Fresh refrigerated Tuber melanosporum truffle: effect of the storage conditions on the antioxidant profile, antioxidant activity and volatile profile

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Abstract

The antioxidant properties of fresh black truffles (*Tuber ~~melanosporum)~~melanosporum*) (T.) refrigerated at ~~4 °C~~ 4 °C under different packaging conditions: air atmosphere ~~(A),~~ (*A*), reduced pressure ~~(V),~~ (*V*), mix 1% O_2/99% N_2 (ON) and mix 40% CO_2/60% N_2 (CN) were evaluated during ~~28 d~~ 28 days of storage. The results were compared with the volatile fingerprint determined by SPME-GC/MS and with the phenolic antioxidants characterized by ~~HPLC-MS/MS.~~ HPLC–MS/MS. The total antioxidant content and the antioxidant activity decreased during the storage, regardless of the packaging method. The principal ~~components~~ component analysis (PCA) showed that the total antioxidant content was well correlated with low storage times, high antioxidant activity and three volatile aldehydes. In contrast, 2-propyl-butanoate and 2-nonen-1-ol inversely correlated with the antioxidant activity, as well as gentisic acid, which was the only phenolic antioxidant which increased during the storage. V, CN and ON were always better strategies than *A* to preserve the freshness of ~~T.~~ *T*. However, *V* is a valid and cheap alternative to normal atmosphere to prolong the shelf life for up to ~~two weeks.~~

~~**Keywords::** black~~

2 weeks.

Keywords: Black truffle, ~~antioxidants~~ Antioxidants, ~~volatile~~ Volatile compounds, ~~storage~~ Storage, ~~modified~~ Modified atmosphere packaging

Introduction

During the storage, alteration phenomena of fresh products are mainly due to loss of texture and ~~colour,~~ color, structure modification, microbial growth and ~~off-flavours~~ off-flavors formation [1]. Black truffles (*Tuber melanosporum* Vitt.) are hypogeous fungi of great value for their unique and peculiar aroma. After collection, the qualitative and sensory characteristics are preserved unaltered for a very limited period. Truffles are seasonal products. Only by applying a suitable preservation technique the ~~shelf-life~~ shelf life of fresh truffles can be extended to satisfy the market requests, and many studies have been made in this direction [2].

The oxidation process decreases consumer acceptability of a food product by forming ~~off-flavour,~~ off-flavor, as well as by degrading nutrients, and it produces toxic compounds and dimers or polymers of lipids and proteins. Oxidation in foods can be reduced by removing pro-oxidant compounds such as free fatty acids, metals and oxidized compounds, and by protecting foods from light, air, by applying reduced pressure or by adding antioxidants [3].

Saltarelli et al. [4] investigated refrigeration ~~(4 °C),~~ (4 °C), freezing ~~(-20 °C)~~ (−20 °C) and sterilization as methods of preservation of the more common truffles: *Tuber magnatum*, *Tuber borchii*, ~~*Tuber*~~ *T. melanosporum* and *Tuber aestivum.* Sugars, proteins, enzymes involved in the metabolism of the fungi and mesophilic bacteria were evaluated and the results showed that the best storage conditions for the preservation of the biochemical and microbiological parameters was the refrigeration at ~~4°C.~~ 4 °C. In 2010, Rivera et al. [5] have investigated the possibility to extend the ~~shelf-life~~ shelf life of the truffle (~~*Tuber*~~ *T. melanosporum* and ~~*Tuber*~~ *T. aestivum*) by combining modified atmosphere packaging (MAP) and refrigeration. Later, Amadoro et al. [6] showed that chitosan and ultrasound treatments, used individually or combined with ~~MAP (Modified Atmosphere Packaging),~~ modified atmosphere packaging (MAP), may be an interesting alternative for the storage and postharvest treatment of truffles (~~*Tuber*~~ *T. aestivum*).

The antioxidant activity of raw truffles (*Terfezia claveryi* and *Picoa juniperi*) was reported by Murcia et al. [7]. *Tuber claveryi* exhibited a more potent activity than ~~*Picoa*~~ *P. juniperi*. Moreover, freezing affected the radical scavenging activity in a limited way and the canning process caused a significant loss of activity. In the ~~2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic~~ 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) test, *Tuber claveryi* showed ~~TEAC (trolox~~ trolox equivalent antioxidant ~~capacity)~~ capacity (TEAC) values of 4.77, 3.57 and 2.52 for raw, frozen and canned samples, respectively, while TEAC values of 3.91, 2.57 and 0.56 were found for raw, frozen and canned ~~*Picoa*~~ *P. juniperi* samples, respectively.

The antioxidant activity of the ethanol crude extract of *Tuber indicum* and its sub-fractions (petroleum ether, ethyl acetate, *n*-butyl alcohol and residue) was also investigated [8]. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) ~~radical-scavenging~~ radical scavenging activity was found to be in the order *n*-butyl alcohol ~~fraction > residue > ethanol crude extract > ethyl acetate fraction > petroleum~~ fraction > residue > ethanol crude extract > ethyl acetate fraction > petroleum ether fraction, with efficient concentration (EC_{50}) values in the range ~~1.53-2.87 mg/mL.~~ 1.53–2.87 mg/mL. The ethyl acetate fraction also exhibited the highest ferrous ion chelating activities, with a EC_{50} value of ~~0.70 mg/mL.~~ 0.70 mg/mL. More recently, Beara et al. [9] investigated the total phenolic content and antioxidant activity of methanol and water extracts of black summer (~~*Tuber*~~ *T. aestivum*) and white (~~*Tuber*~~ *T. magnatum*) truffles. The total phenol content varied moderately between the extracts, from 11.7 to ~~18.7 mg~~ 18.7 mg gallic acid equivalents (GAE) per ~~g~~ gram of dry matter. Differences among the total phenols content and profile in the extracts could be explained by the different extraction conditions applied [10]. The radical scavenging activity of the extracts ranged between inhibition concentration (IC_{50}) values of 149 and ~~192 µg/mL.~~ 192 µg/mL. In another study, ~~*Tuber*~~ *T. melanosporum*, ~~*Tuber*~~ *T. aestivum* and ~~*Tuber*~~ *T. indicum* were investigated for their total phenols content and inhibition of ABAP-induced lipid peroxidation [11]. ~~Analysed~~ Analyzed truffles contained between 1 and ~~2 mg~~ 2 mg of phenols per ~~g~~ gram of dried matter and showed inhibition of lipid oxidation with the following trend: ~~*Tuber*~~ *T. aestivum* ~~> *Tuber indicum* > Tuber~~ > *T. indicum* > *T. melanosporum*.

The aim of this work was to evaluate the effect of different ~~modified atmosphere packaging (MAP)~~ MAP conditions on the antioxidant properties of fresh samples of ~~*Tuber*~~ *T. melanosporum* stored at ~~4 °C~~ 4 °C for ~~28 days.~~ 28 days. The antioxidant properties ~~(Folin-Ciocalteu,~~ (Folin–Ciocalteu, ABTS, DPPH, FRAP, ferric reducing antioxidant power and Fe-chelating assay), the volatile compounds and the phenolic profile were determined.

Materials and methods

Samples

Intact samples of ~~*Tuber*~~ *T. melanosporum* Vitt. were harvested in the hilly areas of the Marche Region (Central Italy) with the collaboration of a private company (Acqualagna Tartufi, PU). The fresh samples were manually cleaned and brushed with a wet soft brush and put in polypropylene (PP) vessels ~~(140×175×46~~

~~mm)~~ (140 × 175 × 46 mm) containing an absorbent pad. Each vessel contained approximately ~~100g~~ 100 g of fresh intact truffles. A thermo-sealing machine mod. Rio 39 from Saccardo (Thiene, Italy) was used to pack the vessels with three different atmosphere ~~composition~~ compositions and under reduced pressure. The package was sealed with an antifog film from Nutripack (Milan, Italy), consisting of a polyester film of ~~12 µm~~ 12 µm adherent to a polypropylene film weldable on PP homopolymer vessels.

The composition of the different atmospheres was the following: air atmosphere ~~(A)~~ (*A*) (control); partial vacuum ~~(V);~~ (*V*); mixture of 1% O_2 ~~/ 99%~~ /99% N_2 (ON), mixture of 40% CO_2 ~~/ 60%~~ /60% N_2 (CN). The sealed vessels were weighed, refrigerated and stored at ~~4 °C~~ 4 °C in the dark until analysis. Samples were investigated at time 0 and after 7, 14, 21 and ~~28 d~~ 28 days of storage.

Chemicals and reagents

All reagents used in this study were purchased from Sigma-Aldrich S.p.A. (Milan, Italy) while solvent of analytical grade were obtained from VWR International s.r.l. (Milan, Italy).

Antioxidant activity

Preparation of extracts

An aliquot ~~(5 g)~~ (5 g) of truffles samples was weighed and blended at slow speed using a laboratory mill (M20 Universal mill, ~~IKA®-Werke~~ IKA®-Werke GmbH & Co. KG, Staufen, Germany). The samples were mixed with ~~10 ml~~ 10 ml of ethanol 96% (v/v) and were kept away from the light at room temperature for ~~24 h.~~ 24 h. Successively, samples were filtered and ethanol was newly added. The mixture was kept in the dark for other ~~24 h.~~ 24 h. After the second filtration, the extracts were gathered into an Erlenmeyer flask and the solvent was removed in a rotary evaporator (BUCHI Italia s.r.l). Finally, the extract was dissolved into ~~5 ml~~ 5 ml of ethanol 96% (v/v).

Determination of total antioxidant content

The total antioxidant content was determined ~~by~~ using the ~~Folin-Ciocalteu~~ Folin–Ciocalteu procedure [12]. Each ethanolic extract was mixed with ~~0.2 mL Folin-Ciocalteu reagent, 2 mL~~ 0.2 mL of Folin–Ciocalteu reagent, 2 mL of water and ~~1 mL~~ 1 mL of 15% Na_2CO_3. After incubation for ~~2 h~~ 2 h at ~~25°C~~ 25 °C, the absorbance was measured at ~~765 nm (UV-Vis~~ 765 nm (UV–Vis Jenway 6300 spectrophotometer, Bibby Scientific ~~Ltd,~~ Ltd., UK). The total antioxidant content was expressed as mg GAE per ~~g~~ gram of dry extract.

$ABTS^{\cdot+}$ ~~decolourization~~ $^{\cdot+}$ decolorization assay

The $ABTS^{\cdot+}$ assay was based on the method of Loizzo et al. [12], with slight modifications. The $ABTS^{\cdot+}$ solution, produced from the reaction of ABTS 7 ~~mM~~ and ~~2.45 mM~~ 2.45 mM potassium persulfate, was stored in the dark at room temperature for ~~12 h~~ 12 h before use and diluted with ethanol to reach an absorbance of ~~0.70 ± 0.05 at 734 nm.~~ 0.70 ± 0.05 at 734 nm. An aliquot of ~~25 µL~~ 25 µL of extracts at different concentrations were added to ~~2 mL~~ 2 mL of diluted $ABTS^{\cdot+}$ solution and the absorbance was measured after ~~6 min~~ 6 min at ~~734 nm.~~ 734 nm. Ascorbic acid was used as positive control.

DPPH· radical scavenging activity assay

The radical scavenging activity was determined according to the technique reported by Loizzo et al. [12]. Extracts at different concentrations were added to an ethanolic solution of DPPH radical (final concentration was ~~0.1 mM).~~ 0.1 mM). The bleaching of DPPH was determined by measuring the absorbance at ~~517 nm.~~ 517 nm. Ascorbic acid was used as positive control.

Fe^{2+} chelating assay

The chelating activity of the samples was measured following the procedure previously described by Loizzo et al. [13]. Briefly, the extract, $FeCl_2$ ~~(2 mM)~~ (2 mM) and FerroZine™ ~~(5 mM)~~ (5 mM) were mixed and left at room temperature for ~~10 min.~~ 10 min. The absorbance of the Fe^{2+}-FerroZine™ complex was measured at ~~562 nm.~~ 562 nm.

FRAP assay

The FRAP reagent, containing ~~2.5 mL~~ 2.5 mL of ~~10 mM~~ 10 mM tripyridyltriazine (TPTZ) solution in ~~40 mM~~ 40 mM HCl, ~~2.5 mL~~ 2.5 mL of ~~20 mM~~ 20 mM $FeCl_3$ and ~~25 mL~~ 25 mL of ~~0.3 M~~ 0.3 M acetate buffer (pH 3.6) was freshly prepared [12]. An aliquot ~~(2.5 mg/mL)~~ (2.5 mg/mL) of samples was dissolved in ethanol and a ~~0.2 mL~~ 0.2 mL of this solution was mixed with ~~1.8 mL~~ 1.8 mL of FRAP reagent. Ethanol solutions of $FeSO_4$ ~~(50-500 mM)~~ (50–500 mM) were used to obtain the calibration curve. The absorbance of the reaction mixture was measured at ~~595 nm~~ 595 nm and the FRAP value was expressed as mM Fe(II)/g. Butylated hydroxytoluene (BHT) was used as positive control.

Volatile aroma profile

SPME-GC/MS was used to characterize the volatile profile of refrigerated black truffle. The procedure [14–17] was adapted to the conditions of the present experiment. Black truffles ~~(1.5 g)~~ (1.5 g) were finely ground using a mill (M20 Universal mill, ~~IKA® Werke~~ IKA®-Werke GmbH & Co. KG, Staufen, Germany). The powder was introduced in a 10-mL vial and closed with a screw cap equipped with an elastomeric septum. The vial was placed in a heating bath at ~~40°C~~ 40 °C for ~~10 min.~~ 10 min. After this time, the SPME ~~fibre (Divinylbenzene/Carboxen/Polydimethylsiloxane, 1 cm, 50/30 µm)~~ fiber (divinylbenzene/carboxen/polydimethylsiloxane, 1 cm, 50/30 µm) from Supelco/Sigma-Aldrich (Milan, Italy) was introduced into the vial and exposed to the headspace of the sample for ~~15 min.~~ 15 min. Thermal desorption of the compounds from the ~~fibre~~ fiber took place in the GC injector at ~~220 °C~~ 220 °C for ~~15 min.~~ 15 min. The injection was performed in the splitless mode (splitless time ~~0.3 min)~~ 0.3 min) at ~~220°C.~~ 220 °C. The ~~GC-MS~~ GC–MS runs were performed with a Varian 3900 ~~gas-chromatograph~~ gas chromatograph coupled to a Saturn 2100T (Varian, Walnut Creek, CA, USA) ion trap mass spectrometer. The chromatographic separation was performed on a TG-5MS capillary column (Thermo Scientific, ~~30m × 0.25mm~~ 30 m × 0.25 mm I.D., film thickness ~~0.25 µm).~~ 0.25 µm). The oven temperature program started at ~~40 °C~~ 40 °C during ~~10 min,~~ 10 min then was raised to ~~180 °C~~ 180 °C at a rate of ~~3°C/min~~ 3 °C/min and reached ~~250 °C~~ 250 °C at a rate of ~~15 °C/min.~~ 15 °C/min. The transfer line and trap temperatures were set at ~~200 °C.~~ 200 °C. The mass spectra were recorded in full scan mode (mass range ~~31-250 m/z)~~ 31–250 *m/z*) with a scan rate of 1 ~~scan/sec.~~ scan/s.

The volatile compounds were ~~analysed~~ analyzed with the Varian Workstation software. The identification was confirmed with the chromatographic retention index and the NIST library mass spectra (Version: 2.0; 2002) of pure standard substances.

Phenolic profile by HPLC-HRMS and ~~HPLC-MS/MS~~HPLC–MS/MS

Sample preparation

The ethanol extracts used for the antioxidant assays were stored at ~~-80 °C.~~ −80 °C. For each sample, a ~~50 mg.mL~~ 50 mg mL^{-1} solution was prepared (in triplicate) in the mobile phase A of the HPLC system and vigorously stirred. No further purification was performed.

~~***HPLC****-HRMS*~~ *HPLC-HRMS analysis*

The HPLC-HRMS system used consisted of a Thermo Sci. Q-Exactive HRMS instrument coupled to an Agilent 1260 HPLC with a 16 channel DAD detector. The separation was carried out at a flow rate of ~~1 mL min~~ 1 mL min^{-1} with a ODS Hypersyl C18 LC column ~~(125 mm × 4.6 mm i.d., 5 µm,~~ (125 mm × 4.6 mm i.d., 5 µm, Thermo Sci.) protected with a HPLC pre-column filter (Thermo Sci.). A contact closure electronic board allowed ~~to interface~~ interfacing the HPLC with the mass spectrometer. The mobile phase consisted of a combination of solvent A ~~(20 mM~~ (20 mM ammonium formate, 0.1% formic acid in water, v/v) and B (sat. ammonium formate, 0.1% formic acid in acetonitrile, v/v). The gradient was as follows: from 5% B at ~~0 min~~ 0 min to 25% B (v/v) at ~~21 min,~~ 21 min, then to 95% B at ~~22 min~~ 22 min until ~~27 min,~~ 27 min, to 5% at ~~28 min,~~ 28 min, followed by a re-equilibration step (5% B) at ~~32 to 35 min.~~ 32–35 min. The DAD recorded spectra from 210 to ~~600 nm~~ 600 nm and provided real-time monitoring at ~~280 nm (+/- 2 nm).~~ 280 nm (±2 nm). A post-column flow splitter was used to feed both analyzers in parallel (DAD and HRMS) at a fixed ratio. For ~~Full-MS~~ full MS analysis, the mass spectrometer was operated in negative ionization mode using the following conditions: sheath gas at 20 (arbitrary units), aux gas at 5 (arbitrary units), aux temperature ~~250~~

~~°C,~~ 250 °C, spray voltage at ~~-3.5 kV,~~ −3.5 kV, capillary temperature at ~~320 °C~~ 320 °C and RF S-lens at 65. The mass range selected was from 100 to ~~1000~~ 1000 *m/z* with a ~~Full-MS~~ full MS set resolution of ~~70000 (@200 m/z),~~ 70,000 (@200 *m/z*), AGC target at 1e6, max. injection time of ~~200 ms.~~ 200 ms. Eighteen substituted phenolic acids, cinnamic acids and flavan-3-ol standards were, singularly and in their mix, analyzed prior to the samples analysis.

HPLC-FullMS-dd-MS/MS analysis

Data dependent ~~HPLC-MS/MS~~ HPLC–MS/MS experiments were run in negative ionization mode: ~~FullMS~~ full MS parameters were kept as shown, MS/MS AGC target 3e5, max. injection time 200, FTMS set resolution ~~17500,~~ 17,500, loop count 5, isolation window ~~3 m/z,~~ 3 *m/z*, isolation offset ~~1 m/z,~~ 1 *m/z*, and normalized collision energy ~~45 eV.~~ 45 eV. For data dependent settings: minimum AGC target 3e3, apex trigger ~~2 to 8 sec,~~ 2–8 s, charge exclusion ~~3 — 8~~ 3–8 and higher, dynamic exclusion ~~10 sec,~~ 10 s, "if idle" setting set to "pick others". Lock masses were included in the instrument method in both positive and negative mode. 18 substituted phenolic acid, cinnamic acids and flavan-3-ol standards were singularly analyzed prior to the samples analysis.

Data acquisition and processing

The ~~HPLC-DAD~~ HPLC–DAD data were collected and analyzed by OpenLab software while the MS data and results were collected and analyzed by Xcalibur 3.1 software and Compound Discoverer (Thermo Sci.). MzCloud ~~on-line~~ online database and fragmentation list prepared by us by standard injection were also employed for the compounds' identification and confirmation.

Statistical analysis

All samples were ~~analysed~~ analyzed in triplicate. Data were expressed as ~~mean ± standard~~ mean ± standard deviation and were ~~analysed~~ analyzed using one-way analyses of variance (ANOVA). Significant differences between groups were determined with ANOVA with Bonferroni correction. A *P* value ~~< 0.05~~ <0.05 was considered significant. Correlation of phenolic antioxidants relative abundances ~~(HPLC-MS~~ (HPLC–MS EIC integrations) with the antioxidant activity assays and volatiles was done by multivariate analysis ~~(PCA)~~ [principal components analysis (PCA)] using Unscrambler 10.4 software (Camo Software, 2016 ed., Oslo, Norway). All the variables were normalized before elaboration.

Results and discussion

Antioxidant activity

Several in vitro methods were developed for measuring the antioxidant status of food products. These tests differ in the generation of radicals ~~and/or~~ or target molecules, and in the way end points are measured. Due to ~~the~~ in vivo oxidative process complexity, the application of a single method for the assessment of the oxidation state is not enough, since the level of a single antioxidant in food does not necessarily reflect the antioxidant activity. For this reason, choosing an adequate assay is critical [18]. The antioxidant activity of truffle samples under different package condition and storage time was assessed ~~by~~ using several in vitro methods, such as ABTS, DPPH, Fe-chelating and FRAP assay. A concentration–response relationship was observed for all tested samples ~~(**Tables 1-3**~~(Tables 1, 2, 3).

The total antioxidant content was evaluated by the ~~Folin-Ciocalteu~~ Folin–Ciocalteu method. The experimental data ~~(**Table 1**~~(Table 1) revealed that the total antioxidant content decreased during the storage regardless of the packaging adopted. The ON samples exhibited the significantly highest total antioxidant content with values ranging ~~48.6-33.3~~ 48.6–33.3 GAE per ~~g~~ gram of dry extract from 7 to 28 ~~d~~ days of storage, respectively. The correlation analysis revealed a Pearson's correlation coefficient of 0.96 for ON samples.

The total antioxidant content on day 7 and day 14 was significantly lower in the ~~A,~~ *A*, *V* and CN samples than ON. Afterwards, on day 21, the antioxidant content was higher in the ON sample than in ~~A,~~ *A*, *V* and CN. On day 28, the ON samples showed the highest total antioxidant content with respect to the others.

There is a limited number of investigations regarding the antioxidant content of truffles. The species studied were *Terfezia boudieri* [19, 20], ~~*Tuber*~~ *T. aestivum* [9, 11, 21], ~~*Tuber*~~ *T. magnatum* [9], ~~*Tuber*~~ *T. melanosporum* and ~~*Tuber*~~ *T. indicum* [11]. Literature data were barely comparable because of different

extraction methods and unit of measurement. At our knowledge, only the paper of Villares et al. [11] reported data about the phenolic content of truffles. Values of 1.88, 1.52, and ~~1.20 mg~~ 1.20 mg GAE per ~~g~~ gram of dried matter were found for ~~*Tuber*~~ *T. aestivum*, ~~*Tuber*~~ *T. indicum* and ~~*Tuber*~~ *T. melanosporum*, respectively.

The radical scavenging ability was evaluated with the DPPH and ABTS tests ~~(**Table 2**~~(Table 2). Generally, a reduction in bioactivity was found during the storage period (from 7 to 28 ~~d~~ days of storage). The significantly highest ABTS radical scavenging ability was observed in the *V* sample after 7 and ~~14 days~~ 14 days of storage (with IC_{50} values of 1.8 and ~~3.4 µg/mL,~~ 3.4 µg/mL, respectively). On day 21, the CN samples showed the significantly highest ABTS radical scavenging ability. Afterwards, on day 28, the *V* samples showed the significantly highest ABTS activity.

Tuber melanosporum packed with CN maintained the radical scavenging activity significantly higher than truffle packed with ON from 7 to 28 ~~d~~ days of storage.

The same trend was registered also in the DPPH assay. The sample *V* on day 7 and 14 showed the highest DPPH radical scavenging activity with IC_{50} value of 55.7 ~~µg/mL~~ and ~~63.1 µg/mL,~~ 63.1 µg/mL, respectively.

The samples in *A* atmosphere showed the significantly lowest ABTS radical scavenging ability and lowest DPPH radical scavenging activity from 7 ~~d~~ to 28 ~~d.~~ days. Among the investigated methods of packaging, the CN samples preserved the antioxidant compounds better than *A* and ON samples.

Truffles packaged in different way were also subjected to Fe-chelating activity assays ~~(**Table 3**~~(Table 3). During the Fenton reaction, iron is involved into redox processes that ~~generates~~ generate oxygen free radical [22]. One of the possible ~~approach~~ approaches to limit ROS is to reduce iron. Generally, the ability of extracts to chelate iron decreased during the storage. The Fe-chelating activity was determined by measuring the formation of the Fe–FerroZine™ complex, and these activities were compared with the chelating activity of EDTA (IC_{50} value of ~~1.3 µg/ml).~~ 1.3 µg/ml). The fresh truffle extract exhibited the highest activity with IC_{50} value of ~~21.7 µg/mL~~ 21.7 µg/mL followed by ON after 7 and 14 ~~d~~ days of storage (IC_{50} value of 29.5 ~~µg/mL~~ and ~~38.9 µg/mL,~~ 38.9 µg/mL, respectively). On days 21 and 28, CN samples showed the significantly highest Fe-chelating activity, whereas the *A* samples showed the significantly lowest activity from 7 to 28 ~~d~~ days of storage.

The redox potential of phytochemicals plays a crucial role in determining the antioxidant properties of the extract. For this reason, the FRAP assay was performed. The fresh truffle extract showed a FRAP antioxidant power 1.4-time higher than those reported for the positive control, BHT. Noteworthy, the ON sample at 7 ~~d~~ days of storage exhibited a FRAP value quite similar to the positive control.

On 14 and ~~21 days~~ 21 days of storage, ~~A ,~~ *A*, *V* and ON samples showed significantly higher FRAP antioxidant power than CN samples. On 28 ~~d~~ days of storage, ON samples showed a significantly higher FRAP antioxidant power than the *V* and CN samples ~~(**Table 3**~~(Table 3).

PCA

The main identified volatile compounds found in the samples were alcohols (1-octen-3-ol, 2-nonen-1-ol), aldehydes (2,2-dimethyl-4-octenal, heptanal, octanal, 2-methyl-3-methylidenecyclopentane-1-carbaldehyde, octenal), a ketone (3-(5-methyl)-heptanone), one ester (2-propyl-butanoate) and benzene derivatives (methoxybenzene, 3-methyl-methoxybenzene, 1,2-dimethoxybenzene) ~~(**Table 4**~~(Table 4).

Besides, five phenolic acids were identified by ~~HPLC-HRMS~~ HPLC–HRMS analysis and are shown in ~~**Table 5**~~Table 5.

The results of the PCA are displayed in ~~**Fig. 1**~~Fig. 1. The biplot of ~~**Fig. 1**~~ Fig. 1 is built on a model accounting for 52% of the total variance (PC1 and PC2). It shows the clustering of the samples and the loading of variables related to the antioxidant activity tests, the phenolic acids and the volatile compounds. The ~~Folin-Ciocalteu~~ Folin–Ciocalteu index correlated with low storage times, with four of the phenolic acids (gallic, ~~*p*-hydroxybenzoic,~~ *p*-hydroxybenzoic, *o*-hydroxybenzoic and a dihydroxybenzoic acid isomer) and with three aldehydes (2,2-dimethyl-4-octenal, 2-methyl-3-methylidenecyclopentane-1-carbaldehyde and octenal). The samples were gradually shifted towards negative values of PC1 according to increasing storage

time. Until the first week, all the samples (but A1) were positioned on the positive side of PC1, which can be associated with freshness. The volatile compounds that inversely correlated with the antioxidant activity of the samples were heptanal, 2-propyl-butanoate and 2-nonen-1-ol. These compounds positively correlated with the normal atmosphere treatment, confirming that this was not the best procedure to store the black truffle; in fact, A1 was the worst sample among those of the first week, as freshness is concerned.

Unexpectedly, gentisic acid (but not its isomer) was the only phenolic acid increasing at week four; thus, its overall trend inversely correlated with the antioxidant activity.

Conclusion

The model built up on the multivariate statistical analysis of volatile compounds, phenolics and antioxidant performance allowed to investigate successfully the effects of the storage conditions on quality of ~~*Tuber*~~ *T. melanosporum*, one of the most expensive fresh food products.

The evaluation of the antioxidant activity of food products is context-dependent since the oxidative stress is a complex process. The capacity and potency of antioxidants, as obtained by various methods, do not necessarily correlate among them [23].

Three volatile compounds can be associated with the high antioxidant properties of freshly packaged truffles. They are all aldehydes: 2,2-dimethyl-4-octenal, 2-methyl-3-methylidenecyclopentane-1-carbaldehyde and octenal. Thus, they can be considered as natural chemical quality markers for the fresh black truffle.

Two volatile compounds can be considered as markers of the decay of the raw black truffles subjected to the storage at refrigerated temperature: 2-propyl-butanoate is an ester produced upon fermentation of food from animal [24] and plant [25] origin; 2-nonen-1-ol is a monounsaturated alcohol commonly present in mushrooms [26] increasing after fermentation [27] or fermented food processing [28]. Both are positively related to the decrease of the antioxidant properties and total antioxidant content of the samples. Gentisic acid (2,5-dihydroxybenzoic acid) along with one of its isomers and *o*- and *p*-hydroxybenzoic acid have been previously found in edible mushroom varieties [29] and in medicinal ones [30]. Moreover, *o*- [31] and *p*-hydroxybenzoic [32] acid derivatives have been also associated to many metabolic inhibition pathways and specifically as inhibitors of tyrosinase enzymatic hydroxylation of phenols into ~~o-diphenols~~ *o*-diphenols and the oxidation of 3,4-catechol derivatives to benzoquinones. Gentisic acid also showed inhibition effects on polyphenol oxidases [33]. The increasing trend of gentisic acid during the storage ~~is however~~ is, however, hard to explain. Tentatively, its oxidation during the storage may be counterbalanced by the degradation of higher molecular weight ~~analogues~~ analogs or by enzymatic hydroxylation of hydroxybenzoic acid derivatives [34].

Compared to the storage of black truffle in a headspace consisting of normal atmosphere, more valid alternatives can be proposed. Reduced pressure seems to be a valid and cheap alternative to the modified atmosphere used in this experiment to prolong the shelf life of raw ~~*Tuber*~~ *T. melanosporum*. This consideration is based on the results related to the extension of the antioxidant properties and native volatile profile of the samples for up to ~~two weeks~~ 2 weeks compared to normal atmosphere. Moreover, the vacuum technology is cheaper than MAP, ~~however~~ however, showing a similar performance.

The extension of the shelf life of raw black truffles from a few days up to ~~two weeks~~ 2 weeks is very important to small and medium enterprises exporting the fresh product to international and overseas markets because it implies lower delivery costs.

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Compliance with ethical standards

Conflict of

~~interest:~~

interest

The ~~Authors~~ authors declare that they have no conflict of interest.

Compliance with ethics

~~requirements:~~

requirements

All authors declare that this article does not contain any studies with human or animal subjects.

References

1. Senesi E, Prinzivalli C, Sala M, Gennari M (2000) Physicochemical and microbiological changes in fresh-cut green bell peppers as affected by packaging and storage. Ital J Food Sci 12:55–64

2. Costa R, Fanali C, Pennazza G, Tedone L, Dugo L, Santonico M, Sciarrone D, Cacciola F, Cucchiarini L, Dachà M, Mondello L (2015) Screening of volatile compounds composition of white truffle during storage by GCxGC-(FID/MS) and gas sensor array analyses. Food Sci Technol-LEB 60:905–913

3. Choe E, Min DB (2009) Mechanisms of antioxidants in the oxidation of foods. Compr Rev Food Sci Food Saf 8:345–358

4. Saltarelli R, Ceccaroli P, Cesari P, Barbieri E, Stocchi V (2008) Effect of storage on biochemical and microbiological parameters of edible truffle species. Food Chem 109:8–16

5. Rivera CS, Blanco D, Salvador ML, Venturini AE (2010) Shelf-Life extension of fresh *Tuber aestivum* and *Tuber melanosporum* truffles by modified atmosphere packaging with microperforated films. J Food Sci 75:E225–E233

6. Amadoro C, Tremonte P, Succi M, Tipaldi L, Pannella G, Coppola R, Sorrentino E, Reale A, Di Renzo T, Di Rienzo M (2013) Chitosano, ultrasuoni e atmosfera modificata per la conservazione del tartufo nero ~~(Tuber~~ (*Tuber aestivum* Vitt.) fresco. Industrie Alimentari 52:25–32

7. Murcia MA, Martínez-Tomé M, Jiménez AM, Vera AM, Honrubia M, Parras P (2002) Antioxidant ~~Activity~~ activity of ~~Edible Fungi~~ edible fungi (Truffles and Mushrooms): ~~Losses~~ losses during ~~Industrial Processing.~~ industrial processing. J Food Prot 65:1614–1622

8. Guo T, Wei L, Sun J, Hou C-L, Fan L (2011) Antioxidant activities of extract and fractions from *Tuber indicum* Cooke & Massee. Food Chem 127:1634–1640

9. Beara IN, Lesjak MM, Cetojević-Simin DD, Marjanović ZS, Ristić JD, Mrkonjić ZO, Mimica-Dukić NM (2014) Phenolic profile, antioxidant, anti-inflammatory and cytotoxic activities of black (*Tuber aestivum* Vitt.) and white (*Tuber magnatum* Pico) truffles. Food Chem 165:460–466

10. Yim HS, Chye FY, Ho SK, Ho CW (2009) Phenolic profiles of selected edible wild mushrooms as affected by extraction solvent, time and temperature. Asian ~~Journal of~~ J Food ~~and Agro-Industry~~ Agro-Ind 2:392–401

11. Villares A, García-Lafuente A, Guillamón E, Ramos Á (2012) Identification and quantification of ergosterol and phenolic compounds occurring in *Tuber* spp. Truffles. J Food Compos Anal 26:177–182

12. Loizzo MR, Pacetti D, Lucci P, Núñez O, Menichini F, Frega NG, Tundis R (2015) *Prunus persica* var. *platycarpa* (Tabacchiera Peach): bioactive compounds and antioxidant activity of pulp, peel and seed ethanolic extracts. Plant Foods Hum Nutr 70:331–337

13. Loizzo MR, Said A, Tundis R, Hawas UW, Rashed K, Menichini F, Frega NG, Menichini F (2009) Antioxidant and antiproliferative activity of *Diospyros lotus* L. extract and isolated compounds. Plant Foods Hum Nutr 64:264–270

14. Bellesia F, Pinetti A, Bianchi A, Tirillini B (1998) The volatile organic compounds of black truffle ~~(Tuber melanosporum.~~ (*Tuber melanosporum.* Vitt.) from middle Italy. Flavour Fragr J 13:56–58

15. Díaz P, Ibáñez E, Señoráns FJ, Reglero G (2003) Truffle aroma characterization by headspace solid-phase microextraction. J Chromatogr A 1017:207–214

16. Cullerè L, Ferreira V, Chevret B, Venturini ME, Sanchez-Gimeno AC, Blanco D (2010) Characterisation of aroma active compounds in black truffles (*Tuber melanosporum*) and summer truffles (*Tuber aestivum*) by gas chromatography-olfactometry. Food Chem 122:300–306

17. Cullerè L, Ferreira V, Venturini ME, Marco P, Blanco D (2013) Potential aromatic compounds as markers to differentiate between *Tuber melanosporum* and *Tuber indicum* truffles. Food Chem 141:105–110

18. Moon JK, Shibamoto T (2009) Antioxidant assays for plant and food components. J Agric Food Chem 57:1655–1666

19. Hamza A, Zouari N, Zouari S, Jdir H, Zaidi S, Gtari M, Neffati M (2016) Nutraceutical potential, antioxidant and antibacterial activities of *Terfezia boudieri* Chatin, a wild edible desert truffle from Tunisia arid zone. Arab J Chem 9:383–389

20. Dundar A, Yesil OF, Acay H, Okumus V, Ozdemir S, Yildiz A (2012) Antioxidant properties, chemical composition and nutritional value of *Terfezia boudieri* (Chatin) from Turkey. Food Sci Technol Int 18:317–328

21. Kruzselyi D, Vetter J (2014) Complex chemical evaluation of the summer truffle ~~(Tuber aestivumVittadini)~~ (*Tuber aestivum* Vittadini) fruit bodies. J Appl Bot Food Qual 87:291–295

22. Puntarulo S (2005) Iron, oxidative stress and human health. Mol Asp Med 26:299–312

23. Pinchuk I, Shoval H, Dotan Y, Lichtenberg D (2012) Evaluation of antioxidants: ~~Scope,~~ scope, limitations and relevance of assays. Chem Phys Lipids 165:638–647

24. Du M, Ahn DU (2001) Volatile substances of Chinese traditional Jinhua ham and Cantonese sausage. J Food Sci 66:827–831

25. Liu C, Liu Y, Yi G, Li W, Zhang G (2011) A comparison of aroma components of pineapple fruits ripened in different seasons. Afr J Agric Res 6:1771–1778

26. Pinho PGD, Ribeiro B, Gonçalves RF, Baptista P, Valentão P, Seabra RM, Andrade PB (2008) Correlation between the pattern volatiles and the overall aroma of wild edible mushrooms. J Agric Food ~~Chemistry~~ Chem 56:1704–1712

27. Ai-Nong Y, Bao-Guo S (2005) Flavour substances of Chinese traditional smoke-cured bacon. Food Chem 89:227–233

28. Plessas S, Fisher A, Koureta K, Psarianos C, Nigam P, Koutinas AA (2008) Application of *Kluyveromyces* ~~marxianus,~~ *marxianus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *L. helveticus* for sourdough bread making. Food Chem 106:985–990

29. Barros L, Montserrat D, Ferreira ICFR, Baptista P, Santos-Buelga C (2009) Phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different portuguese wild mushrooms species. Food Chem ~~Toxicology~~ Toxicol 47:1076–107930

30. Kim MY, Seguin P, Ahn JK, Kim JJ, Chun SC, Kim EE, Seo SH, Kang EY, Kim SL, Park YJ, Ro HM, Chung IM (2008) Phenolic compound concentration and antioxidant activities of Edible and medicinal mushrooms from Korea. J Agric Food Chem 56:7265–7270

31. Zhang JP, Chen QX, Song KK, Xie JJ (2006) Inhibitory effects of ~~salycilic~~ salicylic acid family compounds on the diphenolase activity of mushroom tyrosinase. Food Chem 95:579–584

32. Chen QX, Song KK, Qiu L, Liu XD, Huang H, Guo HY (2005) Inhibitory effects on mushroom tyrosinase by *p*-alkoxybenzoic acids. Food Chem 91:269–274

33. Zhou Xiong Z, Liu W, Zou L (2017) Different inhibition mechanisms of gentisic acid and ~~cyanidin-3-O-glucoside~~ cyanidin-3-*O*-glucoside on polyphenoloxidase. Food Chem 234:445–454

34. Widhalm JR, Dudareva N (2015) A familiar ring to it: ~~Biosynthesis~~ biosynthesis of plant benzoic acids. ~~Molecular~~ Mol Plant 8:83–97

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Fig. 1 PCA of the antioxidant activity (ABTS, DPPH, FRAP, Fe-chelating and FC), identified antioxidants and volatile compounds ~~(circled variables)~~ (*circled variables*) in relation to different storage conditions. ~~A,~~ *A* air atmosphere; ~~V,~~ *V* reduced pressure; ~~ON,~~ *ON* mix of 1% O_2/99% N_2; ~~CN,~~ *CN* mix of 40% CO_2/60% N_2. The days of storage (d) are reported as follows: 0; time 0; 1, 7 d; 2, 14 d; 3, 21 d; 4, 28 d. Abbreviations used for the identified antioxidants: ~~*pOH-BA* =~~ *pOH-BA* p-hydroxybenzoic acid, *OH-BA* ~~=~~ *o*-hydroxybenzoic acid, *GeA* ~~=~~ gentisic acid, *GaA* ~~=~~ gallic acid, *diOH-BA* ~~=~~ other dihydroxybenzoic acid

Table 1 Total antioxidant content (mg GAE per ~~g~~ gram dry extract) of *Tuber melanosporum* at different storage conditions ~~-~~

~~-~~	Period of storage (days)	*F*	Total antioxidant content (mg/g extract)				
~~**Period of Storage (days)**~~			~~**F**~~	*A*	*V*	CN	ON
0~~54.7~~	~~± 0.8~~54.7 ± 0.8						
7		~~36.7 ± 0.3~~36.7 ± 0.3^{b,d}	~~34.0 ± 0.2~~34.0 ± 0.2^{d}~~34.2~~	~~± 0.3~~34.2 ± 0.3^{d}~~48.6 ±~~	~~0.3~~48.6 ± 0.3^{a,b,c}		
14		~~36.0 ± 0.5~~ 36.0 ± 0.5^{b}	~~29.3 ± 0.2~~29.3 ± 0.2^{a,d}	~~32.0 ± 0.1~~32.0 ± 0.1^{b,d}	~~48.0 ± 0.6~~48.0 ± 0.6^{a,b,c}		
21		~~35.3 ± 0.5~~35.3 ± 0.5^{b,c}	~~29.2 ± 0.3~~29.2 ± 0.3^{c,d}~~24.0~~	~~± 0.1~~24.0 ± 0.1^{c,d}	~~38.1 ± 0.3~~38.1 ± 0.3^{b,c}		
28		~~23.3 ± 0.3~~23.3 ± 0.3^{b,d}~~29.2~~	~~± 0.3~~29.2 ± 0.3^{c,d}~~21.3 ±~~	~~0.3~~ 21.3 ± 0.3^{d}	~~33.3 ± 0.2~~33.3 ± 0.2^{a,b,c}		

Data are expressed as ~~means ± standard deviation (n= 3).~~ mean ± standard deviation (*n* = 3). Values within columns followed by different superscript letters ~~(a,b,c,d)~~ (a, b, c, d) differ significantly from each other at ~~*P* < 0.05~~ < 0.05

F fresh truffle, *A* truffles packed in ambient atmosphere, *V* truffles packed under partial vacuum, *CN* truffle packed with 60% N_2 and 40% $CO_{2,}$ 2, *ON* truffle packaged with the 1% O_2 and 99% N_2

Table 2 Radical scavenging ability of *Tuber melanosporum* (IC_{50} μg/mL) ~~38.5 ± 2.442.4 ± 2.2~~

~~-~~	~~-~~	~~**ABTS (μg/mL)**~~				~~-~~	~~**DPPH (μg/mL)**~~				
~~**Period of Storage (days)**~~	Period of storage (days)	ABTS (μg/mL)					DPPH (μg/mL)				
F		*A*	*V*	CN	ON	*F*	*A*	*V*	CN	ON	
0	38.5 ± 2.4					42.4 ± 2.2					
7		~~138.9 ± 2.7~~138.9 ± 2.7^{b,c,d}	~~1.8 ± 0.3~~1.8 ± 0.3^{a,c,d}	~~41.9 ± 1.1~~41.9 ± 1.1^{a,b,d}	~~84.3 ± 1.6~~84.3 ± 1.6^{a,b,c}		~~> 400~~	~~55.7 ± 1.2~~>400	55.7 ± 1.2^{c,d}	~~71.1 ± 1.6~~71.1 ± 1.6^{b,d}	~~207.2 ± 1.9~~207.2 ± 1.9^{b,c}
14		~~350.3 ± 4.8~~350.3 ± 4.8^{b,c,d}	~~3.4 ± 0.8~~3.4 ± 0.8^{a,c,d}	~~57.1 ± 1.8~~57.1 ± 1.8^{a,b,d}	~~93.1 ± 1.3~~93.1 ± 1.3^{a,b,c}		~~> 400~~	~~63.1 ± 1.6~~>400	63.1 ± 1.6^{c,d}	~~89.2 ± 1.8~~89.2 ± 1.8^{b,d}	~~228.5 ± 1.6~~228.5 ± 1.6^{b,c}
21		~~574.0 ± 4.9~~574.0 ± 4.9^{b,c}	~~85.8 ± 1.5~~85.8 ± 1.5^{a,c}	~~60.0 ± 1.6~~60.0 ± 1.6^{a,b}	~~> 400~~> 400		~~> 400~~	~~84.5 ± 1.9~~>400	84.5 ± 1.9^{c,d}	~~107.2 ± 1.8~~107.2 ± 1.8^{b,d}	~~281.7 ± 2.3~~281.7 ± 2.3^{b,c}
28		~~603.3 ± 4.3~~603.3 ± 4.3^{b,c}	~~98.3 ± 2.3~~98.3 ± 2.3^{a,c}	~~305.3 ± 2.9~~305.3 ± 2.9^{a,b}	~~> 400~~> 400		~~> 400~~	~~151.0 ± 2.2~~>400	151.0 ± 2.2^{c,d}	~~108.1 ± 2.1~~108.1 ± 2.1^{b,d}	~~357.4 ± 2.6~~357.4 ± 2.6^{b,c}

Data are expressed as ~~means ± standard deviation (n= 3).~~ mean ± standard deviation (n = 3). Values within columns followed by different superscript letters ~~(^{a,b,c,d})~~ (a, b, c, d) differ significantly from each other at ~~$P < 0.05$~~ < 0.05

Ascorbic acid in ABTS test IC_{50} ~~1.7 µg/mL,~~ 1.7 µg/mL, in DPPH test IC_{50} ~~2.0 µg/mL~~ 2.0 µg/mL

F fresh truffle, *A* truffles packed in ambient atmosphere, *V* truffles packed under partial vacuum, *CN* truffle packed with 60% N_2 and 40% CO_2, *ON* truffle packaged with the 1% O_2 and 99% N_2

Table 3 Ferrous ions and reducing power of *Tuber melanosporum* extract ~~(µg/mL) (µM 2.5 mg/mL)~~

-	-	Period of storage (days)	Fe-chelating activity (µg/mL)								-	FRAP (µM Fe(II)/g at truffle extract concentration of	~~Period of Storage (days)~~	2.5 mg/mL)
F	*A*		*V*	CN	ON	*F*	*A*	*V*	CN	ON				
0~~2 1.7~~	~~± 2.4~~2				~~4 6.6~~	~~± 2.2~~4								
1.7 ± 2.4						6.6 ± 2.2								
7		~~18 1.6 ± 2.8~~81 81.6 ± 2.8^{b,c,d}	~~88.6 ± 1.88~~8.6 ± 1.8^{a,c,d}	~~56.7 ± 1.45~~6.7 ± 1.4^{a,b,d}	~~29.5 ± 1.02~~9.5 ± 1.0^{a,b,c}		~~92.1 ± 1.89~~2.1 ± 1.8^{b,d}	~~75.1 ± 1.27~~5.1 ± 1.2^{a,c,d}	~~89.1 ± 1.68~~9.1 ± 1.6^{d}	~~67.2 ± 1.16~~7.2 ± 1.1^{a,b,c}				
14		~~30 0.5 ± 2.93~~00.5 ± 2.9^{b,c,d}	~~90.4 ± 1.79~~0.4 ± 1.7^{a,c,d}	~~77.3 ± 1.67~~7.3 ± 1.6^{a,b,d}	~~38.9 ± 1.23~~8.9 ± 1.2^{a,b,c}		~~86.7 ± 1.68~~6.7 ± 1.6^{c} ~~83.2 ± 1.6~~	83.2 ± 1.6^{c}	~~103.5 ± 1.91~~03.5 ± 1.9^{a,b,d} ~~88.6~~	~~± 1.98~~8.6 ± 1.9^{c}				
21		~~42 2.6 ± 4.04~~22	~~18 0.1 ± 2.31~~80	~~90.1 ± 1.99~~0.1	~~11 2.4 ± 1.51~~12		~~89.8 ± 1.98~~9.8	~~± 1.28~~9.5 ± 1.	~~11 7.2 ± 1.81~~17	~~92.7 ± 2.09~~2.7				

		.6 ± 4.0[b,c,d]	.1 ± 2.3[a,c,d]	± 1.9[a,b,d]	.4 ± 1.5[a,b,c]		± 1.9[c] ~~89.5~~	2[c]	.2 ± 1.8[a,b,d]	± 2.0[c]
28		~~620.5 ± 4.06~~ 20.5 ± 4.0[b,c,d]	~~198.6 ± 2.91~~ 98.6 ± 2.9[a,c,d]	~~134.5 ± 2.01~~ 34.5 ± 2.0[a,b,d]	~~136.7 ± 2.61~~ 36.7 ± 2.6[a,b,c]		~~113.4 ± 1.81~~ 13.4 ± 1.8[c]	~~121.7 ± 2.01~~ 21.7 ± 2.0[d]	~~128.4 ± 2.21~~ 28.4 ± 2.2[a,d]	~~104.4 ± 2.11~~ 04.4 ± 2.1[b,c]

Data are expressed as ~~means ± standard deviation (n= 3).~~ mean ± standard deviation (n = 3). Values within columns followed by different superscript letters ~~(a,b,c,d)~~ (a, b, c, d) differ significantly from each other at P ~~< 0.05~~ < 0.05

BHT in Fe-chelating assay IC_{50} ~~1.3 μg/mL,~~ 1.3 μg/mL, in FRAP assay ~~63.2 μM Fe (II)/g~~63.2 μM Fe(II)/g

F fresh truffle, *A* truffles packed in ambient atmosphere, *V* truffles packed under partial vacuum, *CN* truffle packed with 60% N_2 and 40% CO_2, *ON* truffle packaged with the 1% O_2 and 99% N_2

Table 4 Main volatile compounds identified in fresh samples of *Tuber melanosporum* stored at ~~4 °C~~4 °C ~~2-propyl-butanoateheptanalmethoxybenzene1-octen-3-oloctanal3-methyl-methoxybenzene2-methyl-3-methylidenecyclopentane-1-carbaldehydeoctenal2-nonen-1-ol1,2-dimethoxybenzene~~

Number	Volatile compounds
1	4-(2,2-dimethyl)-octenal
2	2-Propyl-butanoate
3	Heptanal
4	Methoxybenzene
5	1-Octen-3-ol
6	3-(5-methyl)-heptanone
7	Octanal
8	3-Methyl-methoxybenzene
9	2-Methyl-3-methylidenecyclopentane-1-carbaldehyde
10	Octenal
11	2-Nonen-1-ol
12	1,2-Dimethoxybenzene

Table 5 Phenolic compounds identified in crude ethanolic extracts of *Tuber melanosporum*

Name	~~LC-MS~~ LC–MS r.t. (min)	av. $[M-H]^-$ ~~(m/z)~~ (*m/z*)	Prevalent MS/MS fragments ~~(m/z) (ESI (-), NCE = 15 eV)(**)~~(*m/z*) (ESI (−), NCE = 15 eV)**
p-Hydroxybenzoic acid	6.7	137.0244	137.024 ~~(< 1),~~ (<1), 93.035 (100)
o-Hydroxybenzoic ~~acid(salycilic~~ acid (salycilic acid)	8.5	137.0244	137.024 (69), 93.035 (34)
2,5-Dihydroxybenzoic ~~acid (gentisic~~ acid (gentisic acid)	3.3	153.0193	153.019 (18.2), 109.030 (100)
Gallic acid	2.6	169.0142	169.014 (6.5), 125.024 (100)
Other dihydroxybenzoic ~~acid(*)~~acid*	6.8	153.0193	153.019 (100), 109.030 (51.3)

~~(*) protocatechuic~~ * Protocatechuic acid (3,4 -) r.t. is ~~4.8 min,~~ 4.8 min, so this can only be assigned to 3,5- or 2,3-dihydroxy benzoic ~~acid. (**) Intensity~~ acid

** Intensity expressed in % over the most intense ~~ion. (***) confirmed~~ ion

*** Confirmed only by standard injection (retention time and HRMS matching)