Extraction and trapping of truffle flavoring compounds into food matrices using supercritical CO_2

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Abbreviations: SFE, supercritical fluid extraction; RSM, response surface methodology; UHPSFC-QTOF-MS, ultra-high-performance supercritical fluid chromatography coupled to quadrupole- time-of-flight mass spectrometry.

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Abstract

A supercritical fluid extraction methodology was used to extract flavoring and bioactive compounds from truffles. Some parameters such as CO_2 flow rate (1-3 mg/mL), extraction time (15-90 min) and different trapping food matrices (grape seed oil, gelatin, agar agar and water) were optimized using response surface methodology to enhance extraction and trapping yields. The optimal conditions (2.27 mg/mL CO_2 flow rate, 82.5 min when using 40 ºC and 30 MPa, with 1mL grape seed oil as trapping matrix) obtained with *Tuber melanosporum* were applied to three different truffle species: *Terfezia claveryi*, *Tuber aestivum* and *Tuber indicum*. A total of 32 metabolites were profiled in the extracts using ultra-high-performance supercritical fluid chromatography coupled to quadrupole time-of-flight mass spectrometry. Compounds such as brassicasterol ergosta-7,22-dienol, oleic and linoleic acid were found at similar amounts in all the extracts but other molecules (*e.g.* fungal sterols) showed a particular distribution depending on the specie studied and whether a trapping matrix was used at the SFE outlet.

1. Introduction

Truffles are considered one of the world's most highly prized and valued foods mainly due to their organoleptic properties. Recently, they are also being investigated because of their bioactive compounds with potential to induce beneficial effects on human health (Khalifa et al., 2019; Patel, Rauf, Khan, Khalid, & Mubarak, 2017). Many of the studies were focused on large molecules such as β-glucans (Tejedor-Calvo, Morales, Marco, et al., 2020), however, truffles also include approximately 5–8 % lipids (Harki, Bouya, & Dargent, 2006). Although lipids are present in lower concentrations than polysaccharides, many of them showed interesting biological activities besides their main role in the characteristic truffle flavor (N. Shah, Usvalampi, Chaudhary, Seppänen, & Sandesh, 2020)).

In *Tuber* species, unsaponifiable lipids such as ergosterol (ergosta-5,7,22-trienol) and brassicasterol (ergosta-5,22- dienol) are the main sterols in the hyphal membranes representing approximately respect. 60 and 30 % of total sterols (Harki, Klaebe, Talou, & Dargent, 1996; Tejedor-Calvo, Morales, García-Barreda, et al., 2020; Tejedor-Calvo et al., 2019), whereas in *Terfezia* genus, brassicasterol content reaches 98 % (Weete, Kulifaj, Montant, Nes, & Sancholle, 1985). Other minor sterols were also detected in *Tuber* sp., such as cholesterol, 5-dihydroergosterol, campesterol, 24(28)-dehydroergosterol, stigmasterol, stigmasta-7,24(28)-dienol, fungisterol, lanosterol, β-sitosterol, 24-methlene-24,25-dihy- drolanosterol and 4α-methylergosta-8(9),24(28)-dienol) (Tang, Li, & Tang, 2012). Some of these compounds were associated with a wide range of health-promoting properties, such as antioxidant, anti-inflammatory, hypolipidemic, hypocholesterolemic and hypoglycemic activities (Gil-Ramírez et al., 2013; Roncero-Ramos & Delgado-Andrade, 2017). Additionally, they also contribute to the truffle flavor as they are precursors of the C19-sterols that generate the characteristic boar meat smell (*e.g.* 5α-androst-16-en3α-ol) (Wang, Li, Li, & Tang, 2008).

Apart from sterols, other biologically active compounds such as tocopherols and unsaturated fatty acids were studied in detail for edible mushrooms, but only scarcely in the truffle species. Shah et al (2020) found palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acids as main fatty acids in truffles and only traces of polyunsaturated fatty acids (PUFAs) (N. N. Shah, Hokkanen, Pastinen, Eljamil, & Shamekh, 2020). These fatty acids were detected in several *Tuber* (*T. aestivum, T. brumale, T. indicum, T. gennadii, T. magnatum, T. melanosporum,* and *T. oligospermum*) and *Terfezia* species (*T. arenaria, T. leptoderma, T. magnusii*) together with lower levels of shorter fatty acids, such as C6:0, C8:0, C10:0, C12:0, C14:0, C14:1, C15:0, C15:1 (Tejedor-Calvo, Amara, et al., 2020). Moreover, the fatty acid profile can be used to distinguish between truffles from different geographical regions (Hilszczańska et al., 2016; N. N. Shah et al., 2020; N. Shah, Usvalampi, Chaudhary, Seppänen-Laakso, et al., 2020) showing different and characteristic flavors. In fact, a new taste known as ologustus or pinguis was associated with activation of gustatory neurons by some fatty acids (Reed & Xia, 2015; Running, Craig, & Mattes, 2015).

Supercritical fluid extraction (SFE) is frequently used to obtain lipid enriched fractions. SFE is an environmentally friendly technology that leaves no traces of organic solvents in the extracted material. This technique was recently utilized to obtain aromatic extracts from truffles (Tejedor-Calvo et al., 2021), however, the obtained lipid fraction was not examined in detail and was not associated to truffle flavors. Therefore, in this work, SFE methods were optimized and applied to three different truffles species and the lipids obtained in their extracts were identified and relatively quantified. Depressurization was carried out through different food grade matrices as an attempt to enhance extraction yield trapping aromatic volatiles.

2. Materials and methods

2.1. Biological material

Tuber melanosporum (Vittad.) and *Tuber aestivum* ascocarps were collected at Gúdar-Javalambre woods (Teruel, Spain) and *Tuber indicum* was supplied by Espora Gourmet company (Soria, Spain). *Terfezia claveryi* Chatin was collected from an experimental field in Caravaca de la Cruz (Murcia, Spain). Truffles (20 units/species) were taxonomically authenticated by morphological features (Montecchi & Sarasini, 2000; Riousset, 2001), selected and processed under refrigeration (Rivera, Venturini, Marco, Oria, & Blanco, 2011). Truffles were lyophilized, ground and sieved to obtain a particle size lower than 0.5 mm and stored at -20 ºC until further use.

Grapeseed oil was purchased from Dietisa company (Barcelona, Spain). Food grade agar-agar and powder gelatine were purchased from a local supermarket. The agar-agar proximal composition was 85% fiber, <0.5% carbohydrates, <0.5% lipids, 1.3% proteins, 0.7% salt. Gelatin package indicated 89% protein and 1.2% salt in its nutritional table.

2.2. Reagents

Methyl *tert*-butyl ether (MTBE; 99.8%), MS-grade ammonium formate and ammoniumacetate were provided by Sigma-Aldrich (Steinheim, Germany). Hexane (95%) was obtained from LAB-SCAN (Gliwice, Poland). LC-HPLC grade methanol and water LC-MS grade was provided by VWR (VWR Chemicals, France). MilliQ water was produced using a Milli-Q purification system (Millipore, Billerica, MA). Ultrapure CO_2 was provided by AGA industrial gases (Lidingö, Sweden). All other solvents were of analytical grade.

2.3. Supercritical fluid extraction (SFE)

An analytical SFE system (Waters MV-10, Milford, MA) including a fluid delivery module for pumping CO_2, an oven for placing the extraction vessels, an automated back pressure regulator, a pump, and a fraction collector module was used to conduct the experiments. The heads of the CO_2 pump were cooled using a chiller operated at 4 °C (Figure 1). Samples (1 g) were loaded together with glass beads into a 10 mL stainless steel extraction vessel. After each extraction the system was flushed with a CO_2 flow for 5 min. The system was controlled by ChromScope™ software (Waters, Milford, MA). The collected extracts were stored at −20 °C before further analysis.

Two face centered central composite designs (FC-CCDs) with three center points were created in MODDE 10.1 (Sartorius Stedim Biotech, Malmö, Sweden). Extraction time (15-90 min) and flow rate (1-3 mL/min) were selected as independent factors, with extraction yield as response variable. Pressure and temperature were kept at 300 bar and 40 ºC, respectively (Tejedor-Calvo et al., 2021). The design was adjusted to maximize the extraction yield, considering flow rate and time being of equal importance. In total, 12 experiments were performed in a random order (Table 1). All extracts were recovered in a glass bottle (10 mL) by inserting the SFE outlet into a trapping solution of grapeseed oil (OIL), agar-agar (AA), gelatin (GE) or MilliQ water (W). The AA (2.5 mg/mL) and GE (20 mg/mL) solutions were prepared by heating them at 100 ºC for 10 min. Different matrix volumes were tested (1, 3 and 5 mL). The optimal conditions providing the highest extraction yields were defined by using graphical and numerical analyses based on response surface plots and a desirability function.

2.4. Lipid extraction

Lipid extraction was carried out as described by Matysah et al. (2008) (Matyash, Liebisch, Kurzchalia, Shevchenko, & Schwudke, 2008). Briefly, each SFE extract or residue

(material remaining in the extraction cell after SFE; approximately 40 mg) was mixed with 1300 µl of MTBE:MeOH (10:3, v/v) and stirred for 1 h at room temperature. Then, MS-grade water (250 µl) was added to induce phase separation. After 10 min incubation at room temperature, the sample was centrifuged at 1,200 g for 10 min. The upper (organic) phase was collected, and the lower phase was mixed with 1300 µl of a solvent mixture equivalent to the expected composition of the initial upper phase (MTBE/methanol/water (10:3:2.5, v/v/v)). Afterward, the solution was again stirred, the upper phase collected, pooled together with the first extraction and dried in a vacuum centrifuge. To facilitate evaporation, MS-grade methanol (200 µl) was added to the organic extract after 25 min of vacuum centrifugation. Extracted lipids were dissolved in 200 µl $CHCl_3$/methanol/water (60:30:4.5, v/v/v) and stored at -80 ºC until analysed.

2.5. *UHPSFC/ESI-QTOF-MS*

Lipid extracts were analysed using an ultrahigh performance supercritical fluid chromatography system (UHPSFC; ACQUITY UPC2, Waters, Milford, MA) using an ACQUITY UPC2 Torus DIOL column (130 Å, 1.7 µm, 3 mm x 100 mm, Waters) equipped with a Torus DIOL (130 Å, 1.7 µm, 2.1 mm x 5 mm, Waters) guard column. The column temperature and the active back pressure regulator (ABPR) were set at 50 ºC and 110 bar, respectively. The injection volume was 1 µL and the flow rate was set at 1.6 mL/min. Methanol containing 20 mM ammonium formate was used as mobile phase modifier using the following gradient: 0 min, 1% modifier; 18 min, 50%; 19 min, 50%; 19.5 min 1%; 21 min 1%.

The UHPSFC was connected to a Xevo 2G QTOF-MS (Waters). Two T-pieces (Waters) were used to control the back pressure at the column outlet and to infuse methanol (0.25 mL/min) as a makeup liquid. The MS was operated in positive and negative electrospray

ionization (ESI) mode with a scan range of m/z 80–1200. In positive ESI mode, the capillary voltage was 3.2 kV, the sampling cone voltage 34 V, the source temperature 120 ºC, the drying gas temperature 420 ºC, the cone gas flow 40 L/h and the drying gas flow 690 L/h. In negative ESI mode, the capillary voltage was 2.6 kV, the sampling cone voltage 42 V, the source temperature 120 ºC, the drying gas temperature 390 ºC, the cone gas flow 40 L/h and the drying gas flow 740 L/h. A collision energy ramp between 15 and 55 eV was used to acquire data independent, targeted and data dependent MS/MS data in both ESI modes. Data was acquired using MassLynx v4.1 (Waters). Raw data were processed using the open-source software package MZmine 2.16 (http://mzmine.sourceforge.net/) [31]. Data were generated by targeted peak detection with a *m/z* tolerance of 0.5 mDa or 5 ppm and a retention time tolerance of 0.2 min.

Tentative compound identification was carried out by comparing experimental with theoretical MS data in negative mode using the exact mass of the [M-H]- ion (http://www.lipidmaps.org). Moreover, MS/MS data were compared with spectra in Massbank (https://massbank.eu/MassBank/), CRC, Wiley and reference literature. The data presented for tentatively identified compounds include the name of the compounds, CAS number (sterols) or common abbreviation (fatty acids), molecular formula, retention time, and theorical and experimental m/z data (Table 2). Blank samples, produced from extraction of pure trapping matrices, were analysed using the same method.

2.6. *Statistical analysis*

Differences between groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test *post hoc* in GraphPadPrism version 5.01 (GraphPad Software, San Diego, CA). Experimental designs were evaluated

using multiple linear regression in Statgraphics Centurion XVI (Statpoint Technologies, Warrenton, VA).

3. Results and discussion

3.1. SFE method and relative lipid profile

Firstly, *Tuber melanosporum* was submitted to SFE extractions and according to the obtained results (Table 1), the optimal extraction conditions generating the highest extraction yield (1.44%), was 2.3 mL/min CO_2 flow rate and 82.5 min (Figure 2a). A low flow rate (1 ml/min) yielded the lowest desirability (below 0.7) whereas the highest was found with long extraction times (50 min) and an intermediate flow rate (2-3 mL/min). Time, more than flow rate, was positively influencing the extraction yields (Figure 2b). Same effect was shown in a study using similar SFE conditions in sunflower oil. They also described that the extraction rate was linearly associated with time in the early extraction stages (Salgin, Döker, & Çalimli, 2006). CO_2 extraction abilities depends mainly on parameters such as pressure and temperature therefore by modulating SFE conditions, CO_2 can selectively extract several compounds depending on their specific solubility in this solvent (Salgin et al., 2006). Thus, depending on the targeted compounds, the extraction processes show different extraction yields, extracts compositions etc.

Compare to previous studies, the extraction yield was slightly lower (2.1 %) than those obtained by Tejedor-Calvo et al. (2021) for *T. melanosporum* (Tejedor-Calvo et al., 2021). Similar yields were reported for edible mushrooms, such as *Agaricus bisporus* (1.4-2.1%) (Gil-Ramírez et al., 2013) and *Lentinula edodes* (1.1-1.7%) (Morales, Piris, Ruiz-Rodriguez, Prodanov, & Soler-Rivas, 2018). Differences between truffle batch, SFE parameters (CO_2 flow rate), scale and device designs might be the reason for these

differences.

A total of 32 compounds were detected in the extracts from *T. melanosporum*. Most of the compounds were fatty acids, including DHA and EPA, and fungal sterols (Figure 3). Surprisingly, compounds not previously described in truffles, such as brassicasterone (ergosta-4,22-dien-3-one), ergone (ergosta-4,6,8(14),22-tetraen-3-one, (22E)-), fecosterol (24-methylene-cholest-8-en-3β-ol) and demosterol (3β-cholesta-5,24-dien-3-ol) were also detected.

Some fatty acids might be linked to truffle flavor and smell since many aromatic compounds are produced downstream within the fatty acid metabolic pathways. For instance, the odor generated when a food containing linolenic acid is decomposing is not only ascribed to the fatty acid oxidation, but also to the smell reduction noticed when the fatty acid is transformed into carbonyl compounds (with low odor threshold values), such as (Z)-3-hexenal, (E,Z)-2,6-nonadienal and (Z)-1,5-octadien-3-one (Anklam, 2005). Moreover, food products containing linoleic acid usually include other strong flavoring compounds such as (E)-2-nonenal, trans-4,5-epoxy-(E)-2-decenal and 1-octen-3-one (Anklam, 2005). Aldehydes with exceptionally strong aromas can be produced in foods by the autoxidation of some fatty acids and yield an exceptionally strong smell even when present in very low amounts. An example is octadeca-(Z,Z)-11,15-dienoic acid (the precursor for (Z)-4-heptenal) that can be found in beef and mutton and often in butter (Anklam, 2005). Some of these aromatic compounds were described in truffles species and in truffle extracts obtained using SFE (Campo, Marco, Oria, Blanco, & Venturini, 2017; Tejedor-Calvo et al., 2021). But no reports were found correlating flavor with lipid compounds of fresh truffle.

A heat map was constructed to illustrate the lipid patterns and their variation within the extractions carried out using *T. melanosporum* (Figure 3). Among the identified sterols,

237 brassicasterol, ergone, ergosta-7,22-dienol and fecosterol were present in the obtained
238 extracts in high levels, whereas desmosterol was more abundant in the SFE residues.
239 Although ergosterol is the main constituent of fungal hyphae (Harki et al., 1996), it was
240 detected in low percentage as compared to the other sterols. However, in other SFE
241 studies (Tejedor-Calvo et al., 2021) this compound was the main contributor to the
242 obtained extracts. The different extraction conditions utilized might modulate the CO_2
243 viscosity enhancing the preferential extraction of other compounds before ergosterol.
244 Moreover, only traces of desmosterol were extracted with SFE, with the larger amounts
245 of this compound remaining in the truffle residue. The solubility of desmosterol decreased
246 with increasing polarity of the solvents. This sterol was reported to be more soluble in
247 ethanol and acetone than in strongly polar solvents (Chen, Su, Xing, Yang, & Ren, 2008)
248 and supercritical CO_2 resembled polarities close to polar solvents (dielectric constant 1-
249 .0-1.4) (Leeke et al., 2005; Raveendran, Ikushima, & Wallen, 2005) being then not the
250 most convenient solvent to extract it.
251 Extracts and residues showed a similar fatty acid profile. Oleic (C18:1) and linoleic
252 (C18:2) acid were found as the main compounds in all the obtained fractions
253 independently of the extraction conditions tested, suggesting that no preferential
254 extraction of certain fatty acids was promoted with any condition, they can be extracted
255 but not all the content (Table 3). The fatty acid profile was similar to that observed in
256 previous studies (Shah et al., 2020; Tejedor-Calvo, Amara, et al., 2020) for a raw truffle
257 suggesting that the SFE extraction did not significantly modify the lipid composition.
258 Lipids containing oleate and linoleate groups were also pointed by other authors (Shah et
259 al., 2020; Tang, Li, Li, Wan, & Tang, 2011; Tejedor-Calvo, Amara, et al., 2020) as main
260 truffle constituents. Hence, SFE seems to be a promising technique for production of
261 extracts with high content of bioactive omega-9 and -3 fatty acids. These unsaturated fatty

acids are involved in many health promoting and disease preventing processes (Calder, 2013; Guo, 2009).

The CO_2 flow rate was the only parameter that induced significant alterations in extraction yield. An increase in the flow rate (E10, E11 and E12 figure 3) was beneficial for the extraction of some sterols (*i.e.* brassicasterol, ergone, fecosterol, ergosterol, brassicasterone) and fatty acids (*i.e.* hexadecenoic, stearic and oleic acids, DHA and 11,14-eicosadienoic acid). In SFE, the extraction yield and mass transfer coefficient increase with higher CO_2 flow rate (Salgin et al., 2006). Therefore, a high CO_2 flow rate generally extract a larger number of compounds. Nevertheless, a lower CO_2 flow rate could be particularly used to obtain extracts enriched in *e.g*. erogsta-7,22-dienol.

3.2. Influence of trapping matrices

After the SFE method was optimized, different matrices were tested to trap aromas and flavoring compounds to prevent their release into the atmosphere during CO_2 depressurization. Four matrices with different chemical composition were selected covering the most frequent molecules found in food matrices to test different binding or scavenging mechanisms such as a high fat, high protein, high polysaccharide and aqueous based matrices. Results indicated that a higher trapping capacity was observed when a low quantity of trapping matrix was used (Figure 3). The matrices tested showed significantly different trapping capacities. The highest yield was obtained using GE (1.42 %) followed by OIL (1.16 %); W and AA showed the lowest trapping ability (Figure 4a). Polysaccharides present in the AA were not able to trap lipophilic compounds, being even less effective than a water matrix. Guichard (2002) studied the interactions between flavoring compounds and food ingredients and demonstrated that the physical state of carbohydrates is an important parameter influencing flavor retention (E. Guichard, 2002).

In this work, the AA viscosity was low (liquid) due to the continuous supply of CO_2. Whether a higher viscosity would benefit trapping of lipids remain unknown. Matrices including triglycerides, proteins, polysaccharides and their combinations forming emulsions, can be designed to fine-tune trapping of a wide range of molecules (E. Guichard, 2002; Elisabeth Guichard, 2006; Mao, Roos, Biliaderis, & Miao, 2017). Designing of a specific matrix combination is challenging, being the lipid composition the factor highly influencing the affinity of the other food molecules, such as proteins, to trap volatiles (Roberts, Pollien, & Watzke, 2003; Seuvre, Espinosa Díaz, & Voilley, 2000). Moreover, synergistic and antagonistic effects might also play a major role.

Under the conditions tested, the number of total sterols collected was independent of the trapping matrix, although the contribution of each individual sterol to the total content slightly differed (Figure 5a). For instance, the SFE extract from *T. melanosporum* collected using water as trapping matrix contained almost 50% ergosta-7,22-dienol, values that were significantly higher than the amounts collected without any matrix. However, ergone levels were higher when matrices were used, except water. When grapeseed oil was used, a larger variety of compounds were detected, with a lipid profile differing from that observed in absence of matrix. The observed differences may be due to transformations of some sterols into new derivatives via interactions with the trapping matrix. The fatty acid content was also highly influenced by the trapping matrix. Oleic and linoleic acid, followed by hexadecenoic acid, remained the most abundant fatty acids in the SFE extracts of *T. melanosporum* without trapping material (Table 3). When GE and AA were used as trapping matrix a completely different lipid profile was observed since behenic and linoleic acid were present in high concentrations and oleic acid was lacking. Presumably, GE proteins or AA polysaccharides might easily bind or trap long chain fatty acids but with particular stearic conformation. OIL was the trapping material

showing the highest extraction yield with linoleic acid contributing to almost 60% of the total extract. Water showed the widest selectivity and was able to concentrate hexadecenoic acid, stearic acid, vaccenic acid, 11,14-eicosadienoic acid, cis-erucid acid and lignoceric acid, as compared with extractions conducted in absence of trapping matrix. These results were surprising, as water provides the most hydrophilic trapping solvent. However, it is possible that the hydrophobic extracts were suspended in water forming micelles or other colloidal structure.

3.3. SFE extraction and trapping of flavoring compounds from other truffle species

The optimal extraction conditions defined using *T. melanosporum* were applied to additional truffles species as it was previously indicated (Tejedor-Calvo et al., 2021) that SFE yields and general compound contents of the obtained extracts were similar (including *Terfezia* species)(Tejedor-calvo, Garc, & Sergio, 2022). Extractions were conducted using grapeseed oil as trapping matrix, as this matrix retained most of the flavoring compounds from *T. melanosporum* and only marginally altered its lipid composition. Hence, SFE extractions of *Tuber aestivum, Tuber indicum* and *Terfezia clavergyii* were carried out with and without the oil at the depressurization outlet.

The extraction yields ranged from 1.0 to 1.3% and were independent of placing or not trapping oil (Figure 4b). The extraction yield from *T. claveryi* was higher compared to the other *Tuber* truffles, but lower than those observed for *T. melanosporum* (1.4%). The use of oil at the outlet modified the observed sterol profile for all 3 species (Figure 5b). In the selected species but particularly in *T. claveryi,* a notable increase in the content of ergone concomitant with a reduction of ergosterol and cholesterol was noticed in presence of oil compared to when it was absent. *T aestivum* extracts obtained without oil in the trap showed a wide variety of sterols, with some of them such as ergoste or stigmasterol being

lower in concentration as compared to when oil was used. The sterol profile of the extract from *T. indicum* was less affected by the presence of oil, with only levels of cholesterol and fecosterol being reduced when trapping matrix was used.

When changes in the fatty acid profile of the studied truffles were evaluated, one of the most dramatic changes was noticed in the *T. indicum* extracts. In this truffle presence of oil in the trap increased the yield of behenic acid almost 8-fold compared to when the trap was left empty. In addition, several minor changes in the fatty acid profile were also observed. For instance, levels of lauric acid, myristic acid, 14Z-eicosenoic acid, and cis-erucid acid slightly increased in the presence of oil in the trap. Conversely, levels of hexadecanoic acid, palmitoleic acid, margaric acid, stearic acid, linoleic acid, and DHA decreased in presence of oil. The latter compound is worldwide known as responsible for several important biological functions, including cell membranes structure, protein function, production of lipid mediators and gene expression, thereby potentially contributing to improve human health (Calder, 2018; Guo, 2009; Sande et al., 2019). Moreover, behenic acid is able to bind glycerol-based lipid backbones impairing pancreatic lipase activity and therefore preventing obesity (Moreira, Santos, Gambero, & Macedo, 2017; Silva et al., 2020). Hence, the use of grapeseed oil as trapping material enables the production of SFE extracts with different fatty acid profiles than without it modulating their different bioactivities and therefore their potential applications.

Moreover, the fatty acid profile can also be related to the aroma compounds and their perception (Roberts et al., 2003). Some of these odorous compounds such as linoleic and oleic were detected in SFE extracts from black truffle (*T. melanosporum*) (Tejedor-Calvo et al., 2021) as well as in the other selected species and they were/were not modified when extracted with oil. Apparently, C8 volatiles, such as 3octanone, 1-octen-3-one, 3-octanol, Z-5-octen-1-ol, 1-octen-3-ol were assigned as potential signaling molecules produced by

362 truffle mycelium and fruiting bodies (Splivallo, Bossi, Maffei, & Bonfante, 2007). Some
363 of these compounds were pointed as mushroom-like aroma and considered as major
364 contributors to the characteristic flavor of several mushrooms and truffles, including *T.*
365 *indicum*. These compounds are mainly formed by the oxidation of linoleic and linolenic
366 acid in the presence of enzymes, such as lipoxygenase and hydroperoxidelyase (Assaf,
367 Hadar, & Dosoretz, 1997). Those enzymes play a key role in fruits, vegetables and
368 mushrooms, releasing some odorous compounds like the green-grassy or cucumber-like
369 smelling aldehydes hexanal, (Z)-3-hexenal (leafy aldehyde), (Z,Z)-3,6-nonadienal or
370 mushroom like of l-octen-3-ol (Anklam, 2005). In the obtained extracts, enzymatic
371 reactions might not be involved because the high pressure and temperature utilized for
372 extraction will denature them however, during depressurization, the extract is released
373 sprayed into the air and it might enhance its contact with oxygen promoting autoxidation
374 or transformation of small volatile molecules such as aromas, that might also be the reason
375 that when depressurization is carried out inside a lipid matrix (with less access to oxygen)
376 the extract composition was modified. Therefore, the trapping matrices might also be used
377 to modulate the aromatic profile of the obtained extracts however, more studies are
378 needed to explore all the potential combinations.

379 **4. Conclusions**

380 Supercritical fluid technology allowed an efficient generation of extracts enriched in lipid
381 molecules involved in truffle aroma and flavor. The optimal conditions (2.27 mg/mL CO_2
382 flow rate and 82.5 min extraction) were able to extract fatty acids (mainly oleic and
383 linoleic acid) and sterols (brassicasterol, ergosta-7,22-dienol and fecosterol) from *Tuber*
384 *melanosporum* truffles. The use of trapping matrices such as grapeseed oil placed at the
385 SFE outlet enhanced capture of some key truffle aromatic molecules but also modify their
386 content of sterols and fatty acids. These results were also obtained when other truffles

species were submitted to similar extractions generating their own particular profile of flavoring and beneficial compounds depending on the specie considered and the use or not of a trapping matrix. These results indicated that environmentally friendly extraction and trapping methodologies can be used to produce tailor-made natural truffle aromatic and functional extracts. At the present, further studies are being carried out to test their food stability before commercialization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Eva Tejedor-Calvo: Conceptualization, Investigation, Formal analysis, Data curation, Methodology, Writing - original draft. **Pedro Marco:** Supervision, Validation, Writing - review & editing. **Peter Spégel:** Software, Supervision, Visualization, Writing - review & editing. **Cristina Soler-Rivas:** Visualization, Writing - review & editing.

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574 **TABLES**

575 **Table 1.** Experimental design for SFE extractions from *T. melanosporum* and obtained
576 extraction yield

Independent factors				Response
N	Run order	Time (min)	Flow (ml/min)	Yield (%)
1	4	15	1	0.85
2	7	52.5	1	1.17
3	8	90	1	1.17
4	9	15	2	0.94
5	3	52.5	2	1.35
6	5	52.5	2	1.35
7	11	52.5	2	1.44
8	12	52.5	2	1.35
9	10	90	2	1.44
10	1	15	3	1.13
11	6	52.5	3	1.35
12	2	90	3	1.44

577

578 **Table 2.** Lipid compounds identified in the SFE extract obtained from *T. melanosporum*
579 using the optimized conditions (40 °C, 2.27 ml/min CO_2 and 300 bar). Experimental and
580 theoretical data for sterols in negative $[M-H]^-$ are shown. *CAS Numbers are provided for
581 sterols and abbreviations for fatty acids. RT correspond to retention time.

Compound identity			Negative ESI $[M-H]^-$		
Common name	Other information*	Molecular formula (neutral)	RT (min)	m/z experimental	m/z theoretical
Sterols					
Brassicasterol	474-67-9	$C_{28}H_{46}O$	2.08	395.3353	395.3319
Ergone	19254-69-4	$C_{28}H_{40}O$	2.32	397.2233	397.2257
Ergosta7,22-dienol	96391-64-9	$C_{28}H_{44}O$	2.32	395.2979	395.3392
Fecosterol	516-86-9	$C_{30}H_{50}O$	2.7	411.3095	411.3632
Cholesterol	57-88-5	$C_{27}H_{46}O$	3.47	395.3886	395.3079
Stigmasterol	83-48-7	$C_{29}H_{48}O$	3.54	445.3292	445.3967
Ergosterol	57-87-4	$C_{28}H_{44}O$	3.85	385.3476	385.2955
Desmosterol	313-04-2	$C_{27}H_{44}O$	4.4	383.3319	383.3523
Brassicasterone	4030-92-6	$C_{28}H_{46}O$	4.99	425.3789	425.3633
9,19-cyclolanostane-3,7-diol	91851-80-8	$C_{30}H_{52}O$	5.92	395.3319	395.3884
Fatty acids (FA)					
Caprylic acid	C8:0	$C_8H_{16}O_2$	2.22	143.1078	143.0692
Capric acid	C10:0	$C_{10}H_{20}O_2$	2.22	171.1391	171.6391
Lauric acid	C12:0	$C_{12}H_{24}O_2$	2.22	199.1704	199.1311
Myristic acid	C14:0	$C_{14}H_{28}O_2$	2.22	227.2017	227.1297
Hexadecanoic acid	C16:0	$C_{16}H_{32}O_2$	2.08	255.2311	255.2304
cis-9-palmitoleic acid	C16:1	$C_{16}H_{30}O_2$	2.11	253.2173	253.2159
Margaric acid	C17:0	$C_{17}H_{34}O_2$	2.11	269.2486	269.2483
Stearic acid	C18:0	$C_{18}H_{36}O_2$	2.11	283.2643	283.2607
Oleic acid	C18:1n9	$C_{18}H_{34}O_2$	2.15	281.2486	281.2494
cis-vaccenic acid	C18:1n11	$C_{18}H_{34}O_2$	2.60	281.2486	281.2476
Linoleic acid	C18:2n6	$C_{18}H_{32}O_2$	2.22	279.2313	279.2321
alpha-Linolenic acid	C18:3n3	$C_{18}H_{30}O_2$	2.57	277.2173	277.2169
14Z-eicosenoic acid	C20:1	$C_{20}H_{38}O_2$	2.22	309.2799	309.2797
11,14-eicosadienoic acid	C20:2	$C_{20}H_{36}O_2$	2.29	307.2643	307.2645
5,8,11-eicosatrienoic acid	C20:3n3	$C_{20}H_{34}O_2$	2.32	305.2486	305.2498
Heneicosylic acid	C21:0	$C_{21}H_{42}O_2$	2.74	325.3112	325.2372
EPA	C20:5n3	$C_{20}H_{30}O_2$	2.39	301.2173	301.2170
Behenic acid	C22:0	$C_{22}H_{44}O_2$	2.26	339.3269	339.3265
cis-erucic acid	C22:1n9	$C_{22}H_{42}O_2$	2.29	337.3112	337.3117
DHA	C22:6n3	$C_{22}H_{32}O_2$	2.22	327.2330	327.2158
Lignoceric acid	C24:0	$C_{24}H_{48}O_2$	2.29	367.3582	367.3579
Nervonic acid	C24:1	$C_{24}H_{46}O_2$	2.32	365.3425	365.8224

582

583 **Table 3.** Fatty acid composition of the SFE extracts from selected truffles: no trapping matrix (none), water (W), gelatin (GE), agar-agar (AA),

584 grapeseed oil (OIL). Indicated results are expressed as percentage of each fatty acid from the total fatty acid content.

Truffle specie	***T. melanosporum***					***T. claveryi***		***T. aestivum***		***T. indicum***	
Trap matrix	**None**	**W**	**GE**	**AA**	**OIL**	**None**	**OIL**	**None**	***OIL***	**None**	***OIL***
caprylic acid	0.07	0.06	1.58	0.94	0.00	0.46	0.94	0.37	1	0.8	0.8
capric acid	0.00	0.00	0.18	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
lauric acid	0.12	0.11	0.84	1.25	0.11	0.53	1.25	0.56	1.24	0.71	0.61
myristic acid	0.03	0.04	0.54	0.74	0.08	0.21	0.74	0.17	0.43	0.4	0.54
hexadecanoic acid	4.27	5.66	0.66	0.64	1.55	2.1	0.64	1.37	0.55	3.69	0.78
cis-9-palmitoleic acid	0.48	0.90	0.63	0.12	0.19	0.64	0.12	0.62	0.21	0.52	0.43
margaric acid	0.18	0.26	0.38	0.16	0.09	0.22	0.16	0.19	0.17	0.42	0.5
stearic acid	3.04	8.19	0.38	5.43	1.55	0.99	0.43	0.92	0.33	0.96	0.88
oleic acid	29.23	18.85	10.20	11.53	29.37	35.12	38.64	29.34	28.23	21.34	30.3
vaccenic acid	1.72	8.73	6.02	3.00	0.68	0.31	0.00	3.29	0.00	4.89	0.00
linoleic acid	49.64	34.42	51.19	47.01	59.38	38.56	32.01	43.14	30.2	31.86	31.9
α-linoleic acid	1.74	1.87	1.37	0.26	0.43	0.16	0.26	1.15	0.25	2.66	0.76
14Z-eicosenoic acid	0.70	0.88	0.75	5.17	0.57	3.58	5.17	3.07	4.72	2.93	3.93
11,14-eicosadienoic acid	1.84	6.27	0.75	0.32	0.41	0.8	0.32	1.58	1.05	1.14	1.01
5,8,11-eicosatrienoic acid	0.08	0.46	0.30	0.13	0.04	0.03	0.13	0.1	0.33	0.6	0.5
heneicosylic acid	0.03	0.24	3.49	0.34	0.03	0.09	0.34	0.15	0.69	0.00	0.00
EPA	0.05	0.81	0.20	0.13	0.04	0.03	0.13	0.22	0.15	0.44	0.34
behenic acid	0.30	0.92	17.03	13.33	2.46	1.72	13.33	1.74	26.52	11.29	22.29
cis-erucid acid	0.31	1.37	1.35	0.41	0.11	0.35	0.41	0.35	0.41	0.62	0.72
DHA	5.22	4.96	0.91	8.64	2.70	13	4.53	10.92	2.12	8.24	3.34
lignoceric acid	0.93	4.99	0.89	0.41	0.20	1.07	0.41	0.73	1.27	6.32	0.21
nervonic acid	0.00	0.00	0.33	0.06	0.02	0.03	0.06	0.02	0.14	0.19	0.19

585

FIGURES

Figure 1. Supercritical fluid extraction and trapping system with oil as trapping matrix.

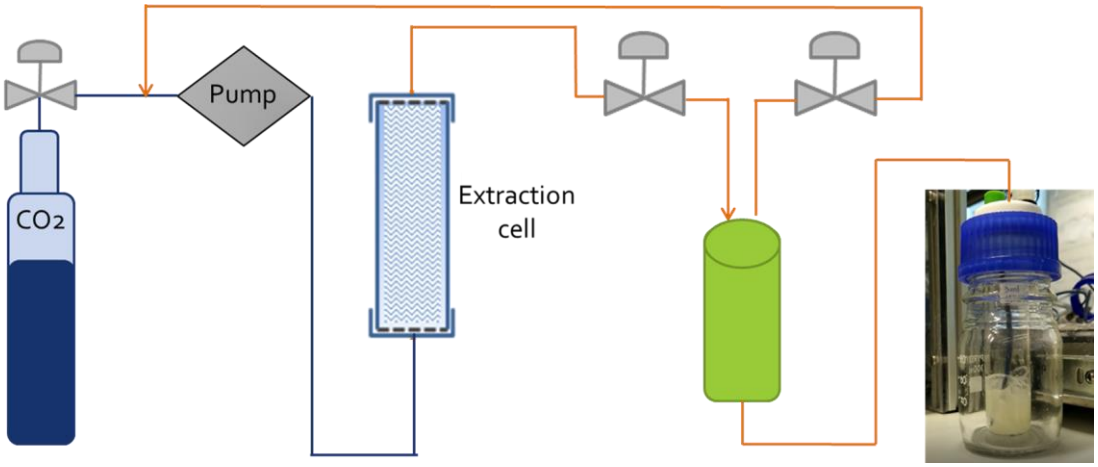
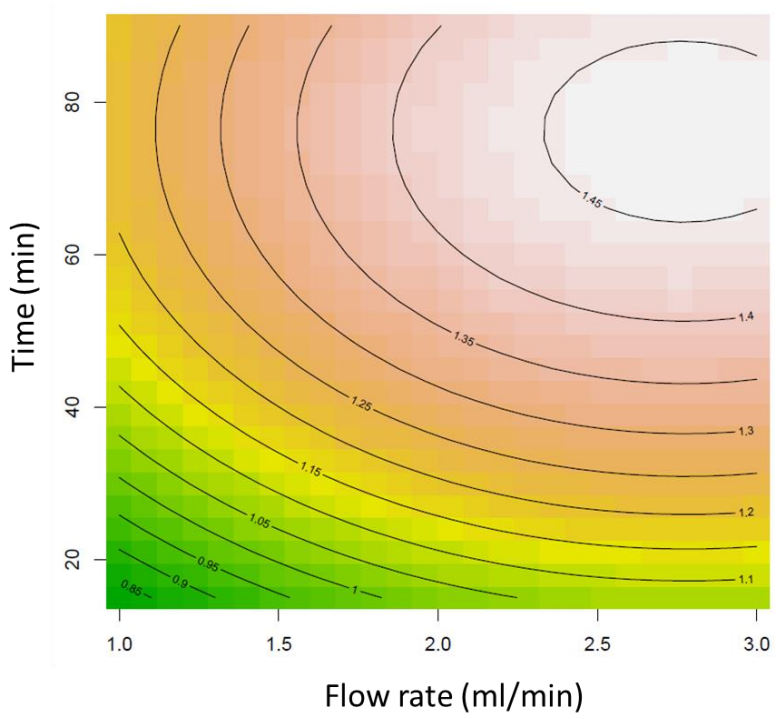


Figure 2. A) 2D response surface plot of SFE extractions from *T. melanosporum*. The responses variable was the extraction yield. B) Effect of time (min) and flow rate (ml/min) on the SFE extraction yield.

A)



B)

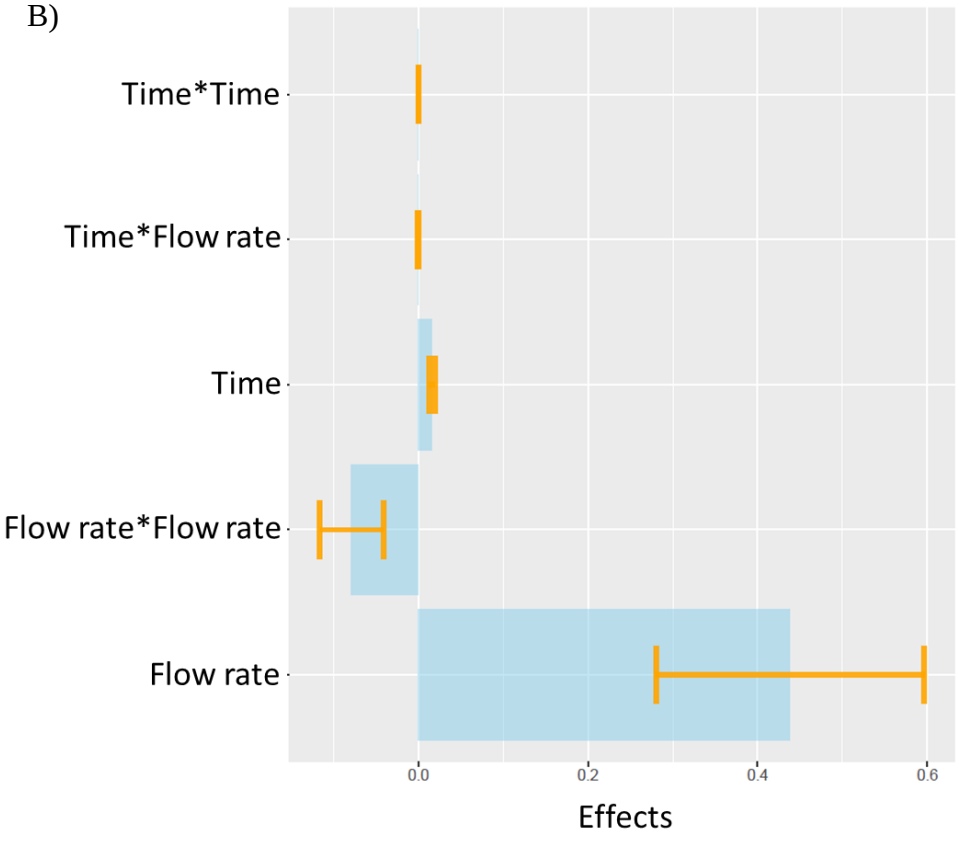
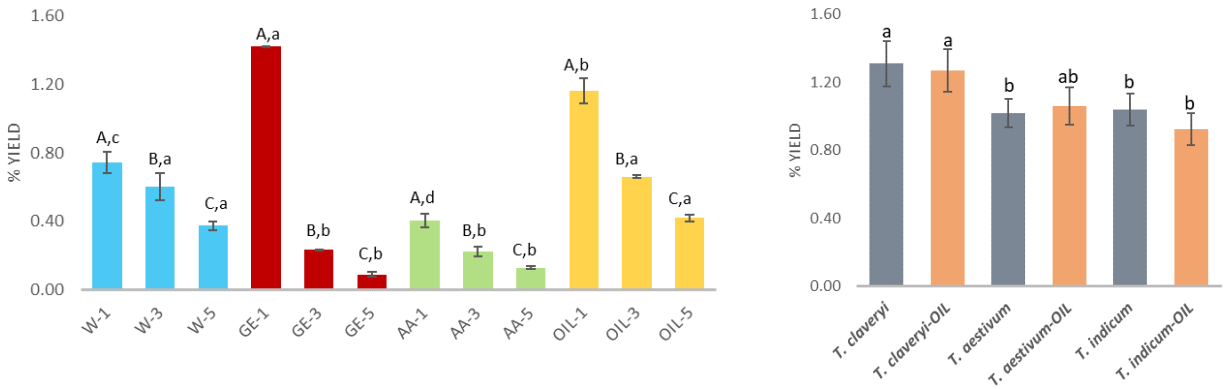


Figure 3. Fatty acids (yellow) and sterols (green) from the SFE extracts (E) and residues remaining in the extraction cell (R) obtained from *T. melanosporum* under the experimental conditions indicated in table 1. Color intensity refers to the percentage (0-100 %) of total sterol and total fatty acids respectively.

Figure 4. Extraction yield (%) of a) *T. melanosporum* extracts obtained at optimal conditions using as trapping matrix water (W, blue), gelatin (GE, red), agar agar (AA, green), and grapeseed oil (OIL, yellow). The number next to the trapping material indicate the volume utilized (mL). b) Extracts from 3 truffle species with (orange) and without (grey) grapeseed oil as trapping material.

a) b)

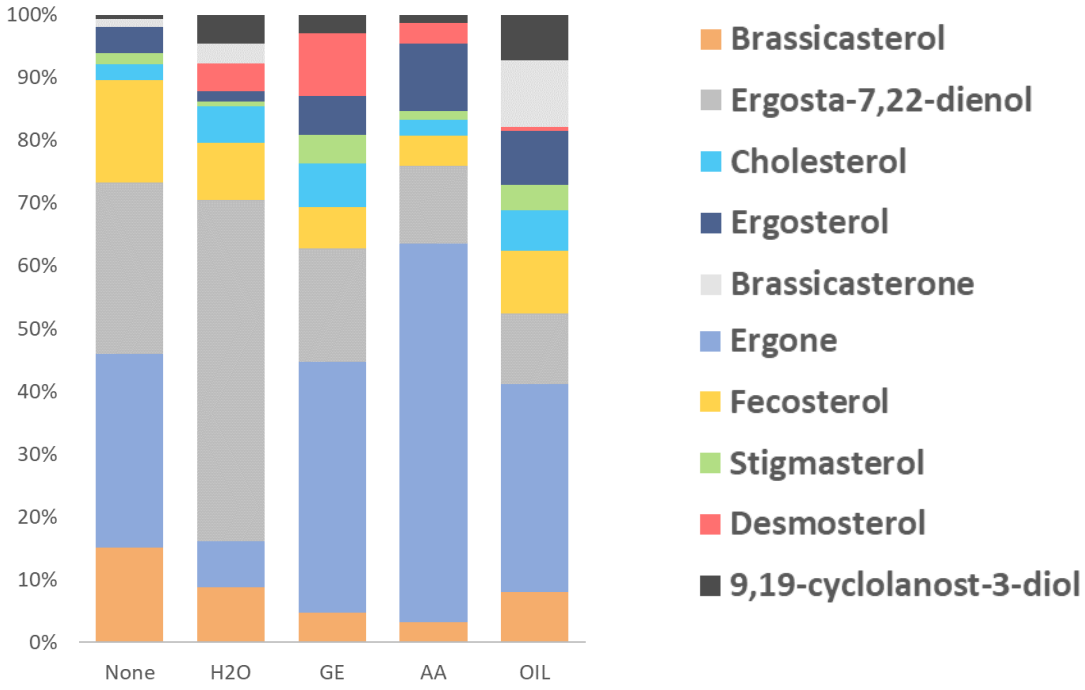


[A, B] Different letters denote significant differences ($p \leq 0.05$) between mL added among the same trapping matrices.

[a, b] Different letters denote significant differences ($p \leq 0.05$) between same volume within different matrices (Figure-a), between the use of oil or not (Figure-b)

Figure 5. Sterol composition (%) of A) *T. melanosporum* SFE extracts obtained in absence of trapping matrix or in presence of 1mL trapping matrix: without matrix (none), water (W), gelatin (GE), agar-agar (AA), grapeseed oil (OIL). B) SFE extracts from three different truffle species with and without oil as a trapping matrix.

A)



B)