profiles obtained after 1h extraction of NC (non-cancerous) and CC (cancerous) soiled bedding at  
 773 three experimental conditions; (T0: before treatment, T2: after 2 weeks doxycycline diet; and T12:  
 774 after 12 weeks doxycycline diet). The correlation circle identifies VOCs showing an absolute  
 775 correlation coefficient > 0.8.

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 777

778 **Table 1** : List of the 17 VOCs identified after simultaneous exposure of two SPME fibers to soiled  
 779 bedding of non-cancerous mice at T0. Only the components not present in the technical controls (clean  
 780 bedding) were retained in this list (Table S1).

781

Compounds	RT <sup>a</sup> (min)	RI <sup>b</sup>	F1	F2	F1 <sup>d</sup>	F2 <sup>d</sup>
			n=7 <sup>c</sup>		%	
Propanoic acid	4,31	707	7	7	12.8 ± 0.7	12.9 ± 0.6
3-methyl-Butan-1-ol	4,89	733	6	6	0.9±0.2	0.8± 0.1
Butanoic acid	6,47	802	7	7	24.1± 2.6	25.2± 2.3
(x)-2-ethyl-Hex-2-en-1-al*	6,68	809	7	7	10. 6± 0.7	9.9 ± 0.7
3-methylButanoic acid	7,98	853	7	7	8.5 ± 0.6	8.3± 0.6
2-methylButanoic acid	8,26	862	7	7	2.2 ± 0.1	2.1± 0.1
Hexan-1-ol	8,45	869	7	7	7.9± 0.3	7.5± 0.2
(x)-2,4,4-trimethyl-pent-2-en-1-al*	8,59	873	7	7	3.2 ± 0.3	3.5 ± 0.5
Benzaldehyde	11,19	956	5	5	2.4± 0.6	2.7± 0.7
2,3-dehydro- <i>exo</i> -Brevicomine*	13,8	1039	7	7	3.5 ± 0.4	3.9± 0.209
3,4-dehydro- <i>exo</i> -Brevicomine	14,06	1047	7	7	9.3± 0.3	9.1 ± 0.4
Fenchone	15,36	1089	2	2	0.5± 0.4	0.6± 0.4
2- <i>sec</i> -butyl-4,5-Dihydrothiazole	15,82	1103	7	7	6.9 ± 0.4	6.1± 0.5
Nopinone	16,79	1135	4	4	0.9± 0.3	1± 0.3
Camphor	17,06	1144	7	7	3.5± 0.2	3.5 ± 0.1
Camphene hydrate	17,25	1150	7	7	1.6 ± 0.1	1.6± 0.2
( <i>E</i> )-3-Pinocamphone	17,53	1160	6	6	1.4 ± 0.3	1.4 ± 0.3

782

783 \*: attempt of identification when the exact configuration could not be determined.

784 <sup>a</sup> retention time

785 <sup>b</sup> calculated retention indices

786 <sup>c</sup> Maximum number of occurrences.

787 <sup>d</sup> relative proportion of the compounds (% , mean +/- SE)

788

789

790 **Table 2** : Results of the permutation F tests based on redundancy analyses linking VOCs composition to health status (NC non-cancerous mice *versus* CC  
 791 potentially cancerous mice).

	random factors	variance explained	explanatory factors	variance explained	F	P
Model 1 : analysis of fibers F1 and F2			Fibers (F1/F2)	97,17%	0.957	0.395
			Time (7samples)		34.185	<0.001
Model 2 : analysis of 24h sample			Exp condition( T0,T2,T12)		2.82	<0,001
			Status (NC/CC)	45,70%	1.39	0.47
			Interactions		1.53	0.08
Model 3 : analysis of 1h and 24h sample			Time ( 1h/ 24h)		14.277	<0.001
			Exp condition(( T0,T2,T12)		3.91	<0.001
			Status (NC/CC)		2.037	0.0461
			Time : exp condition ( T0,T2,T12)	57.4%	0.769	0.734
			Time : status		1.075	0.356
			Exp condition : status		1.86	0.027
			Time :exp condition : status		0.939	0.512

Model 4 : analysis of difference of NC sample at 1h	Fibers + replicates	9.60%	NC	24.87%	4,174	<0,001
Model 5 : analysis of difference of CC sample at 1h	Fibers + replicates	2.60%	CC	57.85%	16,085	<0,001
Model 6 : analysis of NC and CC at T0	Fibers + replicates	10.20%	T0 : NC-CC	21.64%	4,174	0,002
Model 7 : analysis of NC and CC for 3 experimental condition	Fibers + replicates	4.30%	Exp condition (T0,T2,T12)		17,0065	
			Status (NC –CC)	46.31%	3,545	<0.001
			Exp condition : Status		2,23	

792

793 **Table 3** : Relative proportions (% , mean  $\pm$  se) of 17 VOCs identified in mice soiled bedding following 1-h SPME sampling, with reference to health status  
 794 (non-cancerous, NC, cancerous, CC) at three experimental conditions (T0, T2 and T12). VOCs present both in the biological and the control samples are not  
 795 listed here.

Compounds	T0		T2		T12		Reference	
	% CC	% NC	% CC	% NC	% CC	% NC	studies involving house mice	studies targeting cancer in house mice
Propanoic acid+	15.8 $\pm$ 0.7	19.8 $\pm$ 1.5	13.1 $\pm$ 1.2	14.3 $\pm$ 1.2	13.1 $\pm$ 0.5	9.3 $\pm$ 0.5	<b>Faeces</b> : [55] <b>Body</b> : [44]	
3-methyl-Butan-1-ol+	1.3 $\pm$ 0.2	0.2 $\pm$ 0.1	6.5 $\pm$ 0.9	2.9 $\pm$ 0.5	3.1 $\pm$ 0.3	3.7 $\pm$ 0.2	<b>Faeces</b> : [55]	
Butanoic acid	18 $\pm$ 4.3	15.2 $\pm$ 2.9	0.2 $\pm$ 0.03	3.5 $\pm$ 2.2	10.5 $\pm$ 3	5.7 $\pm$ 2.3	<b>Urine and Body</b> : [44]	
(x)-2-ethyl-Hex-2-en-1-al*	6.3 $\pm$ 0.8	6. $\pm$ 0.6	3 $\pm$ 0.4	3.8 $\pm$ 0.4	3.9 $\pm$ 0.4	4.7 $\pm$ 0.6		
3-methylButanoic acid	8.3 $\pm$ 1.2	8. $\pm$ 1.3	6.8 $\pm$ 1.4	4.8 $\pm$ 1.2	10.2 $\pm$ 1.4	3.8 $\pm$ 0.9	<b>Urine</b> : [44] <b>Urine</b> : [78]	
2-methylButanoic acid	1.7 $\pm$ 0.3	1.2 $\pm$ 0.3	2.6 $\pm$ 0.3	1.4 $\pm$ 0.3	2.3 $\pm$ 0.3	0.9 $\pm$ 0.1	<b>Urine</b> : [44]	
<b>Hexan-1-ol</b>	<b>10.8 <math>\pm</math> 4.4</b>	<b>6.2 <math>\pm</math>0.4</b>	<b>45.7<math>\pm</math>2.7</b>	<b>38.1<math>\pm</math>4.5</b>	<b>33. 9<math>\pm</math>3. 1</b>	<b>47.7<math>\pm</math>2.1</b>	<b>Body</b> : [44]	
(x)-2,4,4-trimethyl-Pent-2-en-1-al*	2 $\pm$ 0.5	1.1 $\pm$ 0.6	0	0	0	0		
<b>Benzaldehyde</b>	<b>1.4 <math>\pm</math>0.3</b>	<b>1.5<math>\pm</math>0.6</b>	<b>0.12<math>\pm</math>0.1</b>	<b>0.9<math>\pm</math>0.5</b>	<b>1.9<math>\pm</math>0.2</b>	<b>1<math>\pm</math>0.4</b>	<b>Urine and Body</b> : [44] <b>Urine</b> : [43,50,58,60,78–81]	<b>Breast cancer, urine</b> : [61] <b>Melanoma, urine</b> : [62] <b>Lung cancer, urine</b> : [63]
2,3-dehydro- <i>exo</i> -Brevicomine*	3 $\pm$ 0.5	2.9 $\pm$ 0.6	2.7 $\pm$ 0.7	0.7 $\pm$ 0.2	1.9 $\pm$ 0.3	2.2 $\pm$ 0.2	<b>Urine</b> : [42,44,82]	
<b>3,4-dehydro-<i>exo</i>-Brevicomine</b>	<b>15.5<math>\pm</math>2</b>	<b>11.6<math>\pm</math> 1.8</b>	<b>8.9<math>\pm</math>0.9</b>	<b>8.5<math>\pm</math>0.8</b>	<b>3.1 <math>\pm</math>0.3</b>	<b>5.5<math>\pm</math>0.4</b>	<b>Urine</b> : [43,50,78,79,81,83–85]	
Fenchone +	1.6 $\pm$ 0.2	4.5 $\pm$ 0.5	0	3.3 $\pm$ 0.6	2.1 $\pm$ 0.5	1.6 $\pm$ 0.2		

<b>2-sec-butyl-4,5-Dihydrothiazole</b>	<b>8.5 ±1</b>	<b>8.5±0.7</b>	<b>8.3±1.4</b>	<b>7.9±0.5</b>	<b>5.7±0.6</b>	<b>7.4±0.8</b>	<b>Urine :</b> [42–44,50,60,80–83,85,86] <b>Body :</b> [44]	<b>Lung cancer, urine :</b> [72]
Nopinone+	1. ±0.5	1.7± 0.2	0.8±0.3	1 ±0.2	1.5±0.4	0.9 ±0.2		
Camphor+	2.3 ±0.1	5.9 ± 0.5	0.9±0.3	3.7±0.6	3.5±0.7	2.8±0.2		<b>Colorectal cancer, blood:</b> [75]
Camphene hydrate+	0.7± 0.2	2.3± 0.3	0.2±0.1	1.2±0.3	1.5±0.3	1.3±0.1		
(E)-3-Pinocamphone+	1.9 ± 0.2	3.2± 0.2	0.2±0.2	2.1±0.3	1.9±0.4	1.5±0.3		

796

797 \*: attempt of identification; in some cases the configuration could not be determined.

798 +VOCs differing between CC and NC mice at T0

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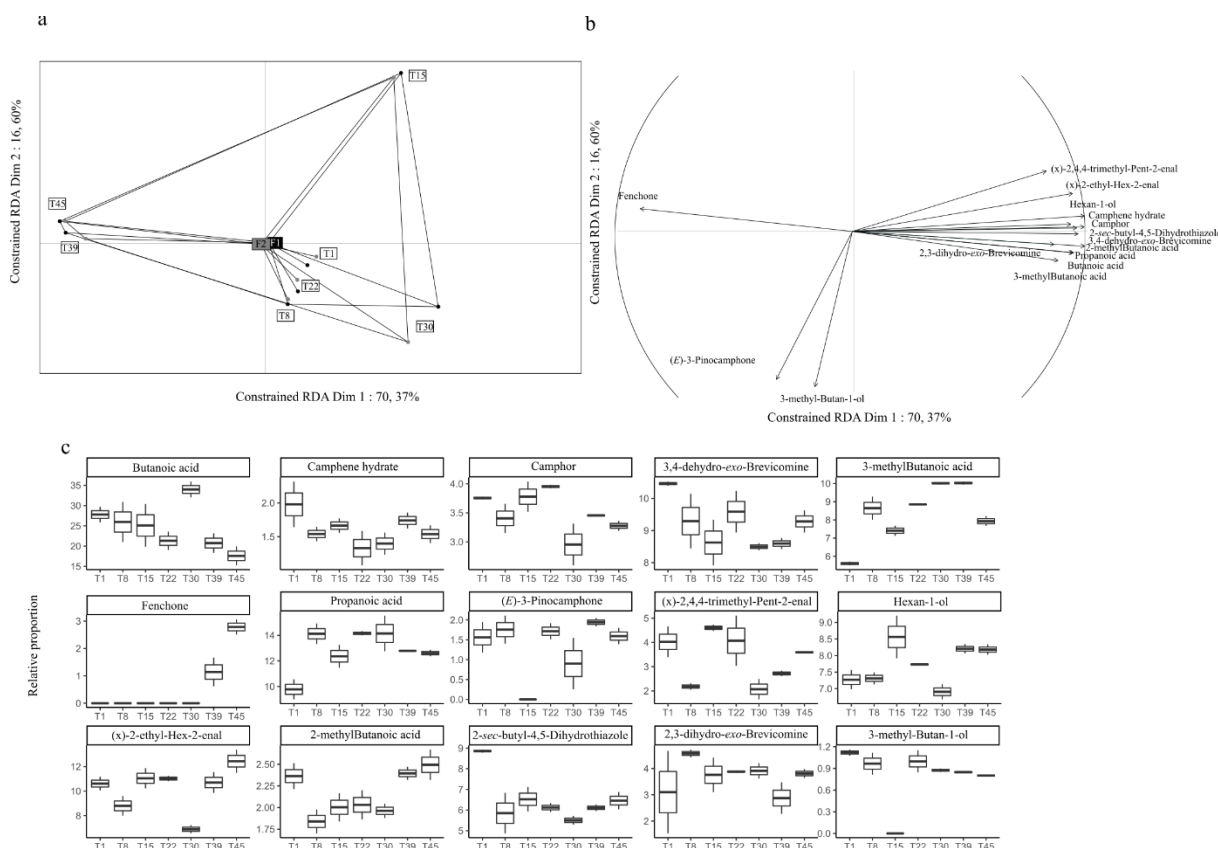
800 **Table 4** : Post-hoc tests following RDA model 7.

	T0_CC	T2_CC	T12_CC	T2_NC
T0_NC	0,622			< 0,001
T2_NC		0,4948		
T12_NC			< 0,001	0,2593
T2_CC	< 0,001			
T12_CC		< 0,001		

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**SUPPLEMENTARY MATERIALS :**

**Fig.S1** : Representation of a ) Score plots of the redundancy analysis (RDA) comparing variation of relative proportion of 17 volatile organic components sampled with fibers F1 and F2 at seven occasions; b) A correlation circle identifying compounds showing an absolute correlation coefficient > 0.8 and c) Variation of the relative proportion of the compounds pinpointed in the RDA analysis.



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815 **Table S1** : List of VOCs identified both in the biological samples and the controls. In blue compounds  
 816 found only in soiled bedding and retained for our analyses. In pink: the n-alkanes used as standards for  
 817 the identification of the compounds.

Compounds	RT <sup>a</sup>	RI <sup>b</sup>
n-Hexane	2,79	600
Acetic acid	2,83	603
Pentanal	4,05	692
n-Heptane	4,16	700
Propanoic acid	4,31	707
3-methylButan-1-ol	4,89	733
NI	4,98	737
Toluene	5,55	762
Pentan-1-ol	5,62	765
Hexanal	6,35	798
n-Octane	6,40	800
Butanoic acid	6,47	802
2-ethyl-Hex-2-enal	6,68	809
3-methylButanoic acid	7,98	853
ethylBenzene	8,12	858
2-methylButanoic acid	8,26	862
nXylène	8,38	866
Hexan-1-ol	8,45	869
(x)-2,4,4-trimethyl-Pent-2-enal	8,59	873
NI	8,90	884
Styrene	9,00	887
Pentanoic acid	9,19	893
Heptanal	9,30	897
n-Nonane	9,39	900
NI	9,50	903
$\alpha$ -Pinene*	10,48	934
Hept-2-enal	11,06	952
Benzaldehyde*	11,19	956
Heptan-1-ol	11,60	969
$\beta$ -Pinene*	11,84	977
Oct-1-en-3-ol*	11,88	978
6-methyl-Hept-5-en-2-one	12,03	983
2-pentylFuran	12,23	989
Hexanoic acid	12,44	996
Octanal	12,53	998
n-Decane	12,58	1000
$\delta$ -3-Carene	12,90	1010
<i>p</i> -Cymene*	13,32	1024
Limonene*	13,48	1029
NI	13,65	1034

2,3-dihydro- <i>exo</i> -Brevicomine	13,80	1039
6-methyl-Heptan-2-one	13,96	1044
3,4-dehydro- <i>exo</i> -Brevicomine	14,06	1047
Oct-2-enal	14,28	1054
vinylHexanoate	14,37	1057
Unknown compound 1	14,69	1067
Octan-1-ol	14,80	1071
Heptanoic acid	15,19	1083
Fenchone	15,36	1089
Cymenene	15,34	1088
n- Undecane	15,72	1100
Nonanal	15,73	1100
2- <i>sec</i> -butyl-4,5-Dihydrothiazole	15,82	1103
Fenchol	16,18	1115
NI	16,60	1129
Nopinone	16,79	1135
' <i>E</i> -Pinocarveol	16,95	1140
Camphor	17,06	1144
( <i>Z</i> )-Verbenol	17,13	1146
Camphene hydrate	17,25	1150
( <i>E</i> )-Non-2-enal	17,45	1157
( <i>E</i> )-3-Pinocamphone	17,53	1160
Pinocarvone	17,60	1162
Borneol	17,79	1168
( <i>Z</i> )-3-Pinocamphone	17,96	1174
4-Terpineol	18,12	1179
NI	18,19	1181
<i>p</i> -Cymen-8-ol	18,27	1184
$\alpha$ -Terpineol	18,37	1187
Unknown Monoterpene derivative 1	18,50	1191
Myrtenal	18,60	1195
Myrtenol	18,70	1198
n-Dodecane	18,76	1200
Decanal	18,80	1201
Verbenone	18,98	1208
2,4-Nonadienal	19,05	1210
Unknown compound 2	19,14	1213
Benzothiazole	19,40	1223
NI	19,63	1231
Cumin aldehyde	19,85	1238
Carvone	19,96	1242
Piperitone	20,27	1253
( <i>E</i> )-Dec-2-enal	20,43	1259
Undecan-2-one	21,31	1290
1-methyl-Naphthalene	21,43	1294

N,N-dibutyl-Formamide	21,60	1300
n-Tridecane	21,60	1300
NI	22,75	1343
$\alpha$ -Terpinyl acetate	22,94	1350
NI	23,22	1361
4- <i>tert</i> -butyl-Cyclohexyl acetate	23,53	1372
NI	23,76	1381
n-Tetradecane	24,27	1400
NI	24,57	1412
Geranyl acetone	25,53	1450
n-Pentadecane	26,81	1500
( <i>E</i> )-Nerolidol*	28,38	1572
n-Hexadecane	29,00	1600
Caryophyllene oxide	29,01	1600
Sesquiterpenol Inconnu	29,89	1641
(6 <i>Z</i> ,9 <i>E</i> )-Heptadeca-6,9-diene	30,60	1673
n-Heptadecane	31,13	1700

818 <sup>a</sup>Calculated retention indices

819 <sup>b</sup>Retention time

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821 **Table S2** : VOCs identified following 24h versus 1h extraction of soiled bedding. Only the components

822 not present in the technical controls (clean bedding) are listed here.

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Compounds	RT <sup>a</sup>	RI <sup>b</sup>	Frequency <sup>c</sup>		Relative proportion (%) <sup>d</sup>		Peak surface area <sup>e</sup>	
			1h	24h	1h	24h	1h	24h
Propanoic acid	4,31	707	18	18	8.8 ± 1.2	1.9 1 ± 0.3	138210±21251	113800±18072
3-methyl-Butanol	4,89	733	18	18	16.4 ± 1.1	2.4 ± 0.2	252880±20666	144730±14082
Butanoic acid	6,47	802	18	18	12.8 ± 1.4	3.6 ± 0.5	199270±20343	211310±29576
2-ethyl-Hex-2-enal	6,68	809	18	18	7.1 ± 0.7	6.1 ± 0.6	107310±11053	356570±26223
3-methylButanoic acid	7,98	853	18	18	2.5 ± 0.4	1.8 ± 0.3	38823± 6761	113740±18092
2-methylButanoic acid	8,26	862	18	18	0.8 ± 0.4	1.6 0.6	10771±5476	88525±29420
Hexan-1-ol	8,45	869	18	18	20.7 ± 3.6	3.5 ± 0.7	328740±65452	231950±51847
(x)-2,4,4-trimethyl-Pent-2-enal	8,59	873	18	18	1.5 ± 0.3	1.6 ± 0.2	21019±5820	98112±9508
Benzaldehyde	11,19	956	18	18	6.3 ± 0.5	2 ± 0.2	93406±7192	127530±14850
Hexanoic acid	12,44	996	/	18	/	19.2 ± 2.6	/	1243400±185210
2,3-dehydro- <i>exo</i> -Brevicomine*	13,8	1039	18	18	1.9± 0.3	0.7 ± 0.5	28640± 3800	65194±47046

3,4-dehydro- <i>exo</i> -Brevicomine	14,06	1047	18	18	8.06 ± 1.	3.6 ± 0.4	121530±16625	208840±19179
vinylHexanoate	14,37	1057	/	18	/	1.5 ± 0.1	/	69453±4453
Unknown compound 1	14,69	1067	/	18	/	1.4 ± 0.2	/	82456±5608
Heptanoic acid	15,19	1083	/	18	/	2.3 ± 0.2	/	144720±16808
Fenchone	15,36	1089	16	18	1.3 ± 0.3	/	21307±5286	41292 ± 4808
2- <i>sec</i> -butyl-4,5-Dihydrothiazole	15,82	1103	18	18	6.3 ± 0.4	8.4 ± 0.7	95078±6289	496870±32187
Nopinone	16,79	1135	18	18	1 ± 0.1	1.4 ± 0.2	14224± 1911	83731±9687
Camphor	17,06	1144	18	18	2 ± 0.3	1.9 ± 0.6	31815±5998	123530±41145
Camphene hydrate	17,25	1150	18	18	0.7 ± 0.2	1.7 ± 0.2		0.000145
( <i>E</i> )-3-Pinocamphone	17,53	1160	18	18	2. ± 0.4	1.4 ± 0.2	10905±2208	87533±10540
<i>p</i> -Cymen-8-ol	18,27	1184	/	18	/	7.1 ± 0.3	27784±4129	91493±12709
Unknown compound 2	19,14	1213	/	18	/	1.6 ± 0.1	/	433270±20811
Carvone	19,96	1242	/	18	/	2.4± 0.2	/	93592±4788
Piperitone	20,27	1253	/	18	/	1.9 ± 0.4	/	142380±9281
4- <i>tert</i> -butyl-Cyclohexyl acetate	23,53	1372	/	18	/	4.8 ± 0.3	/	111140±19920
( <i>E</i> )-nerolidol	28,38	1572	/	18	/	12.9 ± 1.3	/	290280±14621
(6 <i>Z</i> ,9 <i>E</i> )-Heptadeca-6,9-diene	30,6	1673	/	18	/	2.2 ± 0.3	/	845700±147880

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825 <sup>a</sup> retention time

826 <sup>b</sup> calculated retention indices

827 <sup>c</sup> frequency of occurrence of the compounds.

828 <sup>d</sup> relative proportion of the compounds (% , mean +/- SE)

829 <sup>e</sup> relative quantity of the compounds (mean +/- SE)

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