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High resolution mass approach to characterize refrigerated black truffles stored under different storage atmospheres

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S-Adenosyl-homocysteine (PubMed CID: 439155)
Acetyl-carnitine (PubMed CID: 7045767)

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ABSTRACT

Freshly harvested *Tuber melanosporum* samples were packed and stored at 4 °C under reduced atmospheric pressure or modified atmosphere for four weeks. Multivariate analysis was employed to correlate the antioxidant power of the ethanolic extracts of the samples with the chemical composition determined by high resolution mass spectrometry. High performance liquid chromatography coupled with a coularray detector was applied to select the chemical species associated with the antioxidant power. Four classes of chemical compounds were investigated in more detail by a targeted approach: derivatives of glutathione, adenine (such as *S*-adenosyl-homocysteine), oxidized linoleic acid and ergosterol. Adducts containing glutathione and adenine with oxidized linoleic acid were observed in TM for the first time and can be considered markers of freshness of the product. *S*-adenosyl-homocysteine, the acetyl-carnitine adduct with cysteinyl-glycine and several oxidized linoleic acid derivatives were among the markers of degradation.

1. Introduction

Tuber melanosporum (TM) is a valuable food product that can be consumed fresh or processed (Rivera, Blanco, Salvador, & Venturini, 2010). The shelf life of fresh TM can be extended by using an appropriate sealed package under reduced atmospheric pressure (hypobaric packaging) (Savini, Loizzo, Tundis, Mozzon, Foligni et al., 2017). The extraction of mycochemicals may have a strong potential for its application in the food and pharmaceutical industry. One of the most important mycochemicals is ergosterol, the primary sterol of mushrooms; ergosterol has antioxidant, anti-inflammatory and antitumor properties (Akihisa, Nakamura, Tagata, Tokuda, Yasukawa et al., 2007; Guillamón, García-Lafuente, Lozano, D'Arrigo, Rostagno et al., 2010; Villares, García-Lafuente, Guillamón, & Ramos, 2012). According to other authors, ergosterol seems to exhibit hypocholesterolemic effects, like the bioactive phytosterols (Moreno, Heleno, Barros, Barreiro, & Ferreira, 2015). Phenolic compounds of TM have been identified and quantified apparently for the first time by Villares et al. (2012). These authors obtained a phenolic extract in methanol that was characterized by HPLC after separation of proteins, organic acids, sugars and amino acids. The antioxidant properties were evaluated by inhibition of 2,2′-azobis(2-methylpropionamidine) dihydrochloride (ABAP)-induced lipid peroxidation. However, ethanolic extracts may have a wider application in the food and medicinal industry, since ethanol is food grade unlike methanol.

In this work, the whole ethanolic extract of fresh TM stored under

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); AMP, adenosine monophosphate; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FA, formic acid; FRAP, Ferric Reducing Activity Power; GSH, glutathione; 9-HODE, (±)-9-Hydroxy-10E,12Z-octadecadienoic acid; 13-HODE, (±)-13-Hydroxy-9Z,11E-octadecadienoic acid; 9-oxoODE, 9-Oxo-10E,12Z-octadecadienoic acid; 13-oxoODE, 13-Oxo-9Z,11E-octadecadienoic acid; SAH, *S*-Adenosyl-homocysteine; SAM, *S*-Adenosyl-methionine; PCA, Principal component analysis; TM, *Tuber melanosporum*

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different packaging conditions was characterized for the antioxidant compounds by means of HPLC coupled with a coularray detector. Untargeted mass spectrometric approaches have been already employed in the study of truffles (March, Richards, & Ryan, 2006) and other mushrooms (Kalogeropoulos, Yanni, Koutrotsios, & Aloupi, 2013; Miguel, Carvalho, Lourdes, Baptista, Moreira et al., 2014). The statistical analysis is exploited to direct the search of those chemical compounds related particularly to the process under study (e.g. storage, ageing) (Miguel et al., 2014). On the contrary, when the specific classes of compounds have been defined, a more detailed (targeted) approach can give useful insight on a specific aspect. Here, the compounds correlating with the loss of antioxidant capacity determined with HPLC-coularray detector were used to build up a principal component analysis (PCA) model. Successively, the antioxidant compounds significantly contributing to the variance of the PCA model were characterized with HPLC-HRMS/MS. Preliminary targeted approaches focused at studying selected chemicals (volatiles and phenolic compounds) (Savini et al., 2017). In this report, an untargeted approach aimed at probing a wide portion of the chemical species involved in the quality modifications over storage.

2. Material and methods

2.1. 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 Sigma-Aldrich S. p. A. (Milan, Italy) or VWR International s.r.l. (Milan, Italy).

2.2. Samples

Sample preparation and antioxidant assays are described elsewhere (Loizzo, Pacetti, Lucci, Núñez, Menichini et al., 2015; Savini et al., 2017). Briefly, intact fresh samples of *Tuber melanosporum* Vitt. were harvested in the hilly areas of the Marche Region (Central Italy) by a private company (Acqualagna Tartufi, PU). The fresh samples processing was reported in detail in Savini et al. (2017). The polypropylene vessels (140 × 175 × 46 mm) containing the fresh black truffles and filled with the four chosen atmospheres were sealed with an antifog polyester film adherent to a polypropylene film. The composition of the different atmospheres used were: air atmosphere (A) (control); partial vacuum (V), 0.1 bar applied; mixture of 1% O_2/99% N_2 (ON), mixture of 40% CO_2/60% N_2 (CN). Successively, the vessels were weighed, refrigerated and stored at 4 °C in the dark. Ethanolic extracts were made from these samples as described in the next section. They were investigated just at the harvesting (day 0) after 7, 14, 21 and 28 days of storage, all in triplicates.

2.3. Preparation of the extracts

An aliquot (5 g) of TM samples was weighed and blended at slow speed using a laboratory mill (M20 Universal mill, IKA®-Werke GmbH & Co. KG, Staufen, Germany). The samples were mixed with 10 mL of ethanol 96% (v/v) and were kept away from the light at room temperature for 24 h. Samples were then filtered and ethanol was newly added. The mixture was kept in the dark for other 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 extracts were dissolved into 5 mL of ethanol 96% (v/v). The extraction yield ranged 1.2–3.6% w/w.

2.4. Antioxidant assays

The assays were carried out previously and reported elsewhere (Loizzo et al., 2009, 2015; Savini et al., 2017). Briefly, the total antioxidant content was determined by using the Folin-Ciocalteu procedure. Each ethanolic extract was mixed with 0.2 mL Folin-Ciocalteu reagent, 2 mL of water and 1 mL of 15% Na_2CO_3. After incubation for 2 h at 25 °C the absorbance was measured at 765 nm (UV–Vis Jenway 6300 spectrophotometer, Bibby Scientific Ltd., UK). The total antioxidant content was expressed as mg GAE per g of dry extract. The $ABTS^{\cdot+}$ assay was based on the method of Loizzo, Said, Tundis, Hawas, Rashed et al. (2009). The $ABTS^{\cdot+}$ solution, produced from the reaction of ABTS 7 mM and 2.45 mM potassium persulfate, was stored in the dark at room temperature for 12 h before use and diluted with ethanol to reach an absorbance of 0.70 ± 0.05 at 734 nm. An aliquot of 25 μL of extracts at different concentrations were added to 2 mL of diluted $ABTS^{\cdot+}$ solution and the absorbance was measured after 6 min at 734 nm. Ascorbic acid was used as positive control. The radical scavenging activity was determined according to Loizzo et al. (2015). Extracts at different concentrations were added to an ethanolic solution of DPPH radical (final concentration was 0.1 mM). The bleaching of DPPH was determined by measuring the absorbance at 517 nm. Ascorbic acid was used as positive control. The chelating activity of the samples was measured following the procedure previously described elsewhere (Loizzo et al., 2009). Briefly, the extract, $FeCl_2$ (2 mM) and FerroZine™ (5 mM) were mixed and left at room temperature for 10 min. The absorbance of the Fe^{2+}-FerroZine™ complex was measured at 562 nm. The FRAP reagent, containing 2.5 mL of 10 mM tripyridyltriazine (TPTZ) solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer (pH 3.6) was freshly prepared. An aliquot (2.5 mg/mL) of samples was dissolved in ethanol and a 0.2 mL of this solution was mixed with 1.8 mL of FRAP reagent. Ethanol solutions of $FeSO_4$ (50–500 mM) were used to obtain the calibration curve. The absorbance of the reaction mixture was measured at 595 nm and the FRAP value was expressed as mM Fe(II)/g. Butylated hydroxytoluene (BHT) was used as positive control. DPPH, FRAP, $ABTS^{\cdot+}$ and Fe^{2+}-chelation ability are reported as IC_{50}.

2.5. Chemical composition by HPLC-HRMS and HPLC-MS/MS

2.5.1. Sample preparation

The crude ethanol extracts used for the antioxidant assays were vacuum and N_2 dried and then stored at − 80 °C. For each sample, a 50 mg/mL solution was prepared (in triplicate) in the mobile phase A of the HPLC system and vigorously stirred. No further purification was performed before injection for the LC-MS chromatographic analysis.

2.5.2. HPLC with coularray detector

The separation was carried out at a flow rate of 1 mL min^{-1} with a ODS Hypersyl C18 LC column (125 mm × 4.6 mm i.d., 5 μm, Thermo Sci.) protected with a HPLC pre-column filter (Thermo Sci.) on a Ultimate 3000 UHPLC (Thermo Sci.). The mobile phase consisted of a combination of solvent A (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 set as follows: from 5% B at 0 min to 25% B (v/v) at 21 min, then to 95% B at 22 min until 27 min, to 5% at 28 min, followed by a re-equilibration step (5% B) at 32 to 35 min. Eight porous graphite cells of the coularray detector were poised at potentials from + 300 and 860 mV (vs Pd reference electrode) with the increment of 80 mV. The data were recorded and processed with ESA CoulArray 3.1 (Dionex) and Chromeleon software (Thermo Sci.). Retention times were corrected to match the retention time of HPLC-DAD-MS (using the retention times of injected phenolic standards employing the same LC method).

2.5.3. HPLC-DAD-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} with a ODS Hypersyl C18 LC column (125 mm × 4.6 mm