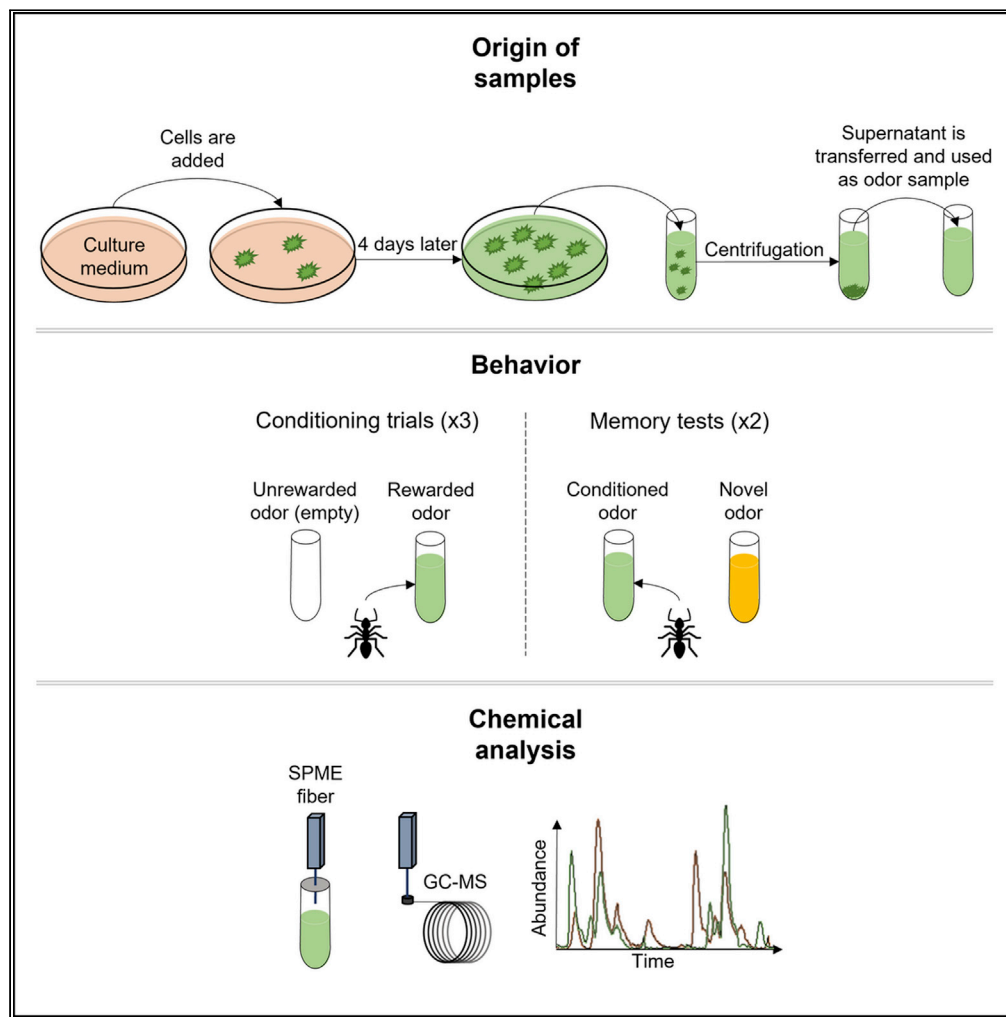


## Article

## Ants detect cancer cells through volatile organic compounds



Baptiste Piqueret,  
Brigitte  
Bourachot, Chloé  
Leroy, Paul  
Devienne, Fatima  
Mechta-Grigoriou,  
Patrizia d’Ettorre,  
Jean-Christophe  
Sandoz

baptiste.piqueret@univ-paris13.  
fr (B.P.)  
d-ettorre@univ-paris13.fr (P.d.)  
sandoz@egce.cnrs-gif.fr (J.-C.S.)

**Highlights**

Ants can rapidly be conditioned to associate the odor of cancer cells with a reward

Ants discriminate between cancerous and healthy cells and between two cancerous lines

Discrimination relies on volatile organic compounds that are specific of cell lines

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## Article

## Ants detect cancer cells through volatile organic compounds

Baptiste Piqueret,<sup>1,\*</sup> Brigitte Bourachot,<sup>2,3</sup> Chloé Leroy,<sup>1</sup> Paul Devienne,<sup>1</sup> Fatima Mechta-Grigoriou,<sup>2,3</sup> Patrizia d’Ettorre,<sup>1,4,6,7,\*</sup> and Jean-Christophe Sandoz<sup>5,6,7,\*</sup>

## SUMMARY

**Cancer is among the world’s leading causes of death. A critical challenge for public health is to develop a noninvasive, inexpensive, and efficient tool for early cancer detection. Cancer cells are characterized by an altered metabolism, producing unique patterns of volatile organic compounds (VOCs) that can be used as cancer biomarkers. Dogs can detect VOCs via olfactory associative learning, but training dogs is costly and time-consuming. Insects, such as ants, have a refined sense of smell and can be rapidly trained. We show that individual ants need only a few training trials to learn, memorize, and reliably detect the odor of human cancer cells. These performances rely on specific VOC patterns, as shown by gas chromatography/mass spectrometry. Our findings suggest that using ants as living tools to detect biomarkers of human cancer is feasible, fast, and less laborious than using other animals.**

## INTRODUCTION

Cancer cells possess specific features such as a deregulated cellular energetic metabolism, the ability to self-sustain themselves with proliferating signals or by exploiting tumor-promoting inflammation factors (Hanahan and Weinberg, 2000, 2011). Their metabolism produces volatile organic compounds (VOCs) that can act as biomarkers for cancer diagnosis using, for instance, gas chromatography or artificial olfactory systems (Krilaviciute et al., 2015; Lavra et al., 2015). However, the results of GC-MS analyses are extremely variable and most of the E-nose systems need to be optimized and are still at the prototype stage (Behera et al., 2019). Millions of years of evolution have shaped animals’ finely-tuned olfactory systems, which detect small odorant concentrations and have the computational power for discriminating among complex odorant blends. Dogs’ noses are well suited for medical diagnosis (Guest and Otto, 2020) and used for the detection of cancer-specific VOCs (Mazzola et al., 2020; Thuleau et al., 2019; Pirrone and Albertini, 2017; Schallschmidt et al., 2015), but training dogs in associative learning paradigms is expensive and time consuming (Pirrone and Albertini, 2017). The conditioning phase, in particular, takes several months and hundreds of trials are needed before the dog is operative. Consequently, studies report low sample sizes both in terms of individual dogs and numbers of tests performed. For instance, in one study, 90.3% of correct identification was achieved using two dogs, 5 months of training, and 1531 conditioning trials to perform 31 memory tests (Thuleau et al., 2019).

Compared to dogs, insects can be easily reared in controlled conditions, they are inexpensive, they have a very well-developed olfactory system (Rössler and Stengl, 2013), and hundreds of individuals can be conditioned with very few trials (Guerrieri et al., 2005; Piqueret et al., 2019; Giurfa and Sandoz, 2012). There is evidence that insects can detect VOCs from cancer cell lines. In fruit flies, for instance, the odors from different cancer cell lines evoked specific olfactory receptor activity patterns in the antenna, suggesting that such insects could be used as cancer biodetectors (Strauch et al., 2014) by employing *in vivo* calcium imaging, a complex and expensive technique. Here, we combined the use of insects (the ant *Formica fusca*) with low-cost, easily transferable, behavioral analysis to provide a robust, yet affordable, bio detector tool for cancer VOCs. We previously demonstrated that individual worker ants of this species quickly learn to associate an olfactory stimulus with a food reward and retain this information for an extensive period of time (several days) (Piqueret et al., 2019). In the present study, individual ants were trained to associate the odor of a cell sample with food reward, and later had to discriminate this learned sample against a new one. The principle is that of classical conditioning, the association of an unconditioned stimulus (US, in our case a reward consisting of sucrose solution) with an initially neutral stimulus (the odor of cancer

<sup>1</sup>Laboratoire d’Ethologie Expérimentale et Comparée UR 4443 (LEEC), Université Sorbonne Paris Nord, 93430 Villetaneuse, France

<sup>2</sup>Stress and Cancer Laboratory, Institut Curie, PSL Research University, Equipe labellisée Ligue Nationale Contre le Cancer, 26, rue d’Ulm, 75248 Paris Cedex 05, France

<sup>3</sup>Inserm, U830, Paris F-75248, France

<sup>4</sup>Institut Universitaire de France (IUF), Paris, France

<sup>5</sup>Evolution, Genomes, Behaviour and Ecology, CNRS, IRD, Université Paris-Saclay, 91190 Gif-sur-Yvette, France

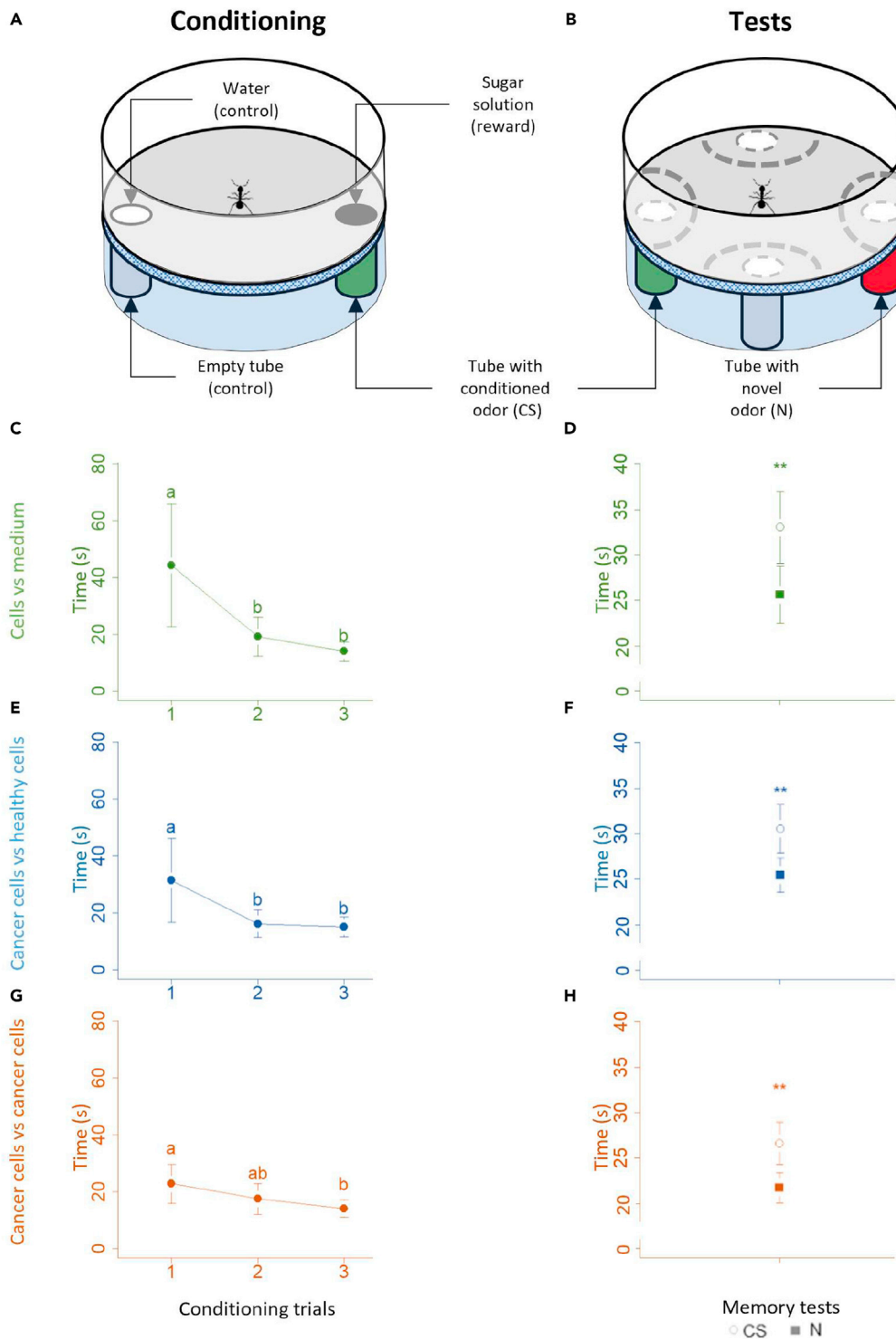
<sup>6</sup>These authors contributed equally

<sup>7</sup>Lead contact

\*Correspondence: baptiste.piqueret@univ-paris13.fr (B.P.), d-ettorre@univ-paris13.fr (P.d.), sandoz@egce.cnrs-gif.fr (J.-C.S.)

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**Figure 1. Behavioral setups and results of the conditioning experiments**

(A) Schema of the experimental arena used during the conditioning of ants. A reward was placed above a tube with the conditioned stimulus (CS), and we recorded the time needed by the ant to find the reward during three conditioning trials. (B) For the memory tests, we used a slightly different setup, where no reward was given, and two odors were present (the CS and a novel odor, N). The time spent by the ant in the vicinity of each odor area (dashed lines) as well as two unscented control areas, was recorded.

**Figure 1. Continued**

(C and D) Ants were conditioned to IGROV-1 (C, n = 36) and underwent memory tests (D).

(E and F) ants were conditioned (E) to MCF-7 cancer cells (n = 25) or MCF-10A healthy cells (n = 22), and tested with these samples (F).

(G and H) Ants were conditioned (G) to MCF-7 (n = 25) or MDA-MD-231 cancer cells (n = 24) and tested with these samples (H).

For the conditioning (C, E, and G), different letters indicate significant differences between trials (LMM,  $p < 0.05$ , after Bonferroni correction). For the memory tests (D, F, and H), open circles (CS area) and squares (N area) represent the mean time, whereas error bars show CIs (95%) of the two successive pooled memory tests. Significant differences between stimuli are indicated with asterisks (LMM, \*\*:  $p \leq 0.01$ ).

See also [Tables S1](#) and [S2](#); [Datas S1](#) and [S2](#).

cells) that becomes a conditioned stimulus (CS) producing the response in the absence of the US during an unrewarded discrimination test.

**RESULTS****Ants can detect cells through olfaction**

Individual ants (n = 36) were subjected to three training trials in a circular arena ([Figure 1A](#)), during which the odor of a human cancer cell sample (IGROV-1, ovarian cancer) cultured in medium (DMEM - Dulbecco modified Eagle's minimal essential medium) was associated with a reward of sugar solution. The time the ants needed to find the reward decreased over the trials ([Figure 1C](#) and [Table S1](#)), indicating that they had learned to detect the presence of cells based on their emitted volatiles. This was confirmed by ants performing two consecutive memory tests in which no reward was present. In a similar circular arena ([Figure 1B](#)), we measured the time spent by the ants investigating two different odors: the odor of the cells (IGROV-1) (conditioned stimulus) and the odor of the culture medium alone (DMEM) (novel odor). Two empty tubes were also present as controls ([Table S2](#)). During these memory tests, ants spent significantly more time near the conditioned odor (cancer cells) than near the culture medium alone ([Figure 1D](#)), demonstrating that ants can recognize the presence of cells in a sample.

**Discrimination between cancerous and healthy cells**

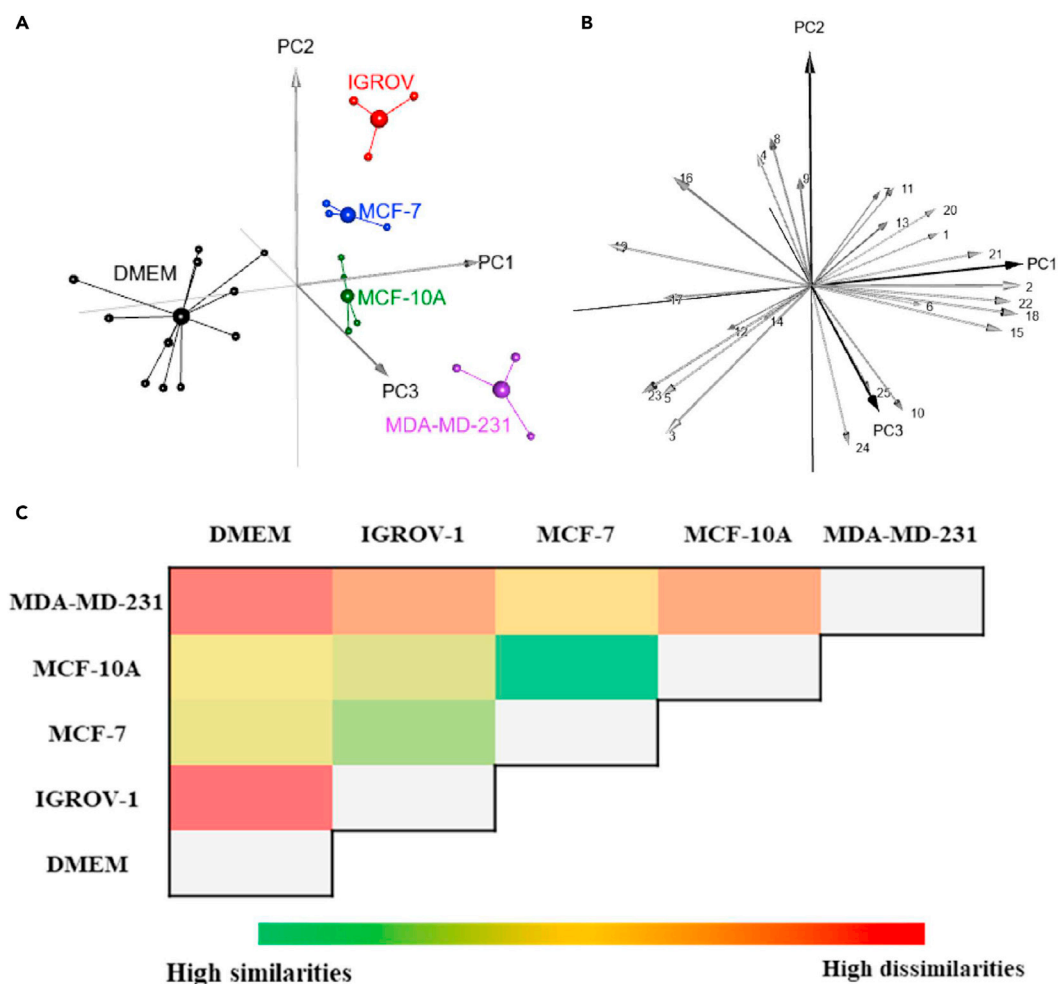
We next investigated whether ants could discriminate cancer cells from healthy ones by using two breast cell lines: an epithelium cancer cell line derived from adenocarcinoma breast cancer, MCF-7 (Luminal-A), and a non-transformed (healthy) breast cell line, MCF-10A. Ants were conditioned to the odor of either the cancer cell line (n = 25) or the healthy one (n = 22, [Figure 1E](#) and [Table S1](#)), and were tested in an arena where two odors were present. For ants conditioned to MCF-7 odor, MCF-10A served as the novel odor and vice versa. Ants spent significantly more time near the conditioned odor ([Figure 1F](#) and [Table S2](#)), demonstrating that they can discriminate a cancerous cell line from a healthy one, and exhibit this ability after a simple, 3-trial olfactory learning protocol.

**Discrimination of two cancerous lines**

Finally, we asked if ants can differentiate between two different cancerous cell lines: the MCF-7 cell line and the MDA-MD-231 breast cancer cell line, an epithelium cell line derived from an adenocarcinoma breast cancer. Contrary to MCF-7, MDA-MD-231 is not a luminal-A subtype, but a so-called triple-negative (TN) subtype. TN cancers are characterized by poorer diagnosis in patients ([Lefort et al., 2014](#)). Ants were either conditioned to the MCF-7 (n = 25) odor or the MDA-MD-231 odor (n = 24, [Figure 1G](#) and [Table S1](#)), and were tested in an arena with the conditioned odor and the novel odor (as above). Ants spent significantly more time near the conditioned odor than the novel one, showing that they can discriminate between two different cancer cell lines ([Figure 1H](#) and [Table S2](#)).

**Discrimination of cells based on VOCs**

To investigate the cues used by ants to discriminate the different cell lines from each other, we analyzed all the cell samples and the culture medium alone with Solid-Phase Micro-Extraction (SPME) coupled with Gas Chromatography and Mass Spectrometry (GC-MS). We found 25 VOCs present across all the samples ([Table S3](#)). The different cell lines as well as the culture medium were characterized by different VOC patterns, as shown in a principal component analysis ([Figures 2A](#) and [2B](#)). This clear differentiation was confirmed by a hierarchical cluster analysis ([Figure S3](#)).



**Figure 2. Principal component analysis and matrix of dissimilarities of cell sample VOC profiles**

(A) Plot of the first three Principal Components (PC), explaining 60.8% of the total variance. Cell line samples are well separated by the Principal Component Analysis (PCA).

(B) Variables used in the PCA showing the correlation between the first three PCs and the original variables. The angle between the vectors represents the correlation between the variables and the PCs. The length of each vector indicates how well the variable is represented in the plot, and consequently its contribution to the discrimination of cell types. Identification of the VOCs: (1) styrene, (2) oxime-, methoxy-phenyl, (3) benzaldehyde, (4) phenol, (5) aromatic compound, (6) decane, (7) 1-hexanol, 2-ethyl-, (8) benzyl-alcohol, (9) benzeneacetaldehyde, (10) hydrocarbon, (11) decane, 4-methyl-, (12) hydrocarbon, (13) acetophenone, (14) undecane, (15) hydrocarbon, (16) nonanal, (17) unidentified VOC, (18) dodecane, (19) decanal, (20) benzaldehyde, 3,4-dimethyl, (21) unidentified VOC, (22) benzene, 1,3-bis(1,1-dimethylethyl)-, (23) decanol, (24) unidentified VOC, (25) 2-undecanone.

(C) Euclidian distances were calculated using the first eight PCs of the PCA (which represent 90.4% of the total variance). Red indicates high dissimilarities, whereas green indicates high similarities between samples.

See also [Tables S3](#) and [S4](#); [Figures S3–S28](#); [Data S3–S5](#).

When quantifying dissimilarity between samples, the pair MCF-10A/MDA-MD-231 was the most dissimilar ([Figure 2C](#)), which could be explained by the difference in aggressiveness between these two types of cancer in the human body (TN/MDA-MD-231 cancers are more aggressive than luminal-A/MCF-10A).

By comparing the factor loadings showing a coefficient higher than 0.6 ([Table S3](#)) with the heatmap ([Figure S3](#)), we observed that styrene (compound 1), oxime, methoxy-phenyl (2), unidentified hydrocarbon (15), dodecane (18), unknown VOCs (21), and benzene, 1,3-bis(1,1-dimethylethyl)- (22) are all more abundant in cell lines than in the medium. On the contrary, benzaldehyde (3), unknown aromatic compound (5), unidentified VOC (17), decanal (19), and decanol (23) are more present in DMEM than in cells, which suggests that they are consumed by the cells. The second and third PCs differentiated among the different cell lines. Although the second PC discriminated MCF-7 and IGROV-1 from MDA-MD-231 and MCF-10A,

the third PC separated MCF-10A and MCF-7 from IGROV-1 and MDA-MD-231. Phenol (4), benzeneacetaldehyde (9), nonanal (16) were more abundant in MCF-7 and IGROV-1, whereas a hydrocarbon (10) and an unidentified VOC (24) were more present in MDA-MD-231 and MCF-10A. Benzyl alcohol (8) is found in higher relative proportions in MCF-7 and MCF-10A than in the other cell lines as indicated by the third PC.

## DISCUSSION

Using a simple conditioning protocol, we show here that *F. fusca* ants can detect the VOCs emitted by cancer cells. A conditioning protocol based on only three training trials was sufficient for ants to associate cell-derived VOCs with a reward. Ants were able to i) perceive the presence of cells in a medium, ii) differentiate cancerous VOCs from non-cancerous ones, and iii) differentiate between two cancerous samples based on VOCs. SPME and GC-MS analysis demonstrated that the different cell lines used in the behavioral study can be chemically characterized and discriminated from each other based on their VOCs.

*F. fusca* ants learn fast and retain a learned association after only three trials. Using ecologically relevant odors (floral, food, or cuticular ant hydrocarbons), such remarkable learning abilities were recently described in the same species (Piqueret et al., 2019) and in other ant species such as *Lasius niger* (Czaczkies and Kumar, 2020; Oberhauser et al., 2019), *Camponotus* spp. (Dupuy et al., 2006; Guerrieri and d'Ettorre, 2008; Josens et al., 2009), and *Linepithema humile* (Rossi et al., 2020). In this last study, the authors used the same conditioning protocol that we developed earlier (Piqueret et al., 2019), and used in the present study, confirming that this protocol yields robust datasets, and can also be used with other ant species. In all our experiments, ants were able to discriminate between the chemical samples, even when the task was potentially arduous (discrimination of two cancerous samples, Figure 2F).

The results of our chemical analyses, shown in a PCA (Figures 2A and 2B), where the first eight principal components explained 90.4% of the total variance (Table S3), provided support for the observed ants' behavior. Indeed, the pattern of VOCs can be used to discriminate one cell sample from another and from the medium alone in a multivariate analysis.

No single common cancer biomarker has ever been identified in the literature (Krilaviciute et al., 2015). As cancer is a complex disease, it is likely that each type of cancer (lung, breast, prostate, etc.) produces its own pattern of biomarkers, and not a single common molecule. The VOCs that we identified from cell samples were already found in several studies investigating potential cancer biomarkers (Lavra et al., 2015; Brooks et al., 2015; Silva et al., 2017; Bajtarevic et al., 2009; Hanai et al., 2012; Liu et al., 2019; Filipiak et al., 2014; Altomare et al., 2013; Amal et al., 2015). In particular, we identified styrene, oxime-methoxyphenyl, benzaldehyde, phenol, decane, 1-hexanol-2ethyl, acetophenone, nonanal, dodecane, and decanal. For example, in the MCF-7 cell line, styrene and dodecane were more present whereas benzaldehyde was less abundant compared to the control. MCF-7 also produced more phenol than the other breast cell lines. This is consistent with the study by Silva et al. (2017), which also focused on MDA-MD-231 cells and found that they expressed, compared to controls, more dodecane and less benzaldehyde, as in our study, but also less styrene, which is in contradiction with our results. However, benzaldehyde was found in lower quantity in MCF-7, MCF-10A, and MDA-MD-231 cells in the study by Lavra et al. (2015), which is in accordance with our results. In the IGROV-1 cell line, we found that styrene and dodecane were more present compared to the control. These VOCs were also found in higher abundance in the breath of patients with ovarian cancer (Amal et al., 2015). In the latter study, they also noted that decanal was less abundant in sick patients, and indeed we find that this compound is consumed by the cancer cells from the medium (Table S4).

Cancers are complex diseases, characterized by different subtypes within the same organ. In our study, we used two different breast cancer cell lines, with one being a luminal-A subtype (MCF-7), and another one (MDA-MD-231) being a triple-negative. These different subtypes differ in the expression of estrogen receptors, with in one hand an overexpression (luminal-A), and in the other, no expression (triple-negative). In addition, even when two studies focus on the same cancer subtype, the acquisition of data may differ, as no standard procedures were established yet. For example, using MCF-7 cell line, and SPME/GC-MS analysis, in one study (Silva et al., 2017), the SPME fiber was left in the headspace of the cell line for 45 min at 37 °C, and then injected into a GC for 10 min at 250 °C, whereas in a similar study (Liu et al., 2019), the fiber was left in the headspace for 30 min at the same temperature of 37 °C, but then injected into a GC at a higher temperature (270°C) for a shorter time (5 min). SPME/GC-MS tools are extremely

powerful for the identification of the VOCs composition of samples, but these methods are still lacking a proper standardization to be broadly used and reliable in real screening situations.

Dogs are the animals most commonly used as bio-detectors of cancer. They notably show high discrimination abilities. They were first tested using cell line samples, as we did with ants in the present study, but dogs were also submitted to body fluids odors, which are more complex (reviewed in [Pirrone and Albertini, 2017](#); [Brooks et al., 2015](#)).

One disadvantage of using dogs is that, despite being efficient, they are slow to learn (few months to year), and require an intensive learning protocol before being ready to discriminate cancer samples from a healthy one. To reduce this training time, one can observe directly if cancer samples elicit a specific response in the brain of the individual, instead of waiting for a behavioral modification. This method was tested with insects, as their brains are easily observable, they can reproduce rapidly, and at a very low cost. For this task, fruit flies were tested ([Strauch et al., 2014](#)). Odors from cancer cell lines were presented to restrained individuals and by using *in vivo* calcium imaging, the researchers were able to demonstrate that individuals were forming specific neuronal patterns for cancer samples that were different from healthy samples. This method was efficient, but we pinpoint two major disadvantages. First of all, individuals have to be sacrificed at the end of the procedure. Secondly, this method requires highly trained technicians and engineers to be performed, which limits the application in terms of money.

In the present study, we managed to combine the advantages of dog training and drosophila brain imaging, as well as limiting the disadvantages of both methods, by providing a protocol that is inexpensive, fast, easily performed, efficient, and does not require intensive academic training for trainers.

Ants are available in great numbers, and collectively choose the right odor with a very high probability ( $p < 0.01$  in all our experiments). Ants are thus equivalent to dogs — the most studied bio-detectors — in terms of detection abilities. In some respects, ants surpass dogs because they need an extremely shorter training time (30 min compared to 6–12 months for a dog) and a reduced cost of training and maintenance (honey and frozen insects twice a week). Our simple conditioning protocol can be implemented by everyone, after a training time of about 3-day (personal observation). Individual *F. fusca* ants can also be used more than once. In a previous study, we showed that with a single conditioning trial, ants could be tested up to nine times before response extinction ([Piqueret et al., 2019](#)). Compared to the already successfully tested insect species (drosophila), trained ants are as efficient, less expensive, and can be performed almost anywhere by anyone, thus representing a method with high potential for implementations at medical institutions.

Ants therefore represent a fast, efficient, inexpensive, and highly discriminant detection tool for detection of cancer cell volatiles. Our approach could potentially be adapted to a range of other complex odor detection tasks including the detection of narcotics, explosives, spoiled food, or other diseases (malaria, infections, diabetes for instance) ([Cambau and Poljak, 2019](#)). With regards to cancer detection, our research will now aim to widen the range of cancer-related odors that can be detected by ants, moving to the detection of body-emitted odors.

### Limitations of the study

Our study was performed on a single population of *F. fusca* ants. Although there are no reasons to believe that this population could be the only one to have developed refined olfactory abilities allowing the detection of human cancer volatiles, it would be of interest to test our protocol with individuals from other populations of *F. fusca*, which can be found in most of the northern hemisphere, as well as with other ant species, such as *Camponotus sp.*, *Linepithema sp.*, and *Lasius sp.*, which can efficiently learn simple and complex odors ([Perez et al., 2015](#); [Rossi et al., 2020](#); [Czaczkas and Kumar, 2020](#)). Concerning the chemical analysis, to date, no standard methods can be found in the literature. We used SPME and GC-MS analyses, as in other studies (e.g., [Lavra et al., 2015](#)) but other extraction methods could be tested and the results compared to propose a standard and efficient way to analyze the volatile emissions of cells. It is also important to consider that some of the VOCs identified in various studies could be contaminants coming from cell culture flasks ([Chu et al., 2020](#)). We did not use culture flasks in our study but we cannot exclude that some of the detected VOCs may still be contaminants. Ideally, the validity of the VOCs as biomarkers could be tested in behavioral experiments in which ants are trained with the full cell odor and tested only with a solution containing the potential biomarkers.

**STAR★METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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  - Data and code availability
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  - Behavioral experiments: conditioning
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- **QUANTIFICATION AND STATISTICAL ANALYSIS**
  - Behavioral experiments: conditioning
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  - Chemical analysis

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2022.103959>.

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**AUTHOR CONTRIBUTIONS**

B.P., P.d.E., and J-C.S. conceived the project and designed the experiments. The conditioning and memory test arenas were built by P.D. with inputs from B.P and B.B. F.M-G. and B.B. proposed the different cell lines, which were cultivated by B.B. and B.P. B.P. performed the behavioral experiments and analyzed the data with the help of P.d.E. and J-C.S., and F.M-G. discussed about experiments and results all along the study. C.L. oversaw the chemistry data acquisition. Chemistry data were analyzed by B.P. with help of C.L. and P.d.E. The manuscript was written by B.P. and revised by P.d.E., J-C.S., B.B., and F.M-G. Final manuscript was approved by all authors.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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## STAR★METHODS

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Biological samples</b>		
DMEM (Dulbecco modified Eagle's minimal essential medium)	GE Healthcare Hyclone	SH30243.01
Fetal bovine serum (FBS)	Biosera	#1003/500
Penicillin - streptomycin	Gibco	#15140122
<b>Chemicals, peptides, and recombinant proteins</b>		
decane	Sigma Aldrich, Saint-Louis, MO, USA	CAS 124-18-5
benzyl alcohol	Sigma Aldrich, Saint-Louis, MO, USA	CAS 100-51-6
Acetophenone	Sigma Aldrich, Saint-Louis, MO, USA	CAS 98-86-2
undecane	Sigma Aldrich, Saint-Louis, MO, USA	CAS 1120-21-4
nonanal	Sigma Aldrich, Saint-Louis, MO, USA	CAS 124-19-6
dodecane	Sigma Aldrich, Saint-Louis, MO, USA	CAS 112-40-3
decanal	Sigma Aldrich, Saint-Louis, MO, USA	CAS 112-31-2
3,4-dimethylbenzaldehyde	Sigma Aldrich, Saint-Louis, MO, USA	CAS 5973-71-7
benzaldehyde	Sigma Aldrich, Saint-Louis, MO, USA	CAS 100-52-7
2-Undecanone	Sigma Aldrich, Saint-Louis, MO, USA	CAS 112-12-9
styrene	Sigma Aldrich, Saint-Louis, MO, USA	CAS 100-42-5
benzene, 1,3-bis(1,1-dimethylethyl)	Sigma Aldrich, Saint-Louis, MO, USA	CAS 140431-85-2
decanol	Sigma Aldrich, Saint-Louis, MO, USA	CAS 112-30-1
<b>Experimental models: Cell lines</b>		
Human: IGROV-1	Curie Institute, Paris, France	RRID: CVCL_1304
Human: MCF-7	Curie Institute, Paris, France	RRID: CVCL_0031
Human: MCF-10A	Curie Institute, Paris, France	RRID: CVCL_0598
Human: MDA-MB-231	Curie Institute, Paris, France	RRID: CVCL_0062
<b>Experimental models: Organisms/strains</b>		
Ants: <i>Formica fusca</i>	Wild: Forest of Ermenonville (France, 49°09'51.5" N, 2°36'49.2" E)	N/A
<b>Software and algorithms</b>		
R software	R Core Team, 2020	<a href="https://www.r-project.org/">https://www.r-project.org/</a>
MSD ChemStation software version E.02.01.1177	Agilent Technologies	<a href="https://www.agilent.com/">https://www.agilent.com/</a>
NIST library	NIST	<a href="https://www.nist.gov/">https://www.nist.gov/</a>
Ethoc software	CRCA	<a href="https://crca.cbi-toulouse.fr/">https://crca.cbi-toulouse.fr/</a>
<b>Other</b>		
SPME fiber (50/30 μm DVB/CAR/PDMS)	Supelco	<a href="https://www.sigmaaldrich.com/FR/fr/product/supelco/57550u">https://www.sigmaaldrich.com/FR/fr/product/supelco/57550u</a>
Agilent Technologies 7890A gas-chromatograph	Agilent Technologies, Les Ulis Cedex, France	<a href="https://www.agilent.com/cs/library/usermanuals/Public/G3430-90011.pdf">https://www.agilent.com/cs/library/usermanuals/Public/G3430-90011.pdf</a>

## RESOURCE AVAILABILITY

## Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Patrizia d'Ettorre ([d-ettorre@univ-paris13.fr](mailto:d-ettorre@univ-paris13.fr)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

- The published article and [supplemental information](#) include all data generated and analyzed during this study. This paper does not report original code.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Insects and origin of colonies

*Formica fusca* is a common ant species found in the Northern Hemisphere. Colonies comprise one queen (monogynous) or several queens (polygynous) and contain several hundred individuals. Fifteen queenright colonies were collected in the forest of Ermenonville (France, 49°09'51.5" N, 2°36'49.2" E) and kept under laboratory conditions ( $25 \pm 2$  °C,  $50 \pm 10\%$  relative humidity, natural day/night cycle) at the Laboratory of Experimental and Comparative Ethology (LEEC, University Sorbonne Paris Nord). Colonies were collected in 2015 (n = 3), 2017 (n = 4), 2018 (n = 1), 2019 (n = 4) and 2020 (n = 3). Tested ants were foragers (ants that leave the nest to search for food) and were individually marked with a dot on the abdomen or thorax using oil-based paint (Mitsubishi Pencil) the day before the experiment. Each ant was used only once, undergoing one conditioning phase and one testing phase, and then put back in their colony.

### Cell cultures

Ovarian cancer (OC), breast cancer (BC) and immortalized (non-tumorigenic) breast cell lines were cultivated at the Curie Institute ('Stress & Cancer Lab', Paris, France). Four human epithelium cancer cell lines were derived from adenocarcinoma ovarian or breast cancers: IGROV-1 (ovarian cancer), MCF-7 (breast cancer, Luminal-A), MDA-MD-231 (breast cancer, triple-negative) and MCF-10A (non-transformed breast cells). The identity of each cell line was checked by Short Tandem Repeat (STR) DNA profiling (Promega, #B9510) and tested for absence of mycoplasma contamination. Cells were propagated in DMEM (Dulbecco modified Eagle's minimal essential medium - GE Healthcare Hyclone SH30243.01) supplemented with 10% fetal bovine serum (FBS - Biosera, #1003/500), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Gibco #15140122). Cells were placed in an incubator at 37 °C and 5% CO<sub>2</sub>. The medium was renewed twice a week.

Cells were cultivated in Petri-dishes with 10 mL of DMEM for the propagation. Before the medium collection, 0.8 to 1 million cells (depending on the cell line) were plated in 10 cm dishes. After four days, the medium was transferred to falcon tubes and then centrifugated (5 min, 1200 rpm, at RT). The supernatant (not containing any cells) was transferred to 4 mL and 15 mL glass vials for the behavioral experiments and chemical analysis respectively. All the samples were frozen at -20°C before being used. A preliminary experiment was performed with fresh cells to prove the feasibility of using cells and ants' olfaction and learning abilities (details in [Supplementary information](#), [Figures S1](#) and [S2](#)).

## METHOD DETAILS

### Behavioral experiments: conditioning

We used olfactory conditioning, in which a single initially neutral odorant (CS – conditioned stimulus) is associated with a reward (US – unconditioned stimulus). Ants were individually placed in a circular arena ( $\varnothing = 12$  cm, height = 3.5 cm) with clean filter paper at the bottom ([Figure 1A](#)). The arena had two holes at the ground level (10 cm between the holes). Two glass vials (4 mL, Supelco, Bellefonte, PA, USA) were each placed below one of the holes with the opening toward the arena. One vial was filled with 4 mL of the supernatant (extracted as explained above, representing the CS), whereas the other was empty. As cells were cultivated at 37°C, they were kept at this temperature throughout the experiment by inserting the vials in a water bath (1.8 L, diameter = 15.5 cm × 14 cm, height = 10 cm, temperature sensitivity of 0.2°C at 37°C, Edvotek, Washington D.C., USA) placed just below the arena. To avoid any effect of heat on ants' learning and memory performances, polystyrene foil (insulating material) was placed between the water bath and the arena. The portion of filter paper above the vials was pierced with an entomological pin to allow natural diffusion of the odor. Small plastic discs ( $\varnothing = 6$  mm) were placed above each hole and received a 1 µL drop of sugar solution (30% w/w) for the CS odor and of distilled water for the other, unscented vial ([Figure 1A](#)).

Due to the presence of these two drops of liquid, the two stimuli were visually indistinguishable. During each conditioning trial, we recorded the time needed by the ant to find the sugar solution (US). The ant was allowed to drink the drop of sugar solution and was then returned to the colony, where it could perform trophallaxis (mouth to mouth exchange of liquid food) with her nestmates. Without trophallaxis, the crop of tested ants would be full in only a few conditioning trials and the ants would not be motivated to find more food. Tested ants were left for about 3 min in the colony (inter-trial interval), during which they terminated trophallaxis and came back spontaneously to the foraging arena, where they were picked up for the next training trial (Piqueret et al., 2019). During this interval, the filter paper at the bottom of the arena and the plastic discs were replaced with clean ones to remove any possible chemical cues left by the ant at the previous trial. The orientation of the arena and the position of the experimenter were also modified between trials to limit the possible use of visual or other spatial cues. Each ant underwent three consecutive conditioning trials.

### Behavioral experiments: memory tests

To test whether ants have learned that the CS is a predictor for reward, we performed memory tests in which the reward was absent. For this unrewarded memory test, four glass vials were used, which were inserted on the four cardinal points of the arena (Figure 1B). One vial contained the CS odor and, on the opposite side, a second vial contained a novel odor (N). On the two vacant positions, the additional glass vials were empty and acted as controls. Empty plastic discs ( $\varnothing = 6$  mm) were placed above the glass vials and circular areas ( $\varnothing = 5.5$  cm) were drawn around each plastic disc, allowing us to record the time spent by the ant in the vicinity of each stimulus for 2 min. Ants underwent two consecutive unrewarded memory tests 15 and 20 min after the end of the last conditioning trial.

Ants' behavior was scored using Ethoc software (CRCA), a behavioral transcription tool. All experiments were also video recorded with a camera (Canon, Legria HFR806) placed above the experimental arena, allowing later inspection and quantification.

### Chemical analysis

VOCs emitted by the supernatant of all cultured cell lines used for behavioral experiments were determined using chemical analysis. Cell metabolism produces compounds that can be found in the culture medium. Cells of different origins do not consume and expel the same compounds, thus producing a unique pattern of VOCs. For all conditions, we used the medium that had previously contained the cells as source of VOCs. In the case of the DMEM, the medium was incubated in the same conditions but not in contact with any cells. For IGROV-1, MCF-7, MDA-MD-231 and MCF-10A, the medium was in contact with these cell types for four days (see cell cultures for details). For each sample, a 15 mL glass vial was filled with 10 mL of the supernatant (or clean medium in the case of the DMEM analysis) and placed at 37 °C using a water bath. A SPME fiber (50/30  $\mu$ m DVB/CAR/PDMS, Supelco) was introduced through the PTFE/silicone 1.5 mm cap for 50 min (Hanai et al., 2012). After that, the fiber was immediately inserted into an Agilent Technologies 7890A gas-chromatograph, equipped with a HP-5MS GC column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m, Agilent Technologies, Les Ulis Cedex, France). The carrier gas was helium (1 mL.min<sup>-1</sup>), and the injection was splitless (250 °C). The oven temperature was programmed at 40°C for 5 min, then increased to 220°C at 7°C min<sup>-1</sup>, and then to 300°C at 15°C min<sup>-1</sup> and held for 3 min. The GC was coupled with a 5975C mass-spectrometer (Agilent Technologies). Mass spectra were recorded with electron impact ionization at 70 eV. Peak areas were integrated with MSD ChemStation software version E.02.01.1177 (Agilent Technologies). Peaks were identified by comparing their ion spectrum to the NIST library (NIST v2.2, 2014) and to standards injected with the same temperature program (decane, benzyl alcohol, acetophenone, undecane, nonanal, dodecane, decanal, benzene, 1,3-bis(1,1-dimethylethyl), decanol, benzaldehyde, styrene, 3,4 dimethylbenzaldehyde, and 2-undecanone, all from Sigma Aldrich, Saint-Louis, MO, USA). We found high consistency between the spectra of the standards and those of the compounds extracted from our cell samples (Figures S4–S28).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Behavioral experiments: conditioning

Data were analyzed using R software (R Core Team, 2020). Significance was fixed at  $\alpha = 5\%$ . All data were analyzed using linear mixed models (LMM, package 'lme4', Bates et al., 2015). The identity of individuals and of the colony were included as nested random factors.

We analyzed the effect of the number of conditioning trials (named trials) on the dependent variable time (continuous variable, the time to find the reward). For the experiment in which several odors were used for training, we analyzed the effect of the conditioning odor (factor with two levels, MCF-7 vs MCF-10A or MCF-7 vs MDA-MD-231). We also looked at the interaction conditioning odor x trials to detect possible differences in the course of ants' acquisition performances depending on the odor stimulus used. On the plots, different letters indicate significant differences between trials (LMM,  $p < 0.05$ , after Bonferroni correction).

### Behavioral experiments: memory tests

First, we checked whether ants spent more time near the vials presenting odors or near the control unscented vials, by analyzing the effect of the independent variable presence of odor (factor with two levels, Yes or No) on the dependent variable time (continuous variable, the time spent near the odors or near the unscented vials). Then, in all experiments, we analyzed the effect of the independent variable stimulus (factor with two levels, CS or N) on the dependent variable time (continuous variable, the time spent in the vicinity of an odor) during the memory tests. Finally, using data subsets, we also analyzed the first and the second memory test in each experiment. Significant differences between stimuli are indicated with asterisks (\*\*:  $p \leq 0.01$ ).

### Chemical analysis

Contaminants (silicate-derived molecules originating from the GC column) were discarded from the analysis. The areas of 25 regularly occurring peaks were standardized by calculating the  $\ln(P_i/g(P))$  (Aitchison, 1982), where  $P_i$  is the area of a peak and  $g(P)$  is the geometric mean of all the peak areas of the sample. We then reduced the number of variables by running a principal component analysis (PCA) on the standardized peak areas and retained the first eight principal components (PCs) to calculate the Euclidean distance between samples (Figure 2C). The VOCs with (positive or negative) factor loading higher than 0.6 on one of the principal components contribute to the discrimination between medium alone and cell lines, and among different cell lines (Tables S3 and S4). Therefore, they are possible candidates as biomarkers.

The PCs variables were also used to construct a heatmap (Figure S3). Combined with that heatmap, the standardized area values of the 25 peaks were used in a Hierarchical Cluster Analysis using Ward's classification method to classify cell samples. The significance ( $p < 0.05$ ) of each node in the cluster was determined by multiscale bootstrap clustering with 10,000 iterations using the 'pvclust' package (Suzuki et al., 2019). The results are visualized as a heat map, where positive PCA scores are in blue, and negative ones are in red. In this analysis, the node separating MCF-7 and MCF-10A samples was significant ( $p < 0.05$ ), as were the nodes grouping MCF-7 samples on the one hand, and MCF-10A samples on the other. The IGROV-1, MDA-MD-231 and corresponding medium (DMEM) samples were each clustered in different groups with well-supported nodes ( $p$ -value  $< 0.1$ ). All the individual samples ( $n = 24$  in total;  $n = 11$  for DMEM,  $n = 3$  for MDA-MD-231, IGROV-1 and MCF-7, and  $n = 4$  for MCF-10A) were correctly clustered, showing their distinctive VOC compositions.

The relative abundance of each peak is also displayed in Table S4.