Characterization of the Volatilome of *Tuber canaliculatum* Harvested in Quebec, Canada

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ABSTRACT: The first detailed characterization of volatile compounds from *Tuber canaliculatum*, a truffle newly grown in Quebec, Canada, was performed with headspace solid phase microextraction (HS-SPME) coupled with gas chromatography–mass spectrometry (GC/MS). A total of 30 compounds were identified, making up more than 98% of the volatile extract. The volatilome of *T. canaliculatum* is dominated by (E)-1-methylthio-1-propene, (Z)-1-methylthio-1-propene, dimethyl disulfide, and 1-octen-3-ol. It also includes six compounds identified for the first time in truffles, namely, 4-hydroxy-4-methyl-2-pentanone, pentyl propanoate, (Z)-1-methyl-2-(prop-1-en-1-yl)disulfide, (E)-1-methyl-2-(prop-1-en-1-yl)disulfide, (Z)-1-methyl-3-(prop-1-en-1-yl)-trisulfide, and (E)-1-methyl-3-(prop-1-en-1-yl)trisulfide. With the growing interest in gastronomy in truffles in North America, it is becoming important to gather knowledge for identification purposes and to delineate the key volatile compounds responsible for the aroma of North American truffles, especially the newly harvested *T. canaliculatum*.

INTRODUCTION

Truffles (*Tuber* sp.) are a type of fungus appreciated worldwide for their complex and unique aroma. Their limited seasonal availability, short shelf life, undersupplied market, and, most importantly, unique gastronomic qualities rank them amongst the most expensive luxury food products.

Truffles are underground ectomycorrhizal fungi that develop in symbiosis with the roots of certain tree species and microorganisms. The cultivation of truffles requires a firm, dark brown with white meandering veins at maturity. Its peridium has a brown-to-cinnamon-colored, warty surface. The spores are dark brown, ellipsoid, and covered with alveoli. The gleba is firm, dark brown with white meandering veins at maturity. The overall morphology of *T. canaliculatum*, including the peridium and gleba, is shown in Figure 3. Despite its growing fame, no scientific study has focused on the molecular composition of its aroma.

Various methods have been used to analyze the olfactory profile of truffles. The most widely used sampling method is headspace solid phase microextraction (HS-SPME). It offers many advantages over other traditional extraction methods. It is simple and solvent-free, requires small quantities of the sample, is particularly fast and sensitive, and provides linear results for a wide concentration of an analyte.

United States and Canada. Its culinary values have been recognized by several chefs, and it is a truffle of significant commercial interest. The aroma has been described as strongly pungent but pleasing. *T. canaliculatum* grows in association with a wide range of host trees but particularly members of the Pinaceae and Fagaceae. Its peridium has a brown-to-cinnamon-colored, warty surface. The spores are dark brown, ellipsoid, and covered with alveoli. The gleba is firm, dark brown with white meandering veins at maturity. The overall morphology of *T. canaliculatum*, including the peridium and gleba, is shown in Figure 3. Despite its growing fame, no scientific study has focused on the molecular composition of its aroma.

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of truffles, it is particularly useful considering their short shelf life, 16 the difficulty of supply, and their high cost, which therefore generally requires a rapid analysis of a small quantity of material. It is often combined with gas chromatography—mass spectrometry (GC/MS) for the separation and identification of the analyte.

Herein, we describe the first detailed characterization of the volatilome of fresh T. canaliculatum harvested in Quebec, Canada, by HS-SPME coupled with GC/MS.

RESULTS AND DISCUSSION

The very first characterization of the volatilome of fresh specimens of T. canaliculatum has been performed. Volatile compounds were extracted by HS-SPME and analyzed by GC/MS using conditions adapted from those reported for the characterization of other truffle species. 17 Macromolecular evaluation and genomics analysis confirmed the identification of Tuber canaliculatum. A typical GC/MS chromatogram for the volatilome of T. canaliculatum is shown in Figure 1, and peak assignments are presented in Table 1. Notably, samples I, II, and III of T. canaliculatum led to very similar chromatograms (Figures S2 and S3 in the Supporting Information).

Thirty compounds that were identified in the chromatograms are shown in Table 1. Results are expressed as the relative percentage obtained from the peak area and represent the mean and standard deviation of three replicates. Our results for each of the three truffles illustrate the variability from one specimen to another. The compounds we identified represent between 97.9 and 98.5% of the extracts (in relative proportions to the total integrated peak areas). Major and novel compounds are illustrated in Figure 2.

The aromatic profile of T. canaliculatum is dominated by (E)-1-methylthio-1-propene (57.2–59.0%) and its cis isomer, (Z)-1-methylthio-1-propene (8.5–10.2%). These compounds are said to have an acrid, strong, garliclike odor. 21 Their di- and trisulfide analogues were also identified, namely, (Z) and (E)-1-methyl-2-(prop-1-en-1-yl)disulfide and (Z) and (E)-1-methyl-3-(prop-1-en-1-yl)trisulfide. Overall, the three specimens presented very similar volatilome chemical compositions. Identification of the major compound (E)-1-methylthio-1-propene was carried out from the mass spectrum (Figure S4 in the Supporting Information) and by comparing its LRI with values from the literature. 20,22,23 Indeed, the LRI reported in the NIST database was slightly different.

Several typical volatile eight-carbon compounds have been identified, namely, 1-octen-3-ol (7.8–17.2%), octan-3-one (1.2–2.0%), 3-octanol (0.3–0.5%), octanal (0.2–0.5%), and 1-octen-3-one (0.1–0.2%). These compounds are typically responsible for the so-called fungal smell. They are ubiquitous in fungi of all species and would have key roles in biological processes, from mushroom initiation to response to pathogens and biotic interactions. 24,25 A high variability of the 1-octen-3-ol concentration was observed in the three specimens of T. canaliculatum studied. Indeed, it was more than twice as large in specimen II as in the other two. This variability in four- and eight-carbon volatile compounds (2-butanone and 2-butanol, 1-octen-3-one, 1-octen-3-ol, and trans-2-octenal) is known for T. aestivum, and these variations could be attributed to genetic variations. 26,27

Several other sulfur compounds have been identified in T. canaliculatum, including dimethyl sulfide (1.2–1.7%), dimethyl disulfide (5.8–12.4%), and dimethyl trisulfide (0.8–2.0%). Significant variability in the relative concentration of these compounds has been observed from one specimen to another. Dimethyl sulfide and dimethyl disulfide have been described as having a cabbagelike, rotten smell and dimethyl trisulfide as having a rotten seaweedlike odor. 28 Despite their questionable description, these compounds constitute an important contribution to the aroma of various fruits and cooked vegetables 29 and they are found in many truffles species. 33 Furthermore, the analysis of altered and older specimens showed a greater proportion of these compounds, which gave them a less pleasant odor than the fresh specimens (data not shown). 2,4-Dithiapentane has been found in small amounts (0.1%) in T. canaliculatum. This compound is considered a key compound responsible for the unique aroma of T. magnatum. 30–33 It has also been identified in traces amount

Figure 1. HS-SPME–GC/MS chromatogram of a fresh T. canaliculatum truffle (sample II) from St-Denis-de-Brompton, Québec, Canada. The labels on the signals correspond to the compound number in Table 1.
in several truffles including *T. sinensis*, *T. sinoalbidum*, and *T. sinexcavatum*. Because of its very high odor activity value, low cost, and low toxicity, it is often added to truffle oil and various flavored food products to artificially strengthen the truffle aroma.

Several other volatile compounds that are common to several truffle species were identified in small amounts in *T. canaliculatum*: acetone, 2,2-dimethylpropanal, 2-butanone, 2-methyl-1-propanol, 2-butanol, 3-methylbutanal, 2-methyl-2-butanol, hexanal, heptanal, octanal, and nonanal. Finally, we have also identified in *T. canaliculatum* 3-methyl-2-butanol, 2,3-butanedione, 2,3-pentanedione, and 2-nitropentane. 2-Nitropentane has so far only been identified in *Tuber rufum*. 2- and 3-methylbutanal are known to have a malty smell and a high odor activity value. They have an important role in the distinctive odor of *T. melanosporum*, in which they are found in significant proportion. 2,3-Butanedione also has a high odor activity value and is known to have a buttery smell.

On the other hand, 4-hydroxy-4-methyl-2-pentanone was identified from *T. canaliculatum* specimens. It is often added to truffle oil and various flavored food products to artificially strengthen the truffle aroma. Because of its very high odor activity value, low cost, and low toxicity, it is often added to truffle oil and various flavored food products to artificially strengthen the truffle aroma.
identified in *T. canaliculatum* and *T. macrosporum* included dimethyl sulfide, 1-octen-3-ol, 3-octanone, 3-methyl-butanal, 2-butanone, dimethyl disulfide, and 2-methylpropanal. In contrast to compounds identified in *T. macrosporum* by Strojnik et al., no aromatic compounds were identified in *T. canaliculatum*. Also, the relative proportions of the compounds identified in the two truffles were very different. Even if some similarities could be highlighted between *T. macrosporum* and *T. canaliculatum*, our results showed that the latter has a volatilome that differs notably from what is currently known for other truffles. Indeed, it comprises an unusually high concentration of 1-(methylthio)-1-propene, a unique proportion of major volatile compounds, and the presence of six compounds not identified until now in truffles. Although it was not possible to assign an olfactory value or a culinary value from the volatilome composition, it was reasonable to presume that *T. canaliculatum* has a distinctive aroma associated with its unique chemical composition of volatile compounds.

The local sampling done for this study hints at the natural variability of the volatilome of *T. canaliculatum* truffles; despite the limited number of specimens, we still do not know much about how the volatilome of *T. canaliculatum* varies according to the geographical position of the samples or their maturity. As well, the olfactory profile of truffles has a direct dependence on the mode of preservation used, and the preservation time of truffles varies from one species to another. The study of the variation of the volatile molecules of a specimen as a function of the age and mode of preservation would make it possible to identify the key molecules involved in the degradation process of *T. canaliculatum* to establish the duration of its preservation and potentially improve the best preservation practices. Such studies have already been carried out on various species of truffles, but this would be the first for *T. canaliculatum*.

Despite its advantages, extraction by HS-SPME also has its shortcomings, particularly that it is highly selective for the volatile compounds with high affinity with the fiber coating, which results in partial extraction of the olfactory profile. In particular, HS-SPME is known to be more selective toward highly volatile compounds. Other methods such as direct solvent extraction/solvent-assisted flavor evaporation (DSE-SAFE) and simultaneous distillation-extraction (SDE) require solvents, larger samples (20 g), and are more time-
consuming than HS-SPME. Nevertheless, they offer the advantage of extracting less volatile, higher-molecular-weight compounds. For this reason, they are considered complementary techniques and should be considered in further studies.

It is also worth noting that the relative proportions presented here were obtained by MS, which is not considered a good detection mode for semiquantitative analysis due to (1) its lack of repeatability over time, (2) the fact that the number of ions produced is specific to each analyte, and (3) the number of ions is often not linearly related to the concentration of the compound. Therefore, these proportions are presented only to give an idea of the order of magnitude and cannot be considered as exact values.

Although this study gives a fingerprint of the volatilome of *T. canaliculatum*, it does not make it possible to determine the real contribution of each individual chemical to the human-sensed aroma. For that purpose, other detection methods will need to be used such as olfactometry and electronic nose. These methods are particularly useful to identify the distinctive smell and contribution of each molecule to the overall aroma of the truffle.

Nonetheless, the detailed characterization of the volatilome of *T. canaliculatum* highlights the main volatile compounds responsible for its characteristic odor. The diversity of truffles in Quebec is still little known. A study on micromammal stools in Quebec has identified at least 12 species of truffles by DNA analysis, including species unknown and/or never listed in the territory. These results suggest that there are many more species yet to be discovered. The present studies will be very useful in comparing the volatilome of other species and demonstrating the richness of fungal diversity in the Quebec region. With the growing interest in truffles worldwide, the current results are significant both scientifically and economically.

**EXPERIMENTAL SECTION**

**Samples.** *T. canaliculatum* specimens were harvested on October 7, 2021 at Arborinnov truffle farm, in St-Denis-de-Brompton, Québec, Canada (N 45°26’49” W 72°2’54”; altitude 270 m). The truffles were found with the help of a Jack Russel dog at a location near an American hazel, *Corylus americana*, and a northern red oak, *Quercus rubra* (Figure S1 in the Supporting Information). Specimens are shown in Figure 3. Freshly found truffles were placed in a paper bag in a cooler filled with ice packs covered with cardboard to avoid direct contact with the ice. The specimens were then stored in a refrigerator at 8 °C and analyzed within 24 h. Three mature specimens of 1.8, 2.9, and 3.7 g, free from alteration, were used. The three truffles (named specimens I to III) are illustrated in Figure 3.

**Identification of Truffles.** Identification of specimens was done by macroscopic evaluation by Mr. Jérôme Quirion, co-owner of Arborinnov, and by genomic analysis.

**DNA Extraction and PCR Amplification.** DNA was extracted from 100 mg (wet weight) of each truffle’s sample using the DNeasy plant mini kit (Qiagen, Mississauga, Ontario). The internal transcribed spacer (ITS) of the nuclear ribosomal DNA (rDNA) was amplified using universal primers ITS-1F (5’-CTTGGTCATTAGAGGAAGTAA-3’50) and ITS-4 (5’-TCCTCCGCTATTGATATGC-3’51). Polymerase chain reaction (PCR) was performed to amplify DNA. A quantity of 0.1 ng of DNA was added to a mix containing 20 mmol·L⁻¹ Tris–HCL, 50 mmol·L⁻¹ KCl, 1.5 mmol·L⁻¹ MgCl₂, 0.2 mM dNTP, 0.1 mg·mL⁻¹ BSA (Roche, Basel, Switzerland), 0.2 μmol·L⁻¹ each primer (Sigma-Aldrich, St-Louis, Missouri), and 0.025 U·μL⁻¹ Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California) for a total reaction volume of 20 μL. PCR amplifications were performed with a PTS-225 thermocycler (MJ Research, Waltham, Massachusetts) under the following cycling parameters: initial
denaturation at 95 °C for 2.5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 55 s, and extension at 72 °C for either 45 s (cycles 1–13), 2 min (cycles 14–26), or 3 min (cycles 27–30), and a final extension at 72 °C for 10 min. PCR products were visualized by gel electrophoresis with 1.5% agarose stained with ethidium bromide. We included a negative control in each PCR batch to confirm the absence of contamination.

**ITS DNA Sequencing and Analyses.** Sanger sequencing was performed on a 3500 Genetic Analyzer (ThermoFisher) at the Plateforme d’Analyses Génomiques of the Institut de Biologie Intégrative et des Systèmes (PAG-IBIS, Université Laval, Québec, Canada). Sequences were submitted to a BLASTN search against GenBank, and alignment results with ≥99% homology were considered a match for species identification. Alignments with <99% allowed the identification of genus or family groups. The unique sequences generated and used in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/nuccore/OM948973).

**Headspace Solid Phase Microextraction (HS-SPME).** Analyses were performed using conditions adapted from Diao et al. SMPE fibers coated with a layer of 50/30 μm divinylbenzene/carboxen/poly(dimethylsiloxane) (DVB/ CAR/PDMS) (Supelco) were used for the analysis. Immediately before the analyses, the specimens were brushed gently and washed with demineralized water. Then, ∼0.5 g of fresh truffles was finely cut using a sharp knife and introduced into a 20 mL SMPE vial sealed with a PTFE (silicon/polytetrafluoroethylene) septum cap. The SMPE fiber was conditioned before analysis according to the supplier’s instructions. The vial was immersed in a water bath at 53 °C for 5 min. Once equilibrium was reached, the fiber was then introduced to the vial and exposed to the headspace for 13.6 min. Each truffle was subjected to three analyses to consider potential instrumental variability.

**Volutilome Analysis by Gas Chromatography/Mass Spectrometry (GC/MS).** Sample volatile compounds were identified using a Thermo Scientific GC/MS (Trace GC Ultra with DSO II detector). The analyses were carried out using a nonpolar phase column (DB-5MS 30 m × 0.25 mm × 0.25 μm). The carrier gas was helium, at a flow rate of 1.0 mL min⁻¹, in constant flow mode. Thermal desorption of the compounds was carried out in the GC injector at 230 °C for 5 min in splitless mode. The temperature program is set as follows: 40 °C for 5 min, then increasing at 3 °C-min⁻¹ to 140 °C, then to 220 °C at 12 °C-min⁻¹, and holding at this final temperature for 5 min. The mass range was 40–450 Da. The ionization energy was 70 eV. Thermo Scientific software Excalibur was used for instrument control and acquisition, and Thermo Scientific QualBrowser was used for processing. Identification of volatile compounds was carried out by comparing their mass spectra and linear retention indices to GC/MS commercial spectral libraries (FFNSC 3 (Wiley) and NIST 14). The alkane standard for the linear retention index determination was prepared from a C7-C30 saturated alkane solution (Millipore Sigma, 49451-U). For the C6 standard for retention time determination, HPLC-grade hexanes were used. Linear retention indices were calculated using the equation from van Den Dool and Kratz. The relative area of each compound in the total extracts is expressed as the percentage from the total chromatogram integration recorded and was calculated with the data obtained from GC/MS analyses and manual peak integration without correction factors.

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