Title

Identification of fungal metabolites from inside Gallus gallus domesticus eggshells by non-invasively detecting volatile organic compounds (VOCs)

Journal

Analytical and Bioanalytical Chemistry, 408(24)

ISSN

1618-2642

Authors

Cumeras, Raquel
Aksenov, Alexander A
Pasamontes, Alberto
et al.

Publication Date

2016-09-01

DOI

10.1007/s00216-016-9778-3

Peer reviewed

HHS Public Access

Author manuscript

Anal Bioanal Chem. Author manuscript; available in PMC 2017 September 01.

Published in final edited form as:

Anal Bioanal Chem. 2016 September ; 408(24): 6649–6658. doi:10.1007/s00216-016-9778-3.

Identification of fungal metabolites from inside *Gallus gallus domesticus* eggshells by non-invasively detecting volatile organic compounds (VOCs)

Raquel Cumeras[1], **Alexander A. Aksenov**[1], **Alberto Pasamontes**[1], **Alexander G. Fung**[1], **Amanda N. Cianchetta**[2], **Hung Doan**[2], **R. Michael Davis**[2], and **Cristina E. Davis**[1,3,*]

[1]Department of Mechanical and Aerospace Engineering, University of California, Davis, One Shields Avenue, California 95616, USA

[2]Department of Plant Pathology, University of California, Davis, One Shields Avenue, California 95616, USA

[3]Center for Nano and Micro Manufacturing (CNM2), University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

Abstract

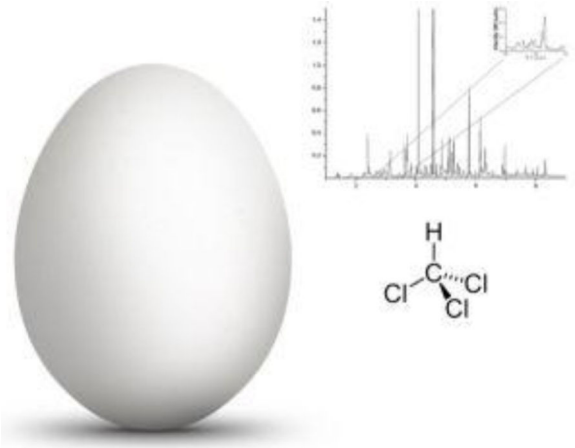
The natural porosity of eggshells allows hen eggs to become contaminated with microbes from the nesting material and environment. Those microorganisms can later proliferate due to the humid ambient conditions while stored in refrigerators, causing a potential health hazards to the consumer. The microbes' volatile organic compounds (mVOCs) are released by both fungi and bacteria. We studied mVOCs produced by aging-eggs likely contaminated by fungi and fresh-eggs using the non-invasive detection method of gas-phase sampling of volatiles followed by gas chromatography/mass spectrometry (GC/MS) analysis. Two different fungal species (*Cladosporium macrocarpum* and *Botrytis cinerea*) and two different bacteria species (*Stenotrophomas rhizophila* and *Pseudomonas argentinensis*) were identified inside the studied eggs. Two compounds believed to originate from the fungi itself were identified. One fungus-specific compound was found in both egg and the fungi: trichloromethane.

Graphical abstract

*Corresponding author cedavis@ucdavis.edu.

Conflict of interest

The authors declare that they have no competing interests.



Keywords

volatile organic compounds (VOCs); fungi; bacteria; hen egg; gas chromatography / mass spectrometry (GC/MS); solid phase microextraction (SPME)

INTRODUCTION

Eggs are a nutritious and inexpensive food and are an important part of the human diet worldwide (1). Though the eggs of other avian species are popular in many countries, chicken (*Gallus gallus domesticus*) eggs are the focus of the United States poultry industry (2). Eggs offer high-quality protein including all nine essential amino acids and numerous essential nutrients in an economical energy package. One large egg provides only 3% of the Daily Value (DV) for energy, but 10% of the DV for protein. Thus, eggs can play a key role in a well-balanced diet (3), and are also a good source of many vitamins and minerals.

The physical structure of an egg is comprised of the yolk, albumen, shell membranes, and shell (4). Microbes are prevented from entering an egg by the cuticle, hard outer shell, inner shell membrane, and outer shell membrane (5). The cuticle itself is a 10 μm thick layer of protein and carbohydrates that coats the outside of the shell, and it provides protection by increasing shell strength and preventing the passage of materials through the shell pores. Other enzymes are also present in the egg white and help defend against pathogens that may infect the developing embryo.

Each shell has approximately 7,000–17,000 pores, whose diameter normally ranges from 9–35μm. The vast majority of eggs (~90%) are microbiologically sterile at oviposition (6), but they are quickly exposed to potential contamination sources such as nesting material, dust, and feces (2, 5, 6). Three potential routes of infection for eggs have been well described in the literature: trans-ovarian infection occurs while the ovum or yolk is still connected to the ovary; oviducal when the albumen is infected as the egg passes through the oviduct (e.g. *Salmonella enteritidis*); and trans-shell when microbes are able to move into the inner surface of the shell (7).

As mentioned, physical pores in the outer shell allow a potential route of entry for either fungi or bacteria, and this can lead to both health hazards and a foul smell and taste of the egg. For example, some species of fungi produce toxins that can affect the central nervous system or cause kidney and liver (8). Detecting contamination is an important aspect of public health protection for consumers. Volatile organic compounds (VOCs) are produced by microbes as they proliferate, and these chemicals are emitted back into the environment through the eggshell. In published studies, 349 bacteria and 69 fungi are known volatile emitters (9). However, there are approximately 100,000 microbe species that have been described or sequences, and potentially millions more that scientists suspect are present, but are not characterized as yet (10, 11). In this work, we show data on gas phase fungal metabolites produced inside hen eggs and their correlation with the fungi species-specific VOCs. Detection of such metabolites may offer a non-invasive approach for detection of pathogen contamination and have important practical implications for the egg and food industry.

MATERIALS AND METHODS

Egg preparation

Six eggs were obtained from the campus facility Meat Lab (University of California Davis, USA). Two of the eggs were fresh while four were stored in a humid ambient refrigerator for forty days. Some of the old eggs presented internal pathogen contamination (Figure 1). Before samplings, eggs were carefully cleaned with deionized water (Evoqua, Denver, CO, USA) and dried gently with Interstate® windshield towels (VWR, Visalia, CA, USA).

Eggs were placed inside Qorpak Kaptclean™ Glass Sample Jars, with a neck size of 70 mm, a cap of PTFE, and a capacity of 240 mL (Coleparmer, Vernon Hills, IL, USA). These wide-mouth Kaptclean® glass jars are pre-cleaned for semi-volatiles, pesticides, PCBs, and metals. A side hole was drilled using a 1.5 mm diamond electroplated drill (UKAM Industrial Superhard Tools, Valencia, CA, USA) in the cap to allow the introduction of the sampling fiber.

The vials were cleaned in a vacuum over at 160 °C for 3 h and then purged with dry air (78–80% N_2, 20–22% O_2, $H_2O \leq 7$ ppm_v) (Airgas; Sacramento, CA, USA). The eggs were placed in jars and sealed with caps. All samples (with and without eggs inside) were taken after flushing the vials with the dry air for 2 min. Once all measurements were done, all eggs were opened to check if the fungus was present and an aseptic sample of each was taken for fungal and bacteria identification.

Egg VOCs sampling and analysis with GC/MS

Solid-phase microextraction (SPME) fibers of 75 μm thickness PDMS/CAR (black hub) (Supelco, Bellefonte, PA USA) were placed inside the vials through the cap holes (Figure 2A). The headspace was statically sampled for 30 min. Prior to sampling, the fibers were conditioned at 300 °C for 1 h. Clean SPME blanks were analyzed to check for artifacts and contaminations.

The VOC samples collected using SPMEs were analyzed with a Varian 3800 Gas Chromatograph / Mass Spectrometer (GC/MS) (Varian, Walnut Creek, CA, USA) with a CombiPAL autosampler system (CTC Analytics; Lake Elmo, MN, USA) and a liquid nitrogen cooling system for cryofocusing. The analytical column was a Factor Four VF-5ms 30 M × 0.25 mm with a film thickness of 0.25 μm. For the analysis, the SPME fibers were inserted into the heated port of the gas chromatograph equipped with Merlin MicroSeal™ and collected compounds were desorbed at 300 °C for 1 min. The GC profile for the analysis was set as follows: cryofocusing for 1 min at temperature −10 °C; then ramp at 50 °C/min to 40 °C; then ramp to 100 °C; then ramp at 5 °C/min to 180 °C; then ramp at 10 °C/min to 250 °C; and finally ramp at 20 °C/min to 280 °C hold for 5 min to bake/purge the column. The helium carrier gas was set to a constant 1 mL/min flow. The scanned mass-to-charge range of the quadrupole trap mass analyzer was set to 35–250 Th.

Fungal VOCs sampling and analysis with GC/MS

Fungal isolates obtained from the interior surface of the eggs shell (see Isolates section below) were placed on 9 cm-diameter Petri plates containing acidified potato dextrose agar (APDA), and sampled after 7 days of incubation at 25 °C to allow for the growth of the colonies. SPME fibers of 75 μm thickness PDMS/CAR (black coating) (Supelco, Bellefonte, PA, USA) were inserted into the Petri-dishes through a small hole on one side (Figure 2B). The headspace was sampled for 30 min in all cases. Prior to sampling, the fibers were conditioned at 300 °C for 1 h. The clean SPME blanks were periodically analyzed to check for artifacts and contaminations. The SPMEs were analyzed using the same GC/MS protocol as for the eggs volatiles analysis.

GC/MS data analysis and interpretation

Data analysis and mass spectral interpretations were carried out using MS Data Review version 6.6 software (12) and automated mass spectral deconvolution and identification system (AMDIS) version 2.64 software with medium resolution and high sensitivity and shape requirements. MS matching was performed against NIST 05 and Wiley 09 databases. The MS fragmentation patterns of deconvolved (if necessary) peaks were matched to library entries. The matches with forward and reverse scores of 800 and above were presumed to be correct; those in 300–800 range were considered “tentative”. In addition, Kovats retention indices were also calculated for each peak for chemical identification confirmation in addition to MS patterns (Kovats mixes of C6-C9 (Test Mix #1) and C10-C25 (DRO Mix) from Restek (Bellefonte, PA USA) were run using the GC analysis protocol described above). For the majority of the identified compounds, the Kovats indices were found to match within ~20 units and below. Two of the compounds identified by MS, although they had high forward and reverse scores, were found to have poor Kovats match (greater than 100 units discrepancy). These compounds were deemed unidentified. Only peaks that appeared exclusively in the eggs or fungi profiles were considered, while any peaks that appeared in any of the blanks (vials, Petri dishes, clean SPMEs) were disregarded. Any compounds that are known to be external contaminants such as siloxanes (column, septa bleed) or phthalates (contaminants from plastic) were also disregarded.

Microbe Isolates

Samples of bacteria and fungi from individual eggs were identified. For fungal isolation, pieces of internal egg tissue (8 mm^3) were placed on 9 cm-diameter Petri plates containing acidified potato dextrose agar (APDA). After the plates were incubated at 25 °C for one week, hyphal tips were transferred to APDA plates to maintain fungal isolates in pure cultures. For bacterial isolation, pieces of internal egg tissue (8 mm^3) were macerated in 500 μL deionized water and streaked onto 9 cm-diameter Petri plates containing King's medium B agar (KB) which were then incubated at 27 °C for one week. Individual bacterial colonies were transferred to obtain pure cultures. All fungal and bacterial isolates were maintained on APDA or KB, respectively, at room temperature (24 ± 1) °C.

Colonies typical of *Cladosporium* and *Botrytis* were frequently observed on APDA. Colonies of *Cladosporium* were gray-olive with conidiophores that were macronematous, erect, oblong, branched, and 1 to 5 septate; ramoconidia were cylindrical or oblong with 0 to 2 septa. Colonies of *Botrytis* were white to grayish with conidia that were light brown and subglobose to broadly ellipsoidal. In addition, black sclerotia formed on the medium after approximately 14 days. Species identification was based on colony morphology and DNA sequence data.

DNA extraction

DNA was extracted from mycelia of fungal cultures on APDA and bacterial colonies on KB. To extract fungal DNA, two tufts of hyphae approximately 15 mg in total from each fungal culture were collected with sterile forceps and placed into a 1.5 mL cryogenic tube containing three sterile glass beads and 500 μL of OmniGenX extraction buffer (E&K Scientific; Santa Clara, CA). To extract bacterial DNA, a sterile bacterial loop was used to transfer cells from one bacterial colony on King's B into 1.5 mL tubes containing the buffer. After the samples were macerated on a Vortexer Genie 2 (VWR; Radnor, PA) at maximum speed for 10 min, the manufacturer's directions for the OmniGenX PureSpin gDNA Purification Kit (E&K Scientific; Santa Clara, CA) solid tissue sample DNA extraction were followed.

Internal transcribed spacer sequences for identification of fungi

The sequence of the internal transcribed spacer (ITS) region was determined for all fungal isolates. ITS primers were: ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') and ITS5 (5' GGA AGT AAA AGT CGT AAC AAG G 3') (13). PCR reactions consisted of 12.5 μL of 2X Mean Green Master Mix (Syzygy Biotech; Grand Rapids, MI), 1 μL of each 10 μM primer and 2 μL of genomic DNA in a 25 μL reaction. Amplification reactions were performed in a thermocycler (PTC-100; MJ Research, Watertown, MA) under the following conditions: 40 cycles of 94 °C for 2 min, 55 °C for 2 min and 72 °C for 2.5 min followed by a final extension at 72 °C for 10 min. A negative control of water template and positive control of known fungal DNA were included in the PCR run. Five μL of each sample were visualized by UV light after electrophoresis on a 1.5% agarose gel and staining with ethidium bromide.

16S sequences for the identification of bacteria

The sequence of the small subunit of the bacterial ribosome, 16S rDNA, was amplified from bacteria cultured from the egg samples. The 16S primers were (14): FP1 (5' AGA GTT TGA TCC TGG CTC AG 3') and RD1 (5' ACG GTT ACC TTG TTA CGA CTT 3'). PCR reactions were prepared and visualized as above and the thermocycler settings were as follows: 95 °C for 30 s followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min.

Sequencing and isolate identification

PCR products were purified with a GeneJET PCR Purification Kit (Thermo Fisher Scientific, Inc.; Waltham, MA) following the manufacturer's instructions. A 24 μL sample of 40–68 ng/μL of PCR product and 12 μL of each 3μM primer, ITS4, and ITS5 or FP1 and RD1, were sent to Quintara Biosciences (Richmond, CA) for sequencing. Sequences were manually checked and identified using NCBI BLAST results. For *Cladosporium* and *Botrytis*, a BLAST search of the 500 bp sequences revealed over 97% similarity with a sequence of *Cladosporium macrocarpum* and *Botrytis cinerea*, respectively. For *Stenotrophoma* and *Pseudomonas*, a BLAST search of the 800 bp sequences revealed over 99% similarity with a sequence of *Stenotrophomas rhizophila* and *Pseudomonas argentinensis*, respectively.

RESULTS

Microbes or fungi volatiles, even if they originated from inside the eggs can be measured by taking advantage of the porosity of the egg shells using a non-invasive technique of volatile detection that avoids breaking the eggs themselves. Volatiles in the head space of the studied eggs were sampled using a SPME fiber for 30 min and analyzed by a GC/MS. All eggs were opened after the VOCs analysis to check if the fungus was present and an aseptic sample of each was taken for fungal and bacteria identification. From the half dozen eggs analyzed, three of the four old eggs stored in humid conditions showed the presence of fungi (Figure 1). Fungal growth was not observed in any of the fresh eggs.

Identification of VOCs from the eggs with GC/MS

As described in the Materials and Methods section, headspace volatiles above the eggs was sampled with SPME fibers placed inside a vial which was flushed for 2 min with dry air. Also, empty vials flushed with the dry air were analyzed, as blank measurements. In Figure 3, the chromatograms for a fresh egg and an egg containing fungi are shown. The abundances of emitted volatiles of the eggs containing fungi are notably higher across the board than the ones without.

In total, up to 60 unique compounds originated from the eggs and were not found either in the chromatograms of the vials blanks or the SPME blanks. From those compounds, 31 have been identified (Table 1) and 29 compounds have been either tentatively identified or not identified. (Electronic Supplementary Material (ESM) Table S1). Table 1 also shows for each compound its presence in the microbes volatile organic compound database (mVOC) (9), and the microbe species (fungi and/or bacteria) that produces it.

The compounds are classified into 6 groups: fungi (present only in the eggs containing fungi); healthy (present only in the eggs not containing the fungi, both fresh and old); all (present in all eggs); old (present only in the old eggs both containing the fungi and not); fresh (present only in the fresh eggs); and other (not exclusive to any of the above groups). Following this classification, the following number of compounds were found: 17 for the "fungi" group, 7 for the "healthy" group, 2 for the "all" group, 24 for the "old" group, 4 for the "fresh" group and 28 for the "other" group. Some of the detected compounds may be of exogenous origin, e.g. from petroleum products. However, since they were not detected in any of the blanks, they are presumed to originate from the samples, although they may not be engendered by the microorganism activity.

To establish which of the compounds reported in Table 1 were originated from the fungal species, we performed a volatile analysis of the fungal isolates grown on Petri-dishes as reported in the Material and Methods section. Only 2 compounds, trichloromethane and p-benzoquinone were found and identified as potential metabolites for *Cladosporium macrocarpum* and *Botrytis cinerea*. Other detected compounds, such as benzene, ethylbenzene, benzonitrile are commonly occurring contaminants and therefore may be of exogenous origin.

DISCUSSION

The low amount of mVOCs detected in comparison with other SPME-GC-MS fungal volatile experiments (15, 16) is expected if we consider that these unique VOCs are detected only when they have passed through the eggshell, which reduces the abundances and total number of compounds that could be detected in the headspace. To counteract this, the experiments were done with eggs stored for a longer period of time than the usual one for egg consumers (~15–20 days), to give more time to any microbe contamination to proliferate inside the eggs to promote more robust metabolites detection.

Metabolites from fungi-infected eggs

Seventeen compounds were found exclusively in the eggs containing fungi. From those, five were identified (Table 1): methanethiol; trichloromethane; dimethyl sulfoxide; hexyl acetate; and valencene. These volatiles are indeed expected to be produced by bacterial and fungal pathogens. Methanethiol has been reported to be produced by different bacteria and fungi species (9) including yeast (17) and produces a smell like rotten cabbage. Dimethyl sulfoxide (DMSO) can be a precursor and/or a degradation product of dimethylsulfide (18). Dimethylsulfide can be formed via lyase enzyme action on 3-dimethylsulfoniopropanoate (DMSP), but it a photochemical route by oxidation of dimethylsulfide is also possible (19). This has been shown to happen for purple phototrophic bacteria such as *Rhodovulum sulfidophilum* (20). Dimethylsulfide was also detected in this study, but it is classified in the "other" category and detected in almost all eggs, with the exception of one fresh egg. As DMSP and its degradation products, including dimethylsulfide, are common metabolites associated with multiple organisms including algae and bacteria, these findings are consistent with microorganism activity. It should be noted that these compounds can be involved in both catabolic and anabolic pathways. For example, both dimethylsulfide and

methanethiol can be used by bacteria to produce sulfur-containing amino acids and proteins. DMSO is assumed to be released as a side product of oxidative radical chemistry within the egg. From the compounds detected, only valencene has been reported to be exclusively produced by different fungi species (9).

The identified microbe species found inside the studied eggs included two fungi species, *Cladosporium macrocarpum* and *Botrytis cinerea*, and two bacterial species, *Stenotrophomas rhizophila*, and *Pseudomonas argentinensis*. *Cladosporium spp.* is a widespread mold with ambient spores found in many areas (21), and it can be allergenic mold in cooler climates (22). It is a well-known trigger for asthmatic attacks (23). There are about 500 species of *Cladosporium* (24), and some are known to produce VOCs associated with distinctive mold odors (25). However, no reports on specific *Cladosporium macrocarpum* volatile metabolites have previously been published in the literature.

Botrytis cinerea found all over the world, with the most prevalent of the species belonging to the genus *Botrytis* (26). *Botrytis cinerea* is found in soil, although it is not usually the most abundant fungi observed in field sampling. *Botrytis cinerea* is a gray mold that can affect soft fruit, like strawberries and raspberries (27), especially during post-harvest food processing. One available report studied the changes of the volatile content of strawberries infected with *Botrytis cinerea* (28), however, the authors did not establish specific volatiles associated with the *Botrytis cinerea* itself. There are reports of the role of volatiles emitted by post-harvested fruits in the development of *Botrytis cinerea* (29) and also of different antifungal activities of essential oils against *Botrytis cinerea* (30); however, to the best of our knowledge, no specific studies on the *Botrytis cinerea* volatiles themselves have been reported.

To investigate which VOCs reported here were specifically produced by the fungi we observed, both fungal isolates were grown on 9-cm Petri dishes and their volatile content was analyzed. Only two distinct metabolites that could be directly attributed to the fungal cultures were identified. Only one of these compounds was found in both eggs infected with fungi and fungal isolates: trichloromethane (also known as chloroform). Even though chloroform is not listed in the microbes database mVOC (9), it has been reported in the headspace of different fungal species (31). In fact, it has been suggested that fungi are important sources of elevated concentrations of chloroform in soil air, although it is still not clear which enzymes are responsible for the production of chloroform and other chlorinated compounds (31). This compound may not be an ideal biomarker in certain circumstances because trichloromethane is commonly used as a solvent and is frequently considered a contaminant. However, in the present study it was not present in any of the blanks, so it is unlikely to come from a contamination and is most likely of biogenic origin. The benzoquinone may originate from exogenous sorces of result from chemical transformations of other fungal metabolites as the quinones in general are naturally occur in fungi. For example, oosporein has been found to confer fungal virulence via inhibiting insect immunity (32).

With regard to our bacterial species that were identified, *Stenotrophomonas spp.* is present in soil, plants and the environment across the world. *S. rhizophila* is one of the

Stenotrophomonas species that can actually be beneficial to plants (33), and it has been reported to have antifungal properties (34, 35). Kai *et al.* (36) reported the volatiles from different *Stenotrophomonas* strains, but only two compounds, β-phenylethanol, and dodecanal were reported produced by *S. rhizophila*. Although neither of these compounds was found in our study, we have observed several similar compounds, including several aldehydes and aromatic/aliphatic alcohols.

Pseudomonas argentinensis was first reported in 2005 and was found in grasses from Córdoba, Argentina (37). However, it has also been identified in a hog farm sprayed field (North Carolina, USA) (38), in animals (cats from Grenada, West Indies) (39), and in humans as a skin infection (Jerusalem, Palestine) (40). No reports on *Ps. argentinensis* volatile metabolites have been published in the literature to date.

Bacterial and fungal species are known to interact, and this can alter their pathogenicity (41). Schoonbeek *et al.* (42) showed the interaction of *B. cinerea* with soil bacteria can reduce *B. cinerea* pathogenicity. One the compounds found in this study, Hexyl acetate, reportedly has a significant inhibitory effect against bacterial strains (43), so it is possible that this compound is affected by a bacterial-fungal interaction. It is well known that multiple microorganisms exchange chemical cues (44), such as quorum sensing to control bacterial colony proliferation. Although outside the scope of our initial study, we speculate that analogous interactions and chemical cue exchanges may occur between species studies in our egg work. Future studies should be planned to examine this possibility.

Metabolites from all eggs

Two common compounds were found and identified in all of the eggs (Table 1): 2-Pentanone and 1-Pentanol. 1-Pentanol was reported in a study from Paraskevopoulou *et al.* (45) where the authors analyzed the headspace of a mixture of egg yolk and whey protein isolate obtained from milk. To further elucidate if those compounds are due to the egg or to the present bacterial contamination, further volatile analysis should be done.

Metabolites from healthy eggs

Up to 7 common compounds were found to belong to the “healthy” eggs (present only in the eggs not containing the fungi, both fresh and old). From those, 4 were also classified as peaks found in “fresh” eggs and are discussed below. From the remaining 3 peaks, 2 were identified (Table 1): 2-butanone and (E)-2-nonen-1-ol. As those compounds are only found in the fungi-free eggs, and not in the fungi-contaminated ones, those mVOCs were most likely released by either the bacteria found in the eggs (*S. rhizophila* and/or *P. argentinensis*), as they are reported to be produced by bacteria (9), or they could also be due to the eggs themselves when they are not fighting a fungal infection. The (E)-2-nonen-1-ol peak was also classified as “old”, meaning that it could be a unique mVOC from the degradation of the egg, bacterial activity or other processes associated with loss of freshness.

Metabolites from fresh eggs

Up to 4 common compounds were found in all of the fresh eggs. From those, only 2 were identified (Table 1): 2-butanol and N,N-dimethylformamide. Even though the 2-Butanol

compound is reported in the mVOC database as produced by fungi, it has also been found to be produced by lactic acid bacteria (46). N,N-dimethylformamide is released by *Paenibacillus polymyxa* (47), a bacterium found in the soil, roots, and rhizosphere of various crop plants. Given that both *S. rhizophila* and/or *P. argentinensis* bacteria are found in the rizhosphere, it is possible that they produce the N,N-dimethylformamide (DMF) compound. However, at the same time, DMF is widely employed in textile and pharmaceutical industries as a ubiquitous solvent so its detection may be of exogenous origin. It was found that some bacterial species are capable of biodegradation of DMF thus removing it from the environment (48). Further studies needed to establish the exact origin of these compounds in our particular samples.

Metabolites from old eggs

Up to 24 common compounds were found in the "old" eggs group. From those, 17 were also classified as "fungi" and 1 as "healthy", which have been discussed above. From the remaining 6 peaks, only three were identified (Table 1): Ethyl 2-methylbutyrate, 3-octanol, and 5-ethenyl-5-methyloxolan-2-one. These compounds are common flavorant compounds and all three have been reported in food products: bilberry, apple and wine for Ethyl 2-methyl butyrate (49); mentha species oils, soybeans and wine for 3-octanol; and for lavender oil (50), grapes and wine (51) for 5-ethenyl-5-methyloxolan-2-one (also referred as lavender lactone). Different microbes are reported to produce those compounds. The volatile ethyl ester ethyl 2-methyl butyrate is produced by different species of bacteria and fungi (9), and the aliphatic alcohol 3-octanol has only been reported from fungi (9). The 5-ethenyl-5-methyloxolan-2-one may be an oxidation metabolite of linalool oxide (furanoid) (52), although it has been obtained by fermenting linalool by a *Pseudomonas* (53). Even though we did not detect linalool, it has been reported to be produced by different bacteria and fungi species (9). These compounds have been found in all the "old" eggs including the fungi-infected and uninfected ones. It is possible for those compounds to originate from various sources, including degradation of the egg itself, such as oxidation of fatty acids or end products of fermentation by bacteria.

Other metabolites

Up to 28 compounds were classified as "other" metabolites, and 18 of those were identified (Table 1). In this group belong the compounds that do not belong exclusively to any of the above groups. Relevant is the presence of the sulfur containing compound dimethylsulfide, which can be produced by the oxidation of methanethiol (one of the identified fungi metabolites) and has been reported from different bacterial and fungi strains (9). Dimethylsulfide has a cabbage-like smell. Other compounds were found in the headspace of a mixture of egg yolk and whey (45): pentanal, heptanal, and phenol. One interesting observation is the occurrence of esters, both in the "old" and in the "other" group. The esters have great biological significance and often occur naturally, as discussed above, albeit predominantly in plant systems. Some of the bacteria, such as *Acinetobacter* species (commonly occurring in soil) are known to utilize wax esters as a storage compound for energy (54). Such compounds would be highly non-volatile and not amenable to detection using gas-phase sampling utilized in this work unless degraded to shorter-chain compounds via oxidation or enzymatic reactions. At the same time, fungi are known to produce small

esters along with other small molecules such as ketones, alcohols, and hydrocarbons (55). The fact that the ester compounds were not particular to the “fungi” group indicates the variability of the VOC distributions between different eggs, likely due to the inherent variability of fungal species, stages of development and, possibly, interaction with other microorganisms. To further elucidate the biological significance and origin of the compounds belonging to the “other” category further experiments must be done with both egg and microbes-microbes co-cultures headspaces.

Final remarks

Different volatile organic compounds released by fungi and bacteria colonies inside unbroken hen eggs were found. Two different fungal species (*Cladosporium macrocarpum* and *B. cinerea*) and two different bacterial species (*S. rhizophila* and *Ps. Argentinensis*) were isolated from inside the studied eggs. These species commonly occur in soil and are saprobic on plants, which may indicate that the eggs were likely trans-shell contaminated while resting in the hen’s nest. One compound, trichloromethane, was found in both egg and fungal isolates grown in Petri dish experiments, and its source, therefore, is presumed to be from fungal activity. Barring potential interference from use of chlorinated solvents, this compound may be a very convenient biomarker of fungal contamination as the halogenated metabolites are distinctly different from other biogenic volatiles and rarely occurs in nature. However, prior to any practical use of this compound by the poultry industry to discriminate old egg batches, further studies to validate these findings should be carried out and also with different storage times to study the evolution of mVOCs while fungal colonization of the eggs, the decomposition process and possible catabolism interferences with fungal contamination. As discussed above, some compounds that are consistent with catabolism have been observed. Also, eggs from different places, which can be in contact with different microbe loads that may result in different volatiles, should be studied to further elucidate the applicability of this methodology. In addition, it is possible that some of the metabolites both may differ at different time points of pathogen contamination and serve as a guide to pinpoint the source of infection. Further studies will be required to assess this as well; mapping of VOC distributions as the eggs proceed from fresh stage to different stages of microbial development under varying conditions (temperature, humidity, initial microbial load etc.) is essential for practical utility of the approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Partial support was provided by the National Institutes of Health (NIH) grant number #UL1 TR000002 [CED] and NSF award #1255915 [CED, AAA]. Opinions expressed in this publication are those of the authors and do not necessarily reflect the view of the funding agency.

References

1. McNamara, DJ. Proceedings of the National Egg Quality School. Raleigh. NC: North Carolina State Univeristy; 2003. Being positive about eggs; p. 230-245.

2. Stadelman, WJ.; Newkirk, D.; Newby, L. CRC Press. 1995. Egg Science and Technology. editor
3. Applegate E. Introduction: Nutritional and Functional Roles of Eggs in the Diet. Journal of the American College of Nutrition. 2000; 19(sup5):495S–498S. [PubMed: 11022998]
4. Romanoff, AL.; Romanoff, AJ. The Avian Egg. New York: John Wiley & Sons I; 1949. editor
5. Mayes FJ, Takeballi MA. Microbial contamination of the hen's egg: a review. J Food Prot. 1983; 46(12):1092–1098.
6. Board RG. The Course of Microbial Infection of the Hen's Egg. Journal of Applied Bacteriology. 1966; 29(2):319–341. [PubMed: 5341100]
7. Board RG, Fuller R. Microbiology of the Avian Egg. Chapman & Hall L. 1994 editor.
8. Bennett JW, Klich M. Mycotoxins. Clinical Microbiology Reviews. 2003; 16(3):497–516. [PubMed: 12857779]
9. Lemfack M, Nickel J, Dunkel M, Preissner R, Piechulla B. mVOC: a database of microbial volatiles. Nucleic Acids Res. 2014; 42(1):D744–D748. [PubMed: 24311565]
10. Blackwell M. The Fungi: 1, 2, 3 … 5.1 million species? American Journal of Botany. 2011; 98(3): 426–438. [PubMed: 21613136]
11. Curtis TP, Sloan WT, Scannell JW. Estimating prokaryotic diversity and its limits. Proceedings of the National Academy of Sciences. 2002; 99(16):10494–10499.
12. Saturn Varian Inc. Saturn Varian Chemstation, version 6.6. Palo Alto, CA, USA: MS Data Review; 2005.
13. White, T.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.; Gelfand, D.; Shinsky, J.; White, T., editors. PCR Protocols: A Guide to Methods and Applications. Academic Press; 1990. p. 315-322.
14. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology. 1991; 173(2):697–703. [PubMed: 1987160]
15. Polizzi V, Adams A, Malysheva SV, De Saeger S, Van Peteghem C, Moretti A, et al. Identification of volatile markers for indoor fungal growth and chemotaxonomic classification of Aspergillus species. Fungal Biology. 2012; 116(9):941–953. [PubMed: 22954337]
16. Van Lancker F, Adams A, Delmulle B, De Saeger S, Moretti A, Van Peteghem C, et al. Use of headspace SPME-GC-MS for the analysis of the volatiles produced by indoor molds grown on different substrates. Journal of Environmental Monitoring. 2008; 10(10):1127–1133. [PubMed: 18843388]
17. Jewison T, Knox C, Neveu V, Djoumbou Y, Guo AC, Lee J, et al. YMDB: the Yeast Metabolome Database. Nucleic Acids Research. 2012; 40(D1):D815–D820. [PubMed: 22064855]
18. Schäfer H, Myronova N, Boden R. Microbial degradation of dimethylsulphide and related C1-sulphur compounds: organisms and pathways controlling fluxes of sulphur in the biosphere. Journal of Experimental Botany. 2010; 61(2):315–334. [PubMed: 20007683]
19. Brimblecombe P, Shooter D. Photo-oxidation of dimethylsulphide in aqueous solution. Marine Chemistry. 1986; 19(4):343–353.
20. Hanlon SP, Holt RA, Moore GR, McEwan AG. Isolation and characterization of a strain of Rhodobacter sulfidophilus: A bacterium which grows autotrophically with dimethylsulphide as electron donor. Microbiology. 1994; 140(8):1953–1958.
21. Adkinson, NFJ.; Bochner, BS.; Burks, AE.; Busse, WW.; Holgate, ST.; Lemanske, RFJ., et al. Middleton's Allergy: Principles and Practice. 8th. Elsevier; 2015.
22. Achatz G, Oberkofler H, Lechenauer E, Simon B, Unger A, Kandler D, et al. Molecular cloning of major and minor allergens of Alternaria alternata and Cladosporium herbarum. Molecular Immunology. 1995; 32(3):213–227. [PubMed: 7898496]
23. Denning DW, O'Driscoll BR, Hogaboam CM, Bowyer P, Niven RM. The link between fungi and severe asthma: a summary of the evidence. European Respiratory Journal. 2006; 27(3):615–626. [PubMed: 16507864]
24. Parr CS, Wilson N, Leary P, Schulz K, Lans K, Walley L, et al. The Encyclopedia of Life v2: Providing Global Access to Knowledge About Life on Earth. Biodiversity Data Journal. 2014; 2:e1079. [PubMed: 24891832]

25. Polizzi V, Delmulle B, Adams A, Moretti A, Susca A, Picco AM, et al. JEM Spotlight: Fungi, mycotoxins and microbial volatile organic compounds in mouldy interiors from water-damaged buildings. Journal of Environmental Monitoring. 2009; 11(10):1849–1858. [PubMed: 19809708]
26. Williamson B, Tudzynski B, Tudzynski P, Van Kan JAL, et al. Botrytis cinerea: the cause of grey mould disease. Molecular Plant Pathology. 2007; 8(5):561–580. [PubMed: 20507522]
27. Jarvis WR. The infection of strawberry and raspberry fruits by Botrytis cinerea Fr. Annals of Applied Biology. 1962; 50(3):569–575.
28. Vandendriessche T, Keulemans J, Geeraerd A, Nicolai BM, Hertog MLATM. Evaluation of fast volatile analysis for detection of Botrytis cinerea infections in strawberry. Food Microbiology. 2012; 32(2):406–414. [PubMed: 22986207]
29. Neri F, Cappellin L, Spadoni A, Cameldi I, Algarra Alarcon A, Aprea E, et al. Role of strawberry volatile organic compounds in the development of Botrytis cinerea infection. Plant Pathology. 2015; 64(3):709–717.
30. Soylu EM, Kurt Ş, Soylu S. In vitro and in vivo antifungal activities of the essential oils of various plants against tomato grey mould disease agent Botrytis cinerea. International Journal of Food Microbiology. 2010; 143(3):183–189. [PubMed: 20826038]
31. Hoekstra EJ, Verhagen FJM, Field JA, Leer EWBD, Brinkman UAT. Natural Production Of Chloroform By Fungi. Phytochemistry. 1998; 49(1):91–97.
32. Feng P, Shang YF, Cen K, Wang CS. Fungal biosynthesis of the bibenzoquinone oosporein to evade insect immunity. P Natl Acad Sci USA. 2015; 112(36):11365–11370.
33. Ryan RP, Monchy S, Cardinale M, Taghavi S, Crossman L, Avison MB, et al. The versatility and adaptation of bacteria from the genus Stenotrophomonas. Nat Rev Micro. 2009; 7(7):514–525.
34. Wolf A, Fritze A, Hagemann M, Berg G. Stenotrophomonas rhizophila sp. nov., a novel plant-associated bacterium with antifungal properties. International Journal of Systematic and Evolutionary Microbiology. 2002; 52(6):1937–1944. [PubMed: 12508851]
35. Schmidt C, Alavi M, Cardinale M, Müller H, Berg G. Stenotrophomonas rhizophila DSM14405T promotes plant growth probably by altering fungal communities in the rhizosphere. Biol Fertil Soils. 2012; 48(8):947–960.
36. Kai M, Effmert U, Berg G, Piechulla B. Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen Rhizoctonia solani. Arch Microbiol. 2007; 187(5):351–360. [PubMed: 17180381]
37. Peix A, Berge O, Rivas R, Abril A, Velázquez E. Pseudomonas argentinensis sp. nov., a novel yellow pigment-producing bacterial species, isolated from rhizospheric soil in Córdoba, Argentina. International Journal of Systematic and Evolutionary Microbiology. 2005; 55(3):1107–1112. [PubMed: 15879241]
38. Arfken AM, Song B, Sung JS. Comparison of Airborne Bacterial Communities from Hog Farm and Spray Field. Journal of microbiology and biotechnology. 2014
39. Hariharan H, Matthew V, Fountain J, Snell A, Doherty D, King B, et al. Aerobic bacteria from mucous membranes, ear canals, and skin wounds of feral cats in Grenada, and the antimicrobial drug susceptibility of major isolates. Comparative Immunology, Microbiology and Infectious Diseases. 2011; 34(2):129–134.
40. Barghouthi SA, Younis K. First time isolation of Pseudomonas argentinensis from human skin infection. Journal of Microbiology and Infectious Diseases. 2014; 4(2):78–83.
41. Frey-Klett P, Burlinson P, Deveau A, Barret M, Tarkka M, Sarniguet A. Bacterial-Fungal Interactions: Hyphens between Agricultural, Clinical, Environmental, and Food Microbiologists. Microbiology and Molecular Biology Reviews: MMBR. 2011; 75(4):583–609. [PubMed: 22126995]
42. Schoonbeek, H-j; Jacquat-Bovet, A-C.; Mascher, F.; Métraux, J-P. Oxalate-Degrading Bacteria Can Protect Arabidopsis thaliana and Crop Plants Against Botrytis cinerea. Molecular Plant-Microbe Interactions. 2007; 20(12):1535–1544. [PubMed: 17990961]
43. Lanciotti R, Belletti N, Patrignani F, Gianotti A, Gardini F, Guerzoni ME. Application of Hexanal, (E)-2-Hexenal, and Hexyl Acetate To Improve the Safety of Fresh-Sliced Apples. Journal of Agricultural and Food Chemistry. 2003; 51(10):2958–2963. [PubMed: 12720377]

44. Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: surviving and thriving in the microbial jungle. Nat Rev Micro. 2010; 8(1):15–25.
45. Paraskevopoulou A, Amvrosiadou S, Biliaderis CG, Kiosseoglou V. Mixed whey protein isolate-egg yolk or yolk plasma heat-set gels: Rheological and volatile compounds characterisation. Food Research International. 2014; 62(0):492–499.
46. Ghiaci P, Lameiras F, Norbeck J, Larsson C. Production of 2-butanol through meso-2,3-butanediol consumption in lactic acid bacteria. FEMS Microbiology Letters. 2014; 360(1):70–75. [PubMed: 25175699]
47. de Lacy Costello BPJ, Evans P, Ewen RJ, Gunson HE, Ratcliffe NM, Spencer-Phillips PTN. Identification of volatiles generated by potato tubers (Solanum tuberosum CV: Maris Piper) infected by Erwinia carotovora, Bacillus polymyxa and Arthrobacter sp. Plant Pathology. 1999; 48(3):345–351.
48. Bromley-Challenor AKC, Caggiano N, Knapp SJ. Bacterial growth on N,N-dimethylformamide: implications for the biotreatment of industrial wastewater. Journal of Industrial Microbiology and Biotechnology. 25(1):8–16.
49. Yannai, S. Dictionary of food compounds with CD-ROM: Additives, flavors, and ingredients. Boca Raton: Chapman & Hall/CRC; 2004.
50. Timmer R, Ter Heide R, De Valois PJ, Wobben HJ. Analysis of the lactone fraction of lavender oil (Lavandula vera D. C). Journal of Agricultural and Food Chemistry. 1975; 23(1):53–56.
51. Burdock, GA. Fenaroli's Handbook of Flavor Ingredients. Fifth. CRC Press; 2004.
52. Gottlieb, OR. Neolignans. In: Herz, W.; Grisebach, H.; Kirby, GW., editors. Fortschritte der Chemie Organischer Naturstoffe / Progress in the Chemistry of Organic Natural Products. Fortschritte der Chemie Organischer Naturstoffe / Progress in the Chemistry of Organic Natural Products. Vol. 35. Springer Vienna; 1978. p. 1-72.
53. Madyastha KM, Bhattacharyya PK, Vaidyanathan CS. Metabolism of a monoterpene alcohol, linalool, by a soil pseudomonad. Canadian Journal of Microbiology. 1977; 23(3):230–239. [PubMed: 851909]
54. Rontani, JF. Production of Wax Esters by Bacteria. In: Timmis, K., editor. Handbook of Hydrocarbon and Lipid Microbiology. Springer Berlin Heidelberg; 2010. p. 459-470.
55. Arora, DK. Handbook of Fungal Biotechnology. 2nd. Marcel Dekker, I., editor. Vol. 592. New York, USA: 2004. pp p

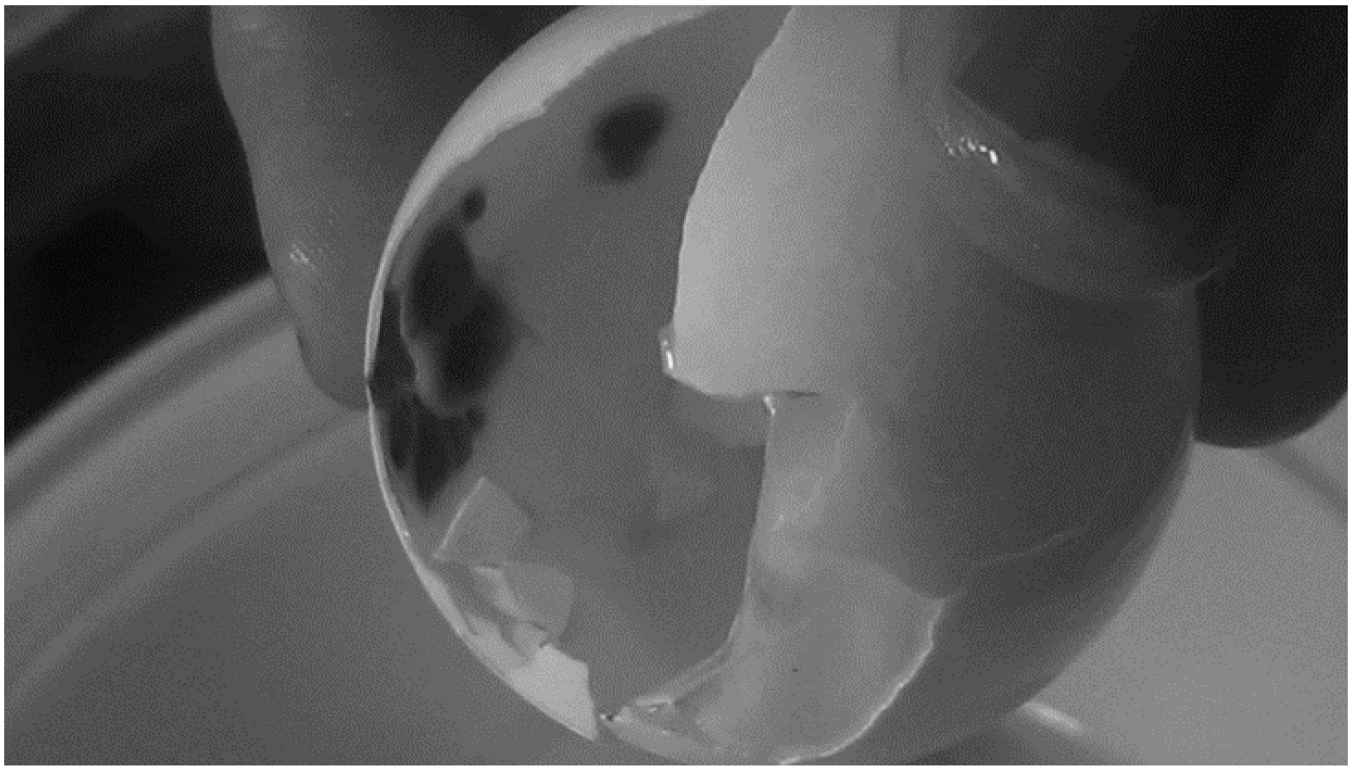


Figure 1.
Fungi found inside a hen eggshell.

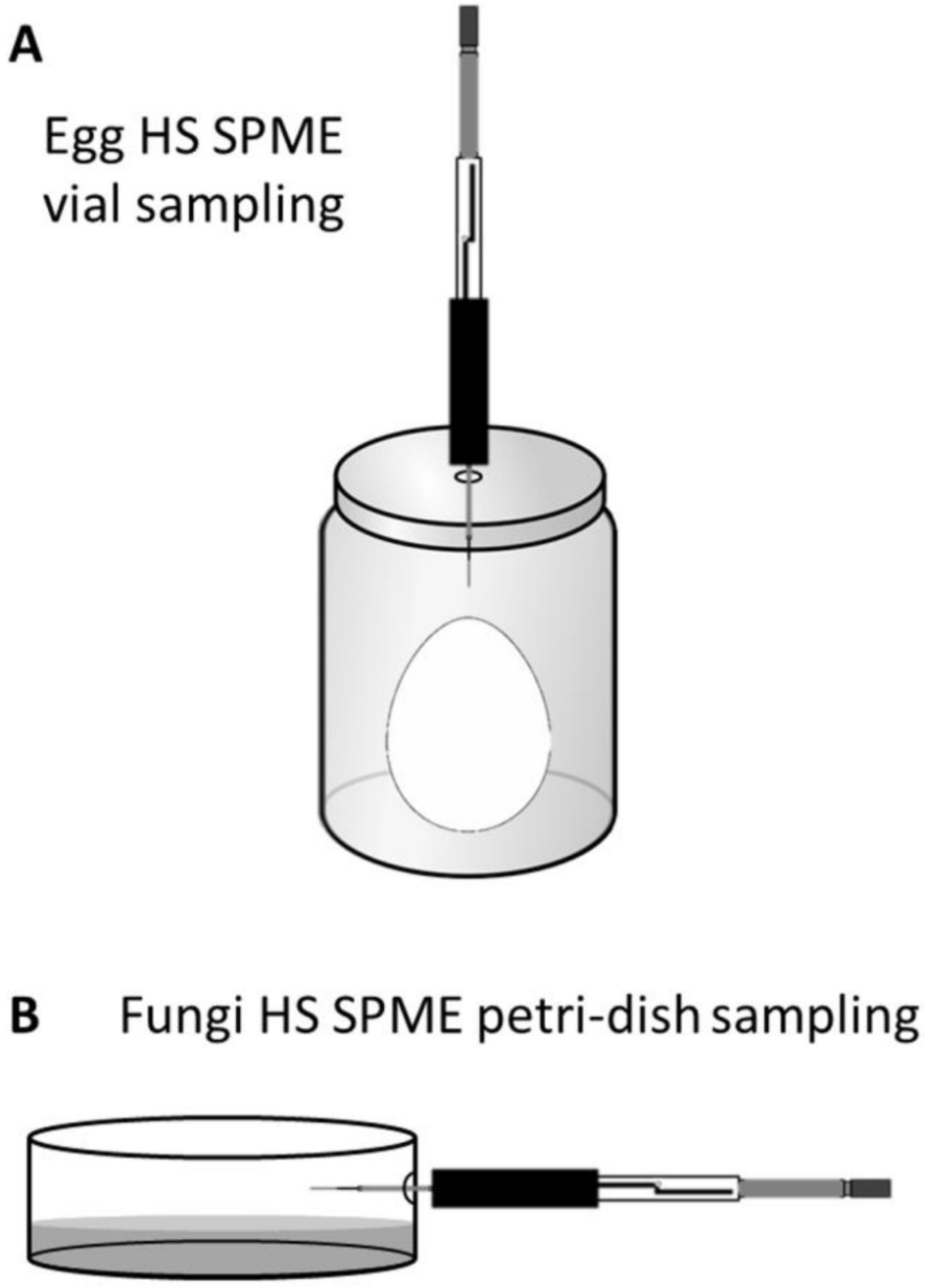


Figure 2.
Schematic representation of the headspace (HS) sampling methodologies with SPME used with A) eggs inside a vial and B) fungi isolates grown on 9 cm-diameter Petri dishes.

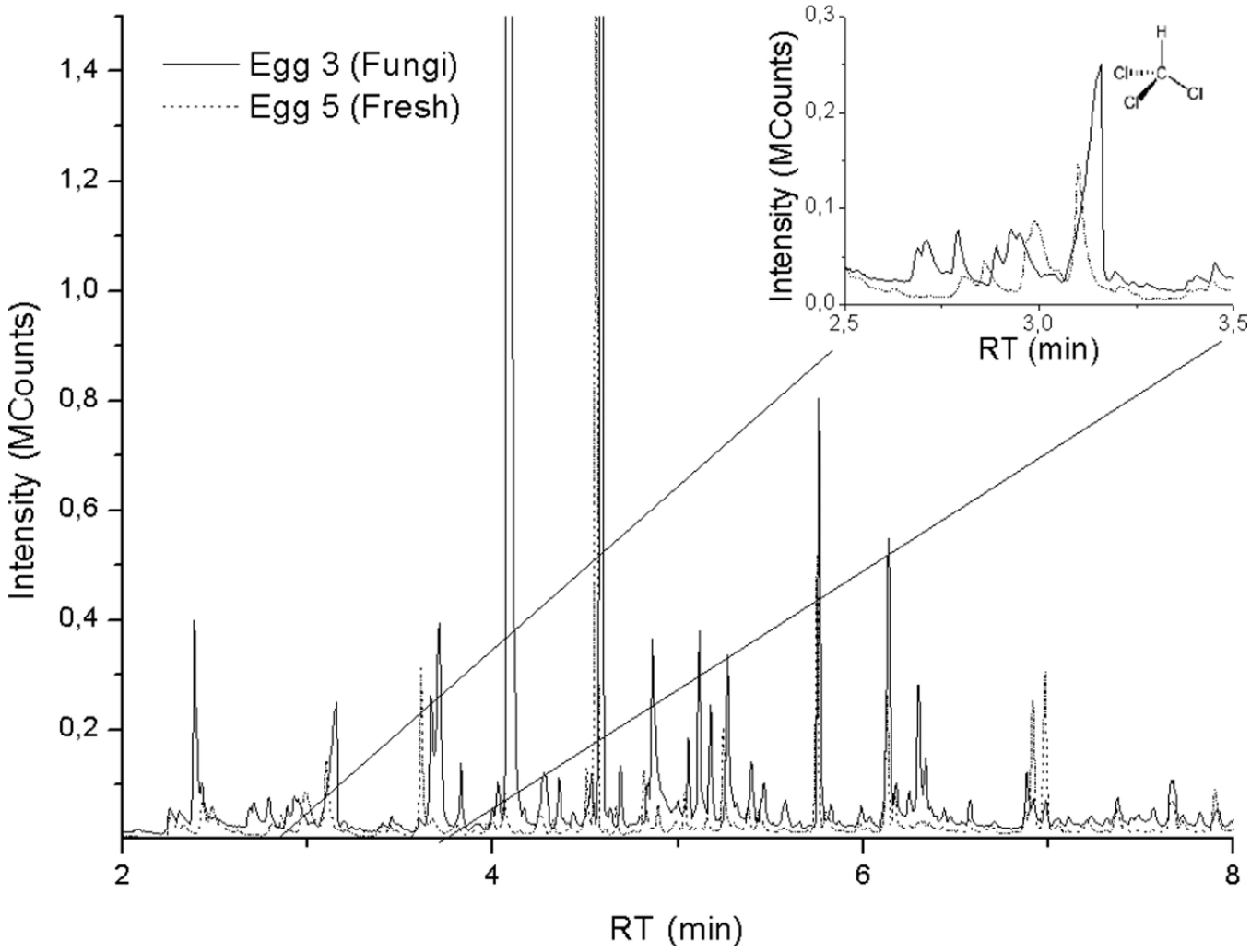


Figure 3.

Representative chromatograms of two analyzed eggs, egg 2 (fungi) and egg 5 (fresh). A zoomed portion of the chromatogram (insert) shows the identified trichloromethane peak for the fungi egg.

Table 1

Compounds identified by HS GC/MS of eggs inside vials, including the group classification, retention time (RT), calculated Kovats retention index (RI, i.u. retention index units), chemical name, chemical formula, CAS number, molecular weight (MW), presence in mVOC database and attribution.

Group	RT (min)	Kovats RI (i.u.)	Chemical Name	Formula	CAS #	MW (u)	mVOC database
FUNGI	1.822	-	methanethiol	CH_4S	74-93-1	48	Fungi/Bacteria
FUNGI	3.148	-	trichloromethane	$CHCl_3$	67-66-3	119	–
FUNGI	4.911	844	dimethyl sulfoxide	C_2H_6OS	67-68-5	78	–
FUNGI	13.742	1382	hexyl acetate	$C_{12}H_{24}O_2$	6378-65-0	200	–
FUNGI	16.372	1497	valencene	$C_{15}H_{24}$	4630-07-3	204	Fungi
ALL	3.560	670	2-pentanone	$C_5H_{10}O$	107-87-9	86	Fungi/Bacteria
ALL	3.948	741	1-pentanol	$C_5H_{12}O$	71-41-0	88	Fungi/Bacteria
HEALTHY	2.820	-	2-butanone	C_4H_8O	78-93-3	72	Fungi/Bacteria
HEALTHY	8.049	1104	2-nonen-1-ol, (e)-	$C_9H_{18}O$	31502-14-4	142	Bacteria
FRESH	3.028	-	2-butanol	$C_4H_{10}O$	78-92-2	74	Fungi
FRESH	4.432	797	n,n-dimethylformamide *	C_3H_7NO	68-12-2	73	–
OLD	4.936	853	ethyl 2-methylbutyrate	$C_7H_{14}O_2$	7452-79-1	130	Fungi/Bacteria
OLD	6.475	994	3-octanol	$C_8H_{18}O$	589-98-0	130	Fungi
OLD	7.232	1039	5-ethenyl-5-methyloxolan-2-one	$C_7H_{10}O_2$	1073-11-6	126	–
other	2.387	-	dimethylsulfide	C_2H_6S	75-18-3	62	Fungi/Bacteria
other	3.613	703	pentanal	$C_5H_{10}O$	110-62-3	86	–
other	3.870	714	ethyl propionate	$C_5H_{10}O_2$	105-37-3	102	Fungi
other	4.217	759	isobutyric acid	$C_4H_8O_2$	79-31-2	88	Fungi/Bacteria
other	4.446	785	butyric acid	$C_4H_8O_2$	107-92-6	88	Fungi/Bacteria
other	5.207	902	propyl butyrate	$C_7H_{14}O_2$	105-66-8	130	Fungi
other	5.583	908	heptanal	$C_7H_{14}O$	111-71-7	114	Fungi
other	6.252	-	phenol	C_6H_6O	108-95-2	94	Fungi/Bacteria

Group	RT (min)	Kovats RI (i.u.)	Chemical Name	Formula	CAS #	MW (u)	mVOC database
other	7.058	1035	benzyl alcohol	C_7H_8O	100-51-6	108	Fungi/Bacteria
other	7.487	1065	octanol	$C_8H_{18}O$	111-87-5	130	Fungi/Bacteria
other	9.129	1163	benzyl acetate	$C_9H_{10}O_2$	140-11-4	150	Bacteria
other	9.562	1187	hexyl butyrate	$C_{10}H_{20}O_2$	2639-63-6	172	–
other	10.456	1460	hexyl 2-methylbutanoate	$C_{11}H_{22}O_2$	10032-15-2	186	–
other	13.012	1349	(1-hydroxy-2,4,4-trimethylpentan-3-yl) 2-methylpropanoate	$C_{12}H_{24}O_3$	74367-33-2	216	–
other	13.533	1372	(3-hydroxy-2,4,4-trimethylpentyl) 2-methylpropanoate	$C_{12}H_{24}O_3$	25265-77-4	216	–
other	16.477	1502	alpha-farnesene	$C_{15}H_{24}$	28973-98-0	204	–
other	18.371	1585	[2,2,4-trimethyl-1-(2-methylpropanoyloxy)pentan-3-yl] 2-methylpropanoate	$C_{16}H_{30}O_4$	6846-50-0	286	–

* Possibly of exogenous origin.