Multi-platform metabolomic approach to discriminate ripening markers of black truffles (*Tuber melanosporum* Vittad.)

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Running head: An untargeted metabolomics study for the evaluation of black truffle ripening

ABSTRACT

Black Périgord truffle (*Tuber melanosporum* Vittad.) is a *Tuber* with a black ascocarp and tiny white veins. This hypogeous fruit body has gained widespread attention among chefs for its aroma. Understanding metabolic variation during ripening of truffles can shed lights on truffle biology. In this work, the comprehensive polar and lipid metabolome along with the volatile organic compounds (VOC) of *T. melanosporum*, were studied at different ripening stages by means of a metabolomic approach using gas chromatography-mass spectrometry (GC-MS). Multivariate statistical data analysis indicated that the polar metabolite profile of truffles changed during ripening and that the metabolites that mostly discriminated truffles in the early ripening stages belonged to the classes of saccharides, while free fatty acids, amino acids, among which precursors of VOC, and others were found at higher levels in the late stages of ripening (December-March). PCA of the volatilome indicated that the modifications of the VOC profile did not have a clear pattern upon ripening. Interestingly, dimethylsulfide and dimethyldisulfide characterized most of the samples collected in December-January, while 1-octen-3-ol was in higher level in samples collected in February-March. No relevant differences were seen in the lipid profile of truffle during ripening. GC-MS based metabolomics can be a powerful tool to study the impact of ripening on polar metabolites and VOC profiles of *T. melanosporum*.

Key Words: ascomycete fungus, XCMS, black truffle, OPLS-DA, *Tuber melanosporum*.

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INTRODUCTION

Truffles are hypogeous ascomycete fungi which live in symbiosis with roots of various angiosperm and gymnosperm species. The black Perigord truffle or black Spanish truffle *Tuber melanosporum* Vittad.) is a highly prized culinary delicacy widely appreciated because of its unique and characteristic aroma. *Tuber melanosporum* truffles can be found in several regions of southern Europe, especially Spain, Italy and France and are harvested with the aid of dogs, which can smell the ripe truffle volatiles underneath the ground surface. Currently, *T. melanosporum* fruiting bodies are obtained from nature and semi-artificial cultivation and have a limited harvesting season during winter.

To increase our biological understanding of this organism and to uncover biomarkers of authenticity, freshness, and maturation, several studies have been carried out focusing on different molecular pools. For their peculiar roles in truffles, VOCs have been amply studied and reviewed (Splivallo et al., 2011; Culleré et al., 2010; Vahdatzadeh & Splivallo, 2018; Vita et al., 2018). Volatiles emitted by truffles mediate the interaction with plants, insects, and mammals, and their content greatly varies during ripening (Splivallo et al. 2011). The volatilome of truffles mainly consists of a blend of aldehydes, alcohols, aromatic and sulfur compounds (Vahdatzadeh et al., 2017). Among the most characteristic volatiles, there are dimethyl sulfide (DMS), 1-octen-3-ol, 2-methylbutanal, 3-methylbutanal, 2-methylbutan-1-ol, and 3-methylbutanol. Metabolites, i.e. the low molecular weight hydrosoluble compounds, in truffles were studied by Mannina et al. (2004) using high-field NMR analytical platform. Longo et al. (2017) proposed an untargeted high-resolution mass spectrometry approach to study metabolites involved in the quality modifications over the storage of *T. melanosporum*. Furthermore, Islam and colleagues (Islam et al., 2013) studied, with a proteomic approach, functional characteristics of proteins in black truffle. Studies of changes in the biochemical characteristics of the black truffle associated to maturation (Harki et al., 2006) and freezing processes (Culleré et al., 2013; Campo et al., 2017) were carried out.

Tuber melanosporum fruiting is a multigene-mediated process that follows organised differentiation patterns and requires several months (Parguey-Leduc et al. 1984, Martin et al. 2010, Zarivi et al. 2015). These patterns can be classified into fruiting induction, typically happening in May-June, sporocarp development (which includes the peridial, veined, ascal and sporal stages), and maturation, typically happening in late autumn and early winter (Montant et al. 1983, Parguey-Leduc et al. 1984, Pacioni et al. 2014, Zarivi et al. 2015). After the mating event, the sporocarp starts to develop and its structure becomes gradually complex as the weight rapidly increases. The sporocarp depends on photosynthetically-derived carbon from the host throughout its development, with the mycorrhizas also playing a key role in nitrogen acquisition of the sporocarp (Hacquard et al

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2013; Le Tacon et al. 2013, 2015). The development of the sporocarp seems to be closely related to tyrosinase expression and cell wall synthesis during the stages with more intense weight increase (Montanini et al. 2011, Zarivi et al. 2011, Balestrini et al. 2012, Hacquard et al. 2013). At the end of the development stage, when the sporocarp has practically achieved its final size, the spores acquire their characteristic pigmentation and the sporocarp develops its unique aroma, with the ensuing senescence processes setting the moment in which dogs can localise the ripe sporocarp.

In this paper, to shed light on truffle biology and on the processes driving aroma production, we studied the comprehensive compositional modifications of black truffle during ripening. To this goal, the polar and lipid metabolome together with the volatilome were characterized by gas chromatography coupled to mass spectrometry followed by multivariate statistical data analysis.

MATERIALS AND METHODS

Chemicals. Analytical standard grade methanol, chloroform, hexane, trichloroacetic acid, pyridine, O-methylhydroxylamine hydrochloride, sodium methoxide 25 wt % in methanol, N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), 2,2,3,3-d_4-succinic acid, potassium chloride, were purchased from Sigma Aldrich (Milano, Italy). Bi-distilled water was obtained with a MilliQ purification system (Millipore, Milan, Italy).

Truffle samples. A total of 55 samples of fresh and healthy ascocarp samples of *T. melanosporum* were collected from October to March (2017-2018) in cultivated truffle-grounds under holm oak trees (*Quercus ilex* L. subsp. *ballota* (Desf.) Samp) in Sarrion (Teruel, Spain), and taxonomically authenticated by morphological features (Riousset et al., 2001). Fresh truffles were washed to remove contaminants. A sample from the inner part of each gleba was taken, lyophilized and kept at -20 °C. For each sample the following data were collected: month of sampling (one sampling every 30 days was performed), the sporocarp development stage according to Zarivi et al. (2011), and whether the dig was found by the dog (aroma is developed at least in one truffle) or the dog passed over and didn´t mark the place, in these cases we carefully excavated the soil until finding the truffles (Tables 1S and 2S).

Sample preparation for GC-MS analysis. Truffle samples were stored into sterile plastic Falcon tubes at -20 °C before analysis. Truffle samples were thawed on ice and ground to a fine powder. Six hundred µL of a mixture of methanol and chloroform (2:1 *v/v*) were added to 100 mg of sample. Samples were sonicated for 15 min and left for 24 h in the dark at room temperature. Further 500 µL of the same mixture were added and samples were ultrasonicated with a Vibracell cell disruptor (Labotal Scientific Equipment, Abu Ghosh, Israel). The ultrasonication was performed twice with 20 s pulses at 60% amplitude (130 W, 20 kHz). Then, 100 µL of aqueous KCl 0.2M and 200 µL of chloroform were added. Samples were vortexed for 10 sec. Samples were then centrifuged at 15294 g for 10 min. A volume of 200 µL of the methanol/water mixture and the organic layer was extracted from each sample and moved into different 1.5 mL sterile glass vials, and dried under a gentle nitrogen stream. The aqueous layer was derivatized adding 40 µL of MSTFA and samples were vortexed. After 30 min at 70 °C, 600 µL of hexane containing 4.15 mg/L of 2,2,3,3-d_4-succinic acid were added as the internal standard and homogenized again before GC-MS analysis (Caboni et al. 2016). Fatty acids of the lipid fraction were methylated using sodium methoxide in anhydrous methanol. To each dried chloroform extract was added 500 µl of hexane and 200 µl of sodium methoxide. The sample was placed in an oven at 55 °C for 30 min. Then, 200 µl of HCl 2N were added, and samples were centrifuged for 5 min at 15294 g. The supernatant was placed in a glass vial for the GC-MS analysis.

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GC-MS analysis. The derivatized samples were analyzed with a Hewlett Packard 6850 Gas Chromatograph, 5973 mass selective detector, and 7683B series injector (Agilent Technologies, Palo Alto, CA), using helium as carrier gas at 1.0 mL/min flow. One µL of each sample was injected in the split-less mode and resolved on a 30 m × 0.25 mm × 0.25 µm DB-5MS column (Agilent Technologies, Palo Alto, CA). Inlet, interface, and ion source temperatures were 250, 250 and 230 °C, respectively. Oven starting temperature was set to 50 °C, final temperature to 230 °C with a heating rate of 5°C/min for 36 min and then for 2 min at a constant temperature. Electron impact mass spectra were recorded from m/z 50 to 550 at 70 eV. Chromatograms in the AIA format were then uploaded to the XCMS Online platform (Tautenhahn, Patti, Rinehart, & Siuzdak, 2012). The output pf XCMS consisted of a list of >4000 features for the aqueous fraction and 60 for the lipid fraction. Each feature corresponded to the area value of an *m/z* ion at a specific retention time value.

The identification of metabolites was performed by mass spectra comparison with analytical standards and NIST14 library database of the National Institute of Standards and Technology (Gaithersburg, MD) and Golm library (http://gmd.mpimp-golm.mpg.de/).

HS-GC-MS analysis. The aromatic profile was analyzed by static headspace (HS) technique by using a Turbomatrix HS16 sampler (PerkinElmer, Massachusetts, USA). Four grams of sliced truffle were placed in 20 mL vials and hermetically closed. Samples were heated at 120 ºC for 20 min and 1 min of pressurization time. The injection was carried out over 6 s at 20 psi and an inlet temperature of 220 ºC. HS Sampler was connected to a Clarus 500 Gas Chromatography system coupled with a Mass Spectrometer (PerkinElmer, Massachusetts, USA) equipped with a DB-Wax capillary column (60m x 0.25mm i.d.x 0.25 µm film thickness) (Agilent Technologies, California, USA). A flow of 1 mL/min was used with helium as a carrier gas. The oven temperature was 45ºC held for 2 min, 45-110ºC at a rate of 7 ºC/min, and finally to 225 ºC at 10 ºC/min, and held for 5 min. The mass spectrometer used the electron impact (EI) mode with an ionization potential of 70 eV and an ion source temperature of 200ºC. The interface temperature was 220ºC. The mass spectrometer scanning was recorded in full scan mode (40-300 m/z). A TurboMass ver. 5.4.2 software was used for controlling the GC-MS system. Peak identification of the volatile components was achieved by comparison of the mass spectra with mass spectral data from the NIST MS Search Program 2.0 library and by comparison of previously reported Retention Index (RI) with those calculated using an n-alkane (C7-C25) series under the same analysis conditions.

Multivariate statistical data analysis (MVA). The GC-MS data were submitted to Multivariate statistical analysis (MVA) as implemented in SIMCA-P+ software (version 14.1, Umetrics, Umeå, Sweden). Prior to MVA, GC features were mean centred and scaled to unit variance column-wise.

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Principal component analysis (PCA) was performed to investigate sample distributions, deviating features and prevailing trends. The partial least squares-discriminant analysis (PLS-DA) and its orthogonal variant (OPLS-DA) were performed for classification of samples and identification of the most discriminant variables. The variable importance in projection (VIP) scores, that summarize the contribution of each variable to the model, were analyzedand only those metabolites having VIP values > 1 were deemed as discriminant between the classes.

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RESULTS

Polar metabolites. At first, GC-MS data of the hydro soluble fraction of truffles were submitted to PCA. Sample distribution in the first 2 PC (score plot Figure 1A) evidenced that samples can be divided into two main classes, one composed by samples harvested in October-November and the other by those harvested from December to March. This sample's behavior suggests that the metabolite levels in truffles mainly depend on the month of harvesting, to note, the influence of other characteristics, such as sporocarp development stage and the ability of dogs to find samples, were unfruitfully explored (see score plots in Figure 1SA and B, supporting information). In the corresponding loading plot (Figure 1B) we highlighted some of the GC-MS features ascribed to the metabolites that mostly influenced sample distribution. The list of detected metabolites is reported in Table 1. An OPLS-DA was carried out comparing the two classes i.e. samples collected in October-November vs those collected from December to March. The metabolites discriminating samples from December to March were mainly organic acids, polyols, free fatty acids, and free amino acids. While the metabolites discriminating samples from October-November were: mono and disaccharides, phenol and a highly discriminant not annotated metabolite at 16.96 min (Table 2). As reported in Tables 1S and 2S, December-March is the best period for harvesting mature truffles (sporocarp developmental stage VI-b,c), probably in these months the polar metabolites reach a well defined profile, different from immature truffles.

VOC analysis. VOC data are reported in Table 3. As we can see, different main classes of compounds were identified, in agreement with the fact that black truffles are regarded as those having the most intense aroma of all the truffles (Wang et al., 2011). A PCA was carried out for the VOC data (Figure 2). The score plot of Figure 2A showed a different sample distribution with respect to that reported for the polar metabolites (Figure 1A). In fact, samples did not form well defined groups; however, most of the samples collected in December-January and in February-March differentiated from the others that, instead, tightly clustered. Interestingly, in the score plot when we used the same color for those samples found in the same dig (Figure 2S), we observed that samples found in the same dig are next to each other, therefore they have a similar VOC profile. This observation supports the theory of a symbiotic relationship between truffle, soil and microbiome, representing the dig as a small ecosystem.

The analysis of the loading plot (Figure 2B) indicated that the December-January samples were characterized, among others, by more dimethyl sulfide (DMS) and dimethyl disulfide (DMDS), key-compounds evoking the aroma typically associated with fresh truffle, while samples collected in February-March by higher levels of 1-octen-3-ol. This eight carbons volatile compound is

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considered a key odorant in mature truffles (Splivallo et al., 2011) and regulates the interactions with the environment during growth (Holighaus & Rohlfs, 2019).

Lipid fraction. The lipid fraction was also investigated by GC-MS. The multivariate statistical analysis did not show any clear sample grouping; therefore, we can state that under our analytical conditions, the profile of the methoxide derivative fatty acids did not consistently changed upon ripening (see supporting information Figure 3S).

Discussion

The discriminant analysis of the polar metabolites indicated that truffles harvested in December-March have higher levels of the free fatty acids: palmitic acid, linoleic acid, oleic acid, and stearic acid. Unsaturated fatty acids in their free form are the preferential targets of lipid oxidation mechanisms. The enzymatic and non-enzymatic oxidation of linoleic acid produces a number of C8 volatile compounds which are important hormone-like factors that regulate the phenotypic *status* of a fungus, i.e. growth, morphological differentiation and secondary metabolite production (Holighaus & Rohlfs, 2019). It has been postulated that in many filamentous fungi these VOC diffuse in the environment and interact with plants, generally exerting at high concentrations toxic effects (Splivallo et al., 2011), and with invertebrates, regulating the response of the ecological communities (Holighaus & Rohlfs, 2019). One of the products of linoleic acid breakdown is the 1-octen-3-ol, which is amongst the most characteristic C8 volatiles found in truffles (Splivallo et al., 2011), in agreement, this VOC was found in higher levels in our samples collected in February-March. Other C8 VOC can be produced by lipid oxidation, some of them were found in our samples.

Mature truffles (December-March) have higher levels of free amino acids, among which, in agreement with Harki et al. (2006), alanine, serine and glutamine. Other amino acids, precursors of VOC through the Ehrlich pathway, were found upregulated in truffles collected in the last stages, among these, isoleucine and valine. Isoleucine is the precursor of 2-methylbutanal, found upregulated among the VOC of samples collected in December-January. This volatile aldehyde has been found to increase during the maturity of truffles (Harki et al., 2006). Valine is involved in the formation of the VOC diacetyl (2,3-butandione) and 2-methyl-1-propanol, found in higher levels in more mature samples.

Cysteine, cystathionine, and homocysteine were found upregulated in the truffles of late harvesting (December-March). These compounds are key steps of sulphur metabolism in *T. melanosporum* fruiting body and are precursors of sulfur-containing volatile compounds, such as dimethylsulfide (DMS) and dimethyldisulfide (DMDS), powerful key odorants characteristic of

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truffles, main attractants of dogs and pigs (Martin et al., 2010; Splivallo et al., 2011). Many sulphur-containing compounds have very low olfactory detection limits and are thus major contributors to the final aroma of truffle fruitbodies (Splivallo et al., 2011). It has been reported that, during fruit body development, the number of sulfur-containing volatile compounds increases, indeed, no sulfur compounds have been identified in completely immature fruit bodies (Zeppa et al., 2004). In agreement, higher levels of DMS and DMDS were found in our samples collected in December-January.

Among VOC we found furfural, this aldehyde probably derived from the soil fungi (Leff et al., 2008). Among GC-MS polar metabolites, we found ergosta-5,7,22-triene. Ergosterol is the primary sterol of mushrooms and is one of the most important mycochemicals in *T. melanosporum* (Harki et al., 1996). Ergosterol has antioxidant, anti-inflammatory and antitumor properties. According to other authors, ergosterol seems to exhibit hypocholesterolemic effects, like the bioactive phytosterols (Longo et al., 2017).

These overall results suggest that at the first stage of ripening lipolytic and proteolytic enzymes acted on the macromolecular classes yielding primary metabolite products (i.e. fatty acids and amino acids), that in turn are broken-down to a plethora of VOC. These bottom down products of the maturation process regulate the interactions with the environment, plants and animals, and also give the peculiar aroma that elevates Black Périgord truffle to one of the most prized and appreciated food delicacy.

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Caption of figures

Figure 1. PCA of hydrophilic metabolites. A) score plot, explained variance 35%; B) loading plot: light blue circles = unk1; dark green circles = disaccharides; dark blue circles = *scyllo*-inositol; dark yellow circles = serine, cystathione and cysteine; violet circles = alanine; red circles = valine; grey circles = polyols. Red 4 point star = phytosterols. Brown boxes = glutamine; light green boxes = linoleic acid. Light blue triangles = erythrose; brown triangles = palmitic acid.

Figure 2. PCA of volatile metabolites. A) score plot, explained variance 48 %; B) loading plot, only discussed metabolites are reported.

352 Table 1. GC-MS characteristics of truffle metabolites.

Metabolite	RT[a]	Mass (*m/z*)		
Unk1 [b]	14.29	72	87	69
Unk2	15.05	147	73	174
2,3-butanediol	15.10	117	147	73
Unk3	15.35	174	130	75
Lactic acid	15.62	117	147	191
Glycolic acid	15.88	147	66	205
Alanine	16.45	116	73	147
Glycine	16.78	102	73	103
Unk4	16.96	130	188	144
β-lactic acid	17.24	147	219	177
β-hydroxybutyric acid	17.54	147	117	191
Unk5	17.71	73	188	144
2-aminobutyric acid	17.76	130	147	204
Unk6	18.12	147	221	117
Unk7	18.47	130	202	174
Valine	18.51	144	218	147
Urea	18.86	147	189	171
Leucine	19.39	158	147	232
Phosphate	19.45	299	73	314
Unk8	19.60	180	136	299
Isoleucine	19.71	158	218	45
Proline	19.75	142	147	102
Succinic acid	19.95	147	75	247
Glyceric acid	20.29	189	292	205
Fumaric acid	20.41	245	147	75
Serine	20.71	204	218	100
Threonine	21.09	218	117	291
Unk9	21.2	261	158	129
3,4-dihydroxybutanoic acid	21.66	233	189	117
Unk10	21.83	57	117	147
Unk11	21.86	273	147	175
Homoserine	21.9	218	128	103
Aminomalonic acid	22.18	179	105	147
Malic acid	22.40	233	245	133
Erythrose	22.72	170	147	217
Unk12	22.73	147	217	205
Pyroglutamic acid	22.76	156	147	258
Aspartic acid	22.81	232	100	218
Unk13	22.84	174	84	75
2,3,4-trihydroxybutyric acid	23.17	292	220	205
Cysteina	23.22	218	220	243
5,2-dihydroxy-4-pyran-4-one	23.46	271	147	45
Unk14	23.82	244	219	103
Glutamine	23.97	246	73	128

Glutamic acid	23.96	246	147	128
Phenylalanine	24.06	192	218	147
Arabinoic acid	24.20	117	189	217
Unk15	24.38	245	147	231
Homocysteine	24.49	234	128	119
Asparagine	24.57	116	231	132
Unk16	24.63	290	129	217
Unk17	24.83	275	117	133
Unk18	25.06	260	217	128
Ketoglutaric acid	25.95	292	103	217
Isocitric acid	26.24	273	147	363
Fructose	26.3	217	204	437
Unk19	26.73	100	204	133
Monosaccharide1 [c]	26.78	189	147	74
Unk20	26.84	218	117	157
Monosaccharide2	26.95	205	147	117
Xilytol	27.30	217	103	205
Mannitol	27.55	319	205	103
Ribitol	27.50	217	103	319
Glucitol	27.58	319	205	217
Altrose	27.90	217	191	319
Monosaccharide3	27.96	191	204	271
Scyllo-inositolo	28.06	318	305	191
Talofuranose	28.14	217	103	191
Palmitic acid	28.30	117	313	132
Monosaccharide4	28.79	147	217	318
Myo-inositol	29.06	217	305	191
Linoleic acid	29.83	337	67	81
Oleic acid	29.86	117	55	129
4,4-dimethyl-N-(2-phenylethyl)androst-2-en-17-amine	29.91	314	160	105
Cystathionine	30.01	218	128	278
Stearic acid	30.09	117	341	132
Unk21	30.89	299	342	227
Disaccharide1	33.04	361	217	103
Disaccharide2	33.44	361	217	103
Disaccharide3	34.33	361	147	217
Ergosta-5,7,22-triene	38.00	69	363	337

a) retention time; b) unk = not identified; c) saccharide = compounds with fragmentation pattern ascribable to mono- or disaccharides.

ha formattato: Francese (Francia)

Table 2. Pair-wise OPLS-DA (1+3 components, $R^2Y = 0.95$, $Q^2Y = 0.87$) of GC-MS data of samples collected in October-November *vs.* December-March. VIP values of discriminant metabolites.

December-March			October-November		
RT	Metabolite	VIP	RT	Metabolite	VIP
27.50	Ribitol	1.88	16.96	Unk4	1.94
15.35	Unk3	1.84	28.79	Monosaccharide4	1.77
24.49	Homocysteine	1.81	15.35	unk3	1.70
21.66	3,4 di-hydroxybutanoic acid	1.79	15.17	Phenol	1.63
18.12	Unk6	1.76	33.04	Disaccharide1	1.54
23.22	Cysteine	1.75	34.33	Disaccharide3	1.50
28.30	Palmitc acid	1.73			
22.72	Erythrose	1.68			
29.83	Linoleic acid	1.67			
30.89	Unk21	1.67			
23.97	Glutamine	1.64			
23.03	2,6-di-tert butyl phenol	1.61			
27.58	Glucitol	1.60			
29.86	Oleic acid	1.59			
22.18	Amino malonic acid	1.55			
15.88	Glycolic acid	1.50			
20.71	Serine	1.49			
16.45	Alanine	1.47			
20.29	Glyceric acid	1.46			
22.73	Unk12	1.33			
17.24	beta-lactic acid	1.31			
19.71	Isoleucine	1.31			
30.09	Stearic acid	1.30			
18.51	Valine	1.26			
30.01	Cystathionine	1.25			

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363 Table 3. VOC profile by HS-GC/MS of truffle samples. Area?

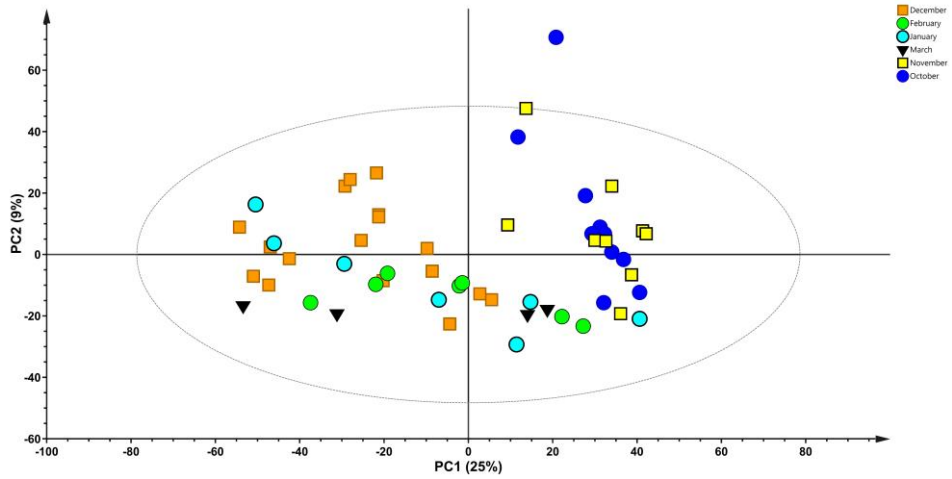
			October		November		December		January		February		March	
RT	CAS	compound	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Acids														
16.80	64 19 7	Acetic acid	242308	251381	1251611	1245438	224003	131624	266257	372080	640073	320268	727626	582577
18.11	79 09 4	Propanoic acid	7693	10554	7047	9225	21002	33332	36649	47489	183025	136229	89745	6559
18,60	79 31 2	Isobutyric acid	157958	205055	85074	92791	490478	621202	579534	750682	2242188	1345699	1191754	756373
19.92	116 53 0	2-methylbutanoic acid	216205	208573	184337	148740	1117251	1731619	860002	892395	5471132	3932371	2373925	2504057
		Sum	624163		1499712		1852734		1742442		8536419		4383049	
Alcohols														
7.21	64 17 5	Ethanol	16661903	11474348	9034105	11879060	20604236	17161874	38988319	39598689	26302718	43538180		
8.62	71 23 8	1-propanol	161622	117474	82765	101471	1130625	1472642	2103466	2108929	3081885	1343922	1470108	1036805
9.92	78 83 1	2-methyl-1-propanol	831961	890801	692469	690014	11745220	17228722	23737632	26141223	66282175	29723397	44038750	########
12.49	123 51 3	3-methyl-1-butanol	158598	240328	20804	32615	546293	1671051	53974	81759	16181	26040	1927	
8.31	78 92 2	2-butanol	68234	65703	69453	65914	4826562	5109181	5573921	5284964	10680038	6594760	5291305	1504136
11.82	71 36 3	Butanol			7887	3012	7577	3915					1159	
11.84	137 32 6	2-methylbutan-1-ol	46506	26300	37645	20585	105752	183936	35125	9653	2168	2234	753	680
6.51	625 31 0	4-penten-2-ol	23085556	16617594	14435286	19014262	22936174	19573992	50360748	51410788	83628450	19615888	72920200	
10.24	6032 29 7	2-pentanol			83397	108644	228073	265362	551745	486818	572292	226973	406724	376121
13.35	71 41 0	1-pentanol	7338	4330	11226	10426	12615	10592	26321	23446	2526	2103		
11.75	107 18 6	Allyl alcohol			10068	14728	17741	24655			98613	50778	48761	43527
15.22	111 27 3	1-hexanol	109905	44421	71941	62464	170136	131907	502870	505111	1086748	815852	962893	52534
12.73	626 93 7	2-hexanol							3468173	3941080	6190731	3269192	3017013	3152532
16.17	13231 87 7	3-methyl-1-hexanol	11597	9781	6290	5110	6660	7513	10288	8087	48267	42107	18234	13567
15.87	589 98 0	3-octenol	52553	31566	30063	23125	27785	30645	38546	48043	132115	86341	66052	41073
16.78	3391 86 4	1-octen-3-ol	172669	109781	178985	91766	197393	159792	52793	33792	520759	272267	227687	68941
18.32	111 87 5	1-octanol	3955	2474	9840	13972	12818	9797	80349	178527	47106	41257	81303	46166
		Sum	41239890		24750142		62006493		123498212		185532129		92091224	
Aldehydes														

3.98	75 07 0	Acetaldehyde	136338420	87780137	228754156	97989715	248156694	60045052	282681000	48799332	153897475	121357369	239338000	########
5.27	123 38 6	Propanal	75973	65000	80360	54780	1188410	1930536	683530	692520	2187070	1820272	199513	210302
5.52	78 84 2	Isobutyraldehyde	770597	230862	2286958	2218224	11786337	12720582	9287697	4963657	9036758	5878871	2047815	689365
5.88	123 72 8	Butanal			3959	1840	270577	317204	387621	358221	382236	158144	345127	123779
6.12	96 17 3	2-methylbutanal	897935	563276	623344	481359	11395622	18859558	2715425	2150193	13201601	8753348	3111645	2722722
6.19	590 86 3	Butanal-3-methyl	592567	346720	447246	266881	2188054	2366133	1385467	1080248	4396446	2908513	1517155	132519
13.45	97 96 1	2-ethylbutanal	43029	28535	44866	27839	27582	19816	30835	25734	6227	5234		
7.86	110 62 3	Pentanal	23420	10977	38161	27590	54034	34590	59044	27570	52176	32965		
9.56	66 25 1	Hexanal	462628	180003	454925	153623	695435	432461	359041	150552	1068697	460806	403358	17898
11.84	111 71 7	Heptanal	125924	51324	94268	54470	98151	72588	80330	44622	55496	34319	3873	
14.64	124 13 0	Octanal			74247	22611	52149	37472	43972	27905	65180		265024	165938
15.77	124 19 6	Nonanal			4031	7698					37862	20963	11677	
17.81	2463 53 8	2-nonenal	7391	7981	4337	2883	2112	1535			11853	8634	4765	
18.27	100 52 7	Benzaldehyde	6470	6351	12918	11055	99381	90640	54205	70969	137333	45901	152869	165573
		Sum	139335009		232822142		275176078		297755603		184372333		247390663	
Ketones														
11.98	110 43 0	2-heptanone	66571	52363	84800	40009	87779	52798	70738	11398				
13.99	111 13 7	2-octenone	13461	11514	12311	4631	19398	15533	10268	7103	19802	10033	10325	2848
14.42	513 86 0	3-hydroxybutan-2-one	103276	165530			377487	704063	432959	858273	3981284	2128113		
5.52	67 64 1	2-propanone	631917	149983			11033679	11609860	9252007	4869735	8614743	6532302	2346275	410822
5.55	67 64 1-2	Acetone			2327305	2231751					1085820		2346275	410822
5.89	78 93 3	2-butanone	93079	46127	168999	70361	8112888	10821776	13662113	12641176	19450355	9108301	22132200	
7.79	431 03 8	2,3-butadione	208518	74709	270879	70298	1049281	762117	1360577	679068	1891010	913538	743202	531261
8.61	107 87 9	2-pentanone	77005	68113	36512	48666	615165	790700	850823	828787	2386308	1070558	1070971	769586
9.33	600 14 6	2,3-pentadione			36019	26413	465397	578954	928861	731339	1289858	556850	715054	553546
10.72	625 33 2	3-penten-2-ona			44859	54829	50733	66477	33516	32968	274142	172690	18971	7456
16.69	32064 72 5	2-nonen-4-one	4018	4877	3967	3094	2564	5239	688	315	22166	20782	872	
18.20	4643 27 0	2-octen-4-one	508	500	4576	4781			9542	16520	1948	1347	1370	697
		Sum	1042367		2969447		20954705		26532077		36560508		18318977	
Esters														

	6.81	589 402	1-methylpropyl formate	66446	65655	167650	176360	11053949	15329979	4406537	3997119			
	9.42	108 645	Ethyl-3-methylbutanate	6599	5637	3797	3096	4276	4192	7616	1436	11573	7252	16840	1664
	9.79	97 85 8	2-methylpropyl-2-methylpropanoate	38519	15485	28482	7466	35014	24855	13752	5800	7734	4727	5975	1745
	12.46	2445 67 2	Isobutyl-2-methylbutanoate	792874	938319	273515	595306	7580234	23703742	8829090	14891499	396812	450910	57224	40192
	12.72	2050 01 03	Isoamyl isobutirate	386993	760794	706573	862028	16938		24755440	49505707	87776836	50636219	37434810	########
	14.38	2762530	Isopentyl-2-methylbutanoate	470	172	1078	736	2106	2860	1988	2110	24993	24329	1851	593
	5.52	108 22 5	Isopropenyl Acetate	787384	237845	2356672	2302041	11947480	12867628	9509641	5083343	9329920	5950001	2180850	655078
	8.99	7452 79 1	Ethyl-2-methylbutanoate	17441	12825	21388	11955	325287	391010	331632	219161	633150	286466	374693	394971
			Sum	1855248		3043786		28759064		37242957		98179084		40072242	
Phenols															
	16.78	100 84 5	3-methylanisol	363	358	254	196	4835	5741	37575	40495	30361	42308	3095	3967
	15.13	100 66 3	Anisole					0				250966	134775	274982	286536
	19.34	99 71 8	4-sec-butylphenol	2254	2581			1938	3838	30742	67008	19921	25617	1081	71
			Sum	2618		254		4596		62950		301247		279158	
Furans															
	12.72	3777 69 3	2-pentylfuran	236425	305477	91342	46808	113337	90031	116248	50146	16728	17671	13853	17688
	14.19	70424 14 5	2-pentenylfuran			189	52	94	87	147	15	6246	13651	128	
	17.19	98 01 1	Furfural	13176	9529	13223	9576	17859	9507	36121	38563	78812	74456	64616	6836
	19.84	98 00 0	Furfuryl alcohol	199233	174565	115496	80863	98371	64388	173948	198864	469769	297788	656811	87492
			Sum	377907		199910		222501		326379		567122		735344	
Sulfur compounds															
	4.02	74 93 1	Methanethiol	2084762	175005	1821922	550385	7175281	15597020	175076	185889	23867975	9474193	6523334	7911629
	4.97	75 18 3	Dimethyl sulfide	75480	57205	113568	139079	17779407	13794841	32689384	24047857	9758283	4633220	17231985	########
	9.54	624 92 0	Dimethyl disulfide	522	444	1675	1900	88704	140588	25875	20299	114199	116162	51774	53585
			Sum	2160712		1936421		25037848		32890335		33740457		23807093	

Hydrocarbons														
4.42	504 60 9	1,3-pentadiene	742	464	1097	869	8810	11523	13105	14798	61806	95564	7859	6803
8.24	563 79 1	2,3-dimethyl-2-buten	8314	9781	34250	35348	3978	5057			9080	2595		
14.86	691 37 2	4-methyl-1-pentene	5149	3792	21884	41793	9991	19032	1200	1164	71100	41896	91895	13953
14.97	63830 6	4-octen-2,3,6-trimethyl			416	379	801	916	158	30	2680	2812		
		Sum	8504		29268		17416		14008		131703		99754	
Aromatic hydrocarbons														
21.17	151 10 0	Dimethoxybenzene					385	265	1312	1575	3883	5446	111	
21.76	494 99 5	3,4-dimethoxytoluene			131	20	255	118	1073	439	2453	3807	110	4
25.12	6443 69 2	3,4,5-trimethoxytoluene					1045	1977	1575	2173	5343	4509	316	43
		Sum			131		1233		2947		11372		481	

Figure 1A and B



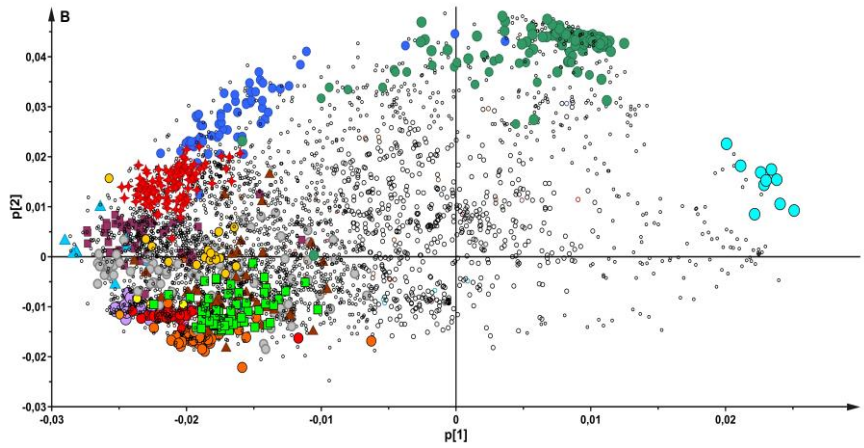
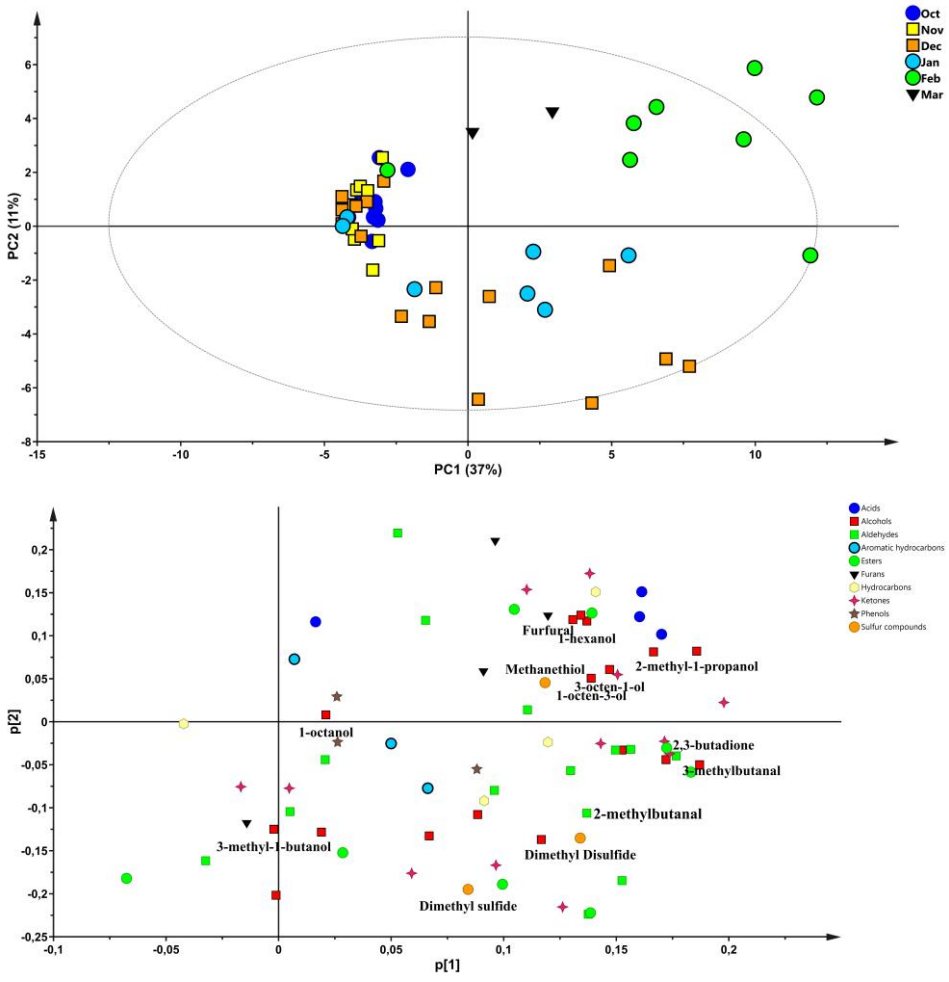


Figure 2A and B



Supporting information

Table 1S. Truffle sample characteristics.

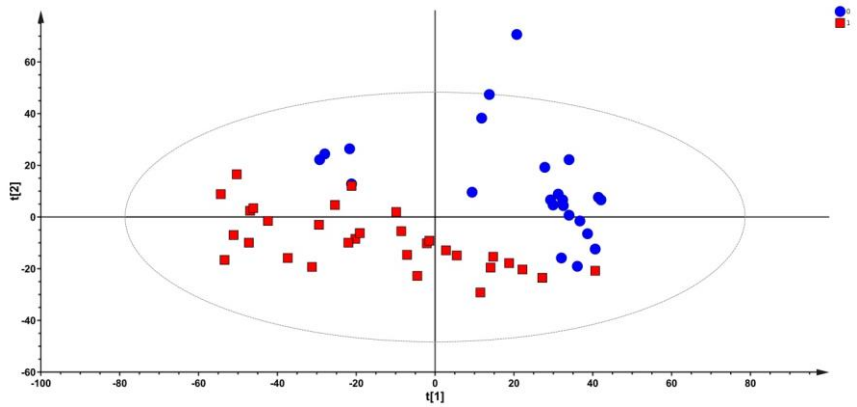
Month	# samples	# detected by dog	sporocarp developmental stages
October	10	0	IV;V;VI-a
November	9	0	VI-a
December	17	13	VI-a;VI-b,c
January	7	7	VI-b,c
February	7	7	VI-b,c
March	4	4	VI-b,c

Table 2S. Truffle sample characteristics.

Stage	# samples	# detected-dog	Description
IV	1	0	Tissue with asci and without spores
V	4	0	Asci with spores in which intracellular content can be observed
VI-a	20	5	Spores in which intracellular content cannot be observed (developed episporium) hyaline to light yellow (not fully melanized)
VI-b,c	30	30	Approx. 80% of the spores are fully melanized (brown coloured)

Figure 1S: GC-MS polar metabolites: PCA score plot, samples colored by A) detected (1) or not detected (0) by dogs; B) sporocarp development stages: IV (4), V (5), VI-a (6), VI-b,c (7). See Table 1 for details.

A



B

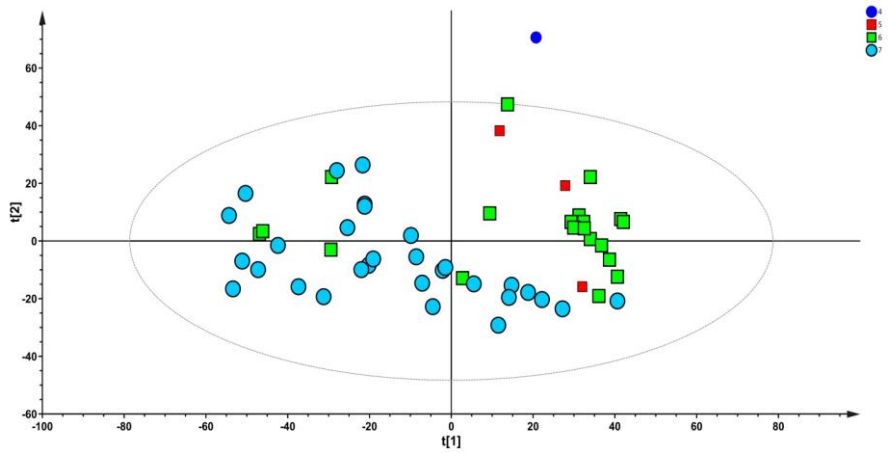


Figure 2S: GC-MS volatile metabolites: PCA score plot, truffle samples found in the same dig have same colors. bottom DMS

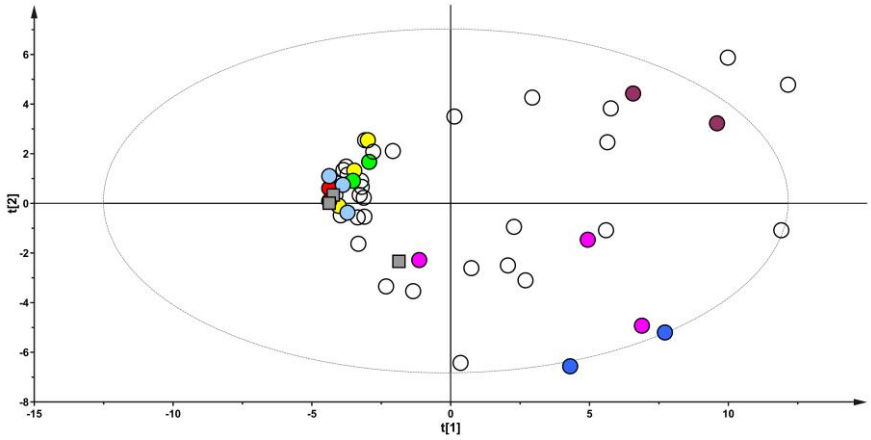
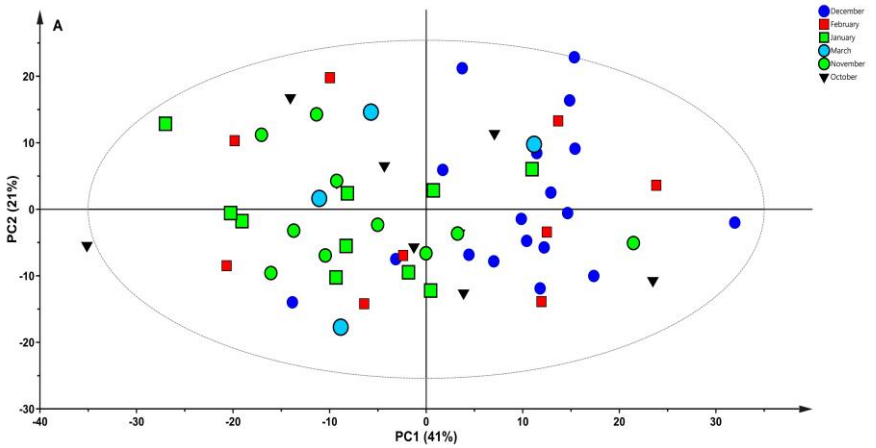
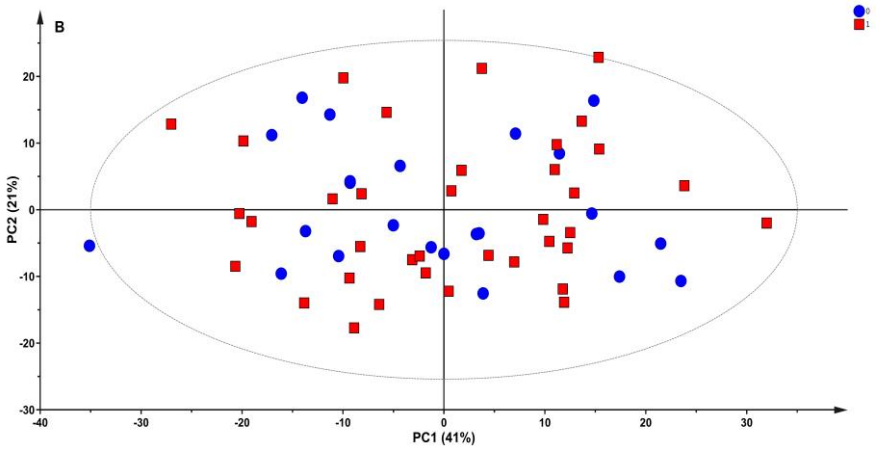
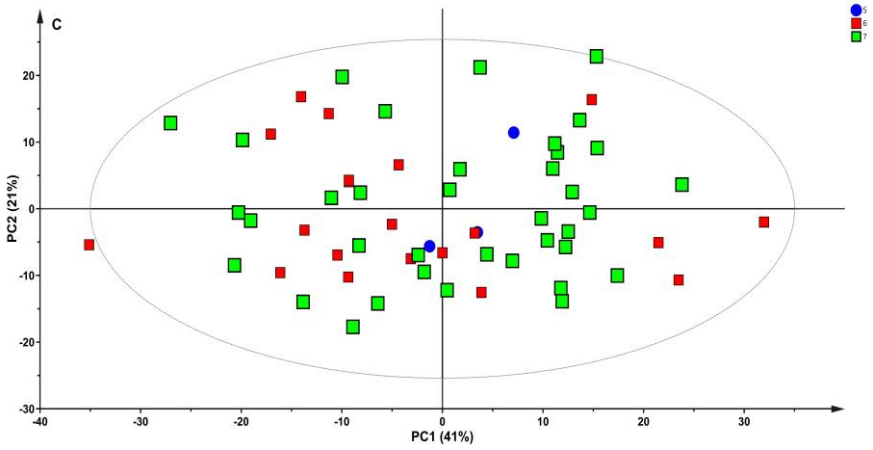


Figure 3S: GC-MS lipid fraction: PCA score plot, samples colored by A) month of harvesting; B) detected (1) or not detected (0) by dogs; C) sporocarp development stages: IV (4), V (5), VI-a (6), VI-b,c (7), see Table 1 for details.






C
PC2 (21%)
PC1 (41%)
20
10
0
-10
-20
-30
-40
-30
-20
-10
0
10
20
30