Supercritical CO_2 extraction method of aromatic compounds from truffles

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Abstract

Truffles are a well-known worldwide product mainly appreciated by their unique aroma, which is composed by more than 50 volatile compounds. However, to this day, no one has accomplished to find the aromatic key that evokes the real aroma of truffles for its use as food flavoring. Among them, black truffle was selected for extraction with supercritical fluids using CO_2 as solvent recovering natural truffle aroma fraction. To achieve the optimal extraction ratio, time, pressure and grapeseed oil addition to the separators were evaluated. Aroma from black truffle powder, extracts obtained, and residual cakes fractions were characterized by headspace gas chromatography-spectrometry and olfactometry techniques. The results indicated that optimal extraction conditions were 30 MPa for 3 h. Also, grapeseed oil addition enhanced trapping some key truffle aromatic compounds as 2,3-butanodione, 2-methyl-1-butanol, octanal and dimethyl disulphide. Olfactometry study showed the aromatic profile of the extracts indicating the molecules ethyl pentanoate (fruity), 1-hexen-3-one (metallic) and ethyl hexanoate (fruity) as the main compounds of extracts samples. For the first time, a natural truffle aroma has been obtained using low-value truffles. After aromatic extraction, carbohydrates, proteins, and phenolic compounds were analysed within the residues, showing a potential source of bioactive compounds.

1. Introduction

Truffles are one of the most valued fungi because of its excellent organoleptic characteristics, especially their aroma. According to UNECE Standard FFV-53 (2017), truffles are categorized in three classes (Extra, I, II) based on their weight, morphological and physical aspects, but the most important attribute, their aromatic quality, is not included in this classification (Garcia-Barreda, Marco, Martín-Santafé, Tejedor-Calvo, & Sánchez, 2020). The aromatic profile of truffles are a complex mix of many volatile organic compounds (VOCs), in which hydrocarbons, alcohols, aldehydes, esters, ketones, benzene derivatives and sulphur compounds have an important role depending on the species (Culleré et al., 2010; Culleré, Ferreira, Venturini, Marco, & Blanco, 2013; Hilszczańska et al., 2016). Because of their elevated price and their unique aroma, in the last decade, the use of truffles species for enhance the added value of products has been increased in the food markets and restaurants. The mainly processed truffle products are fat-based such as cheese, pates, sauces, and oils, among others (Beara, Majkić, & Torović, 2021; Wernig, Buegger, Pritsch, & Splivallo, 2018). However, food processing or preservation technologies dramatically change the truffle flavor due to aromatic losses or chemical reactions (Campo, Marco, Oria, Blanco, & Venturini, 2017), precluding the use of truffles as a natural aromatic enhancer.

Nowadays, there is no evidence of natural or artificial aroma which integrate several aromatic compounds to evoke the aromatic profile of different truffle species. The compound 2,4-dithiapentane or bis(methylthio)methane, is commonly used as truffle aroma substitute (Campo et al., 2018; Pacioni, Cerretani, Procida, & Cichelli, 2014; Torregiani et al., 2017) despite of being the characteristic molecule of white truffle (*Tuber magnatum*), but it is not present in the black truffle (*T. melanosporum*) aromatic profile

(Wernig et al., 2018). Therefore, no natural or artificial aromatic extract that successfully mimics truffle fresh aroma are available.

Supercritical fluid extraction (SFE) is an environmentally friendly advanced technology with many potential applications for the food industry. It uses non-toxic and/or GRAS solvents, such as CO_2, leaving no solvent traces in the extracted fractions. Supercritical CO_2 is frequently used to extract compounds such as fatty acids (Villanueva-Bermejo, Calvo, Castro-Gómez, Fornari & Fontecha, 2019), sterols (Morales, Piris, Ruiz-Rodriguez, Prodanov & Soler-Rivas, 2018), phenolic compounds (Fernández-Ponce et al., 2016) and other molecules that are usually solubilized in organic solvents. SFE has been used successfully to obtain volatile aromatic fractions from spices (Győri, Varga, Fábián & Lázár, 2019), brandy (Señoráns, Ruiz-Rodríguez, Ibáñez, Tabera & Reglero, 2003), plants (Moldão-Martins, Palavra, Beirão da Costa & Bernardo-Gil, 2000) and cheese (Larráyoz, Ibáñez, Ordóñez, Torre & Barcina, 2000). Therefore, this technique could be a good proceeding to extract aromatic compounds from truffles.

Truffles contain other valuable compounds *i.e.* β-glucans, or specific fugal sterols (Tejedor-Calvo et al., 2019) with interesting biological activities such as immunomodulatory and hypocholesterolemic properties (Patel, Rauf, Khan, Khalid, & Mubarak, 2017). These molecules could remain in the residual cake after the extraction of the aromatic compounds as a byproduct, and they could be also extracted to design novel functional foods.

Thus, in this study, a preliminary screening of aromatic compounds was carried out in three truffle species to determine the one which has the most enriched aromatic profile. Then, the aim of the investigation was, for first time, to extract the aromatic fraction from truffles using supercritical fluids as an extraction method. For that, low-valued truffles were used considering that despite their appearance, they contain similar chemical

compounds and aromatic profile than marketable truffles. As a potential extraction method improvement, grapeseed oil (oil-trap) was added into the separators, where extracts were collected, testing it as lipid matrix to trap the aromatic fraction. The aroma of obtained extracts and remaining cakes were analysed by semi-instrumental techniques: headspace gas chromatography mass spectrometry (HS-GC-MS) and gas chromatography-olfactometry (GC-O). Moreover, the presence of other bioactive compounds was also determined to evaluate the potential valorization of products remaining after SFE.

2. Materials and methods

2.1 Biological material

Tuber melanosporum (Vittad.) and *Tuber aestivum* ascocarps were collected at Gúdar-Javalambre county woods (Teruel province, eastern Spain) and *Terfezia claveryi* Chatin was collected from an experimental plantation in Caravaca de la Cruz (Murcia, Spain). Then truffles (20 units/species) were taxonomically authenticated by morphological features (Montecchi & Sarasini, 2000; Riousset, 2001), selected and processed under refrigeration as described by Rivera, Venturini, Marco, Oria & Blanco (2011). After that, only *T. melanosporum* truffles for subsequent analysis (section 2.3) were lyophilized, ground and sieved to obtain particle size lower than 0.5 mm and were stored at -20 ºC until further use. Grapeseed oil was purchased from Dietisa company (Barcelona, Spain).

2.2 Reagents

Solvents such as hexane (95%), chloroform (HPLC grade), methanol (HLPC grade) were obtained from LAB-SCAN (Gliwice, Poland) and absolute ethanol, sodium carbonate, sodium sulphate and sulphuric acid from Panreac (Barcelona, Spain). Potassium

hydroxide, ascorbic acid, 2,6-Di-*tert*-butyl-*p*-cresol (BHT), bovine serum albumin (BSA), acetylacetone, *p*-dimethylaminebenzaldehyde, HCl (37%), phenol, D-glucose, D-glucosamine hydrochloride, gallic acid, fluorobenzene, n-alkanes series and standards for MS identification (all standards of purity higher than 95%) were purchased from Sigma-Aldrich (Madrid, Spain). All other reagents and solvents were used of analytical quality grade.

2.3 Instrumental aroma analyses by HS-GC-MS

The VOCs profile of different truffles species was analysed by static HS-GC-MS using a Turbomatrix HS16 HeadSpace sampler (PerkinElmer, Massachusetts, USA) coupled to a GC-MS following Caboni et al. (2020) method with modifications. For that, fresh samples (2 g) were placed in 20 mL vials mixed with 1µL fluorobenzene, as internal standard, and hermetically closed. Afterwards, they were heated at 120 ºC for 15 min and 1 min of pressurization time. The injection was carried out for 6 s at 20 psi with an inlet temperature of 220 ºC. Further analysis was carried out on a Clarus 500 GC system coupled to a MS (PerkinElmer, Massachusetts, USA). GC was carried out using a DB-Wax capillary column (60m x 0.25mm i.d. × 0.25 µm film thickness) (Agilent Technologies, California, USA) and a flow of 1 mL/min with helium as a carrier gas. The oven temperature was 45 ºC held for 2 min, 45-200 ºC at a rate of 4 ºC/min, and finally to 225 ºC at 10 ºC/min, and held for 5 min. The MS used the electron impact mode with an ionization potential of 70 eV and an ion source temperature of 200 ºC. The interface temperature was 220 ºC. The MS scanning was recorded in full scan mode (35-250 *m/z*). A TurboMass software was used for controlling the GC-MS system. Peak identification of the VOCs was achieved by comparison of the mass spectra with mass spectral data from the NIST MS Search Program 2.0 library, and by comparison of previously reported Retention Index (RI) with those calculated using an n-alkane series (C_6-C_{20}) under the

same analysis conditions. Semiquantification was done by integrating the area of one ion characteristic of each compound and normalization by dividing the data with the internal standard. Measurements were referred to the sample weight. This allowed comparison of each eluted compound between samples.

2.4 Supercritical fluid extraction

Black truffle powder (TP) (15 g) was mixed with 4.76 mm (∅) stainless steel spheres and placed in the 0.5 L extraction cell of an SFE pilot-scale plant (model SF2000, TharTechnology, Pittsburgh, PA). Pressurized CO_2 was forced to reach supercritical state and injected in the loaded extraction cell. The extracted material was collected in two different separators (separator 1 (S1) and separator 2 (S2)) each of 0.5 L capacity with independent control of temperature and pressure. Extraction was carried out at two different pressures, at 30 MPa (high pressure, HP) and at 12 MPa (low pressure, LP) and 40 ºC in the extraction cell (Table 1). Separators pressures were maintained at 15 and 6 MPa in S1 and S2 respectively in HP extraction, and 6 MPa in both separators in LP extraction. The temperature was 40 ºC in both separators in all conditions tested. The CO_2 flow was set at 2.4 kg/h during a total extraction time of 3 h for LP extraction and 2, 3, 4 and 5 hours for HP extraction. The solvent was recirculated. Moreover, 4 mL grapeseed oil of 100% purity were added into the separators in some trials before depressurization of the 3h extractions. Grapeseed oil was selected as a fat matrix and by their odorless properties (previously analysed by HS-GC-MS). Extracts collected in both separators at the end of the extraction processes were dragged with ethanol and immediately dried on a rotary vacuum evaporator. Extracts from separator 1 (ES1) and separator 2 (ES2) and non-extracted remaining material (RM) at the extraction cell were stored at -20 ºC until further analysis. Also, these samples were analysed by HS-GC-MS (see section 2.3).

2.5 Semi-instrumental aroma analyses by SPME-GC-O

The methodological approach was based on works carried out by Culleré, Ferreira, Ventuini, Marco & Blanco (2012) with modifications. A solid phase microextraction (SPME) was selected to extract the aromatic compounds. For that, a fused silica fiber coated with a 50/30 mm layer of divinylbenzene/carboxen/polydimethylsiloxane from Supelco (Barcelona, Spain) was chosen. The samples (0.5 grams of TP, residues, and extracts) were placed in a 20 mL glass vial closed with a septum and conditioned at 53 ºC for 5 min. The fiber was then exposed to the headspace of the truffle for 30 min. In all cases GC-O analysis was carried out immediately after sampling. A total of three SPME extracts were prepared per sample, one per GC-O judge. The judges (one women and two men, ranging from 22 to 38 years of age) have long experience in olfactometry performed the sniffing analysis. Previously, standard compounds from truffles were used for the judges training.

The GC-O analysis was carried out in a gas chromatograph HP 4890 (Termoquest, Milan, Italy) with a flame ionization detector (FID) and an olfactometric port ODO-I supplied by SGE (Ringwood, Australia). This instrument was equipped with a capillary column DB-WAX (polyethylene glycol) supplied by J&W Scientific (Folsom, CA) of 30 m, 0.32 mm i.d., 0.5 μm film thickness, and a precolumn (3 m; 0.32 mm i.d.) from Supelco (Bellefonte, PA). The chromatographic conditions were nitrogen as the carrier (3.5 mL/min); splitless injection (splitless time 60 s); injector and detector temperature 220 ºC. The oven temperature program was: 40 ºC for 5 min, then raised at 6 ºC/min to 220 ºC, maintained during 15 min for cleaning purposes.

The data processed were a mixture of the intensity and the frequency of the odorants detected/identified (Campo et al., 2017). This parameter is known as “modified

192 frequency" (MF) and is calculated by the following formula MF (%) = $[F (\%)*I (\%)]^{1/2}$,
193 where F (%) is the detection frequency of an aromatic odorant expressed as a percentage
194 of the total number of judges and I (%) is the average intensity expressed as a percentage
195 of the maximum intensity. The odorants were identified by comparison of their odors and
196 chromatographic retention index in a DB-WAX column with those of pure reference
197 compounds, when available. Additionally, the identity of compounds was checked by
198 comparing the sequence of LRI with that of other published databases.
199
200 *2.6 Determination of carbohydrates, proteins, and phenolic compounds*
201 The total carbohydrate content was determined in TP and RM using the phenol-sulfuric
202 acid method as indicated by Morales et al., (2018). Chitin content was quantified as
203 described by Tejedor-Calvo et al. (2019). Standard curves of D-glucose and glucosamine
204 hydrochloride were used for quantification of carbohydrates and chitins, respectively. The
205 β-glucan content (50 mg) was evaluated by a β-glucan determination kit specific for
206 mushrooms and yeasts (Megazyme®, Biocom, Barcelona, Spain).
207 Soluble protein concentration (10 mg/mL) was also evaluated in TP and RM using the
208 Bradford method reagents (Sigma-Aldrich, Madrid, Spain) according to Bradford (1976)
209 method. The phenolic compounds (10 mg/mL) were evaluated by the Folin-Ciocalteu
210 method following Ramírez-Anguiano, Santoyo, Reglero & Soler-Rivas (2007). BSA and
211 gallic acid were used as standards for quantification.
212
213 *2.7 Statistical analysis*
214 Differences between data were evaluated at a 95% confidence level ($p < 0.05$) using a
215 one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test.
216 Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad

Software, San Diego, CA). Principal Component Analysis (PCA) was also performed and visualized in RStudio 1.2.1335 (RStudio Team, 2019) using R version 3.6.1 and the factoextra package (Kassambara and Mundt, 2017).

3. Results and discussion

3.1 Screening of VOCs within different truffle species

Firstly, the concentration of several interesting VOCs was determined within selected truffles species to point out the one with more quantity of volatile compounds and selecting it for further studies. In total, 22, 16, and 45 compounds of more than 0.05 mg/100g of truffle were identified in *T. claveryi*, *T. aestivum* and *T. melanosporum*, respectively (Table 1). *T. claveryi* truffles were mainly composed by 2-methyl-1-butanal, propanone, 2-butanone, methanethiol and, 2,3-butanodione. The compound propanone stood out, which have a characteristic fruity odor, raising the highest value (68 mg/100 g truffle). The highest values of VOCs in *T. aestivum* truffles were 2-methyl-1-butanol, 1-methylpropyl formate and propanone, however the number of compounds identified were the lowest. According to that, Culleré et al. (2010) revealed that summer truffle emits is up to 100 times lower than that of black truffles. This fact can explain the high number of compounds identified in *T. melanosporum* (Table 1). The highest values of VOCs in black truffle were achieved by 2-methyl-1-butanal, 3-methyl-1-butanal, propanone and methanethiol (41, 67, 82 and 32 mg/100 g truffle respectively). Thereby, *T. melanosporum* has been selected as a source for optimization the extract of the aromatic fraction agreeing to other studies of black truffle aroma (Campo et al., 2017; Lee et al., 2020; Strojnik, Grebenc, & Ogrinc, 2020),

3.2 Supercritical fluid extractions of black truffles

Supercritical fluid extractions from *T. melanosporum* ascocarps were carried out using different pressure and time conditions, to enhance the aromatic extraction yield using CO_2 (Table 2).

3.2.1 Influence of extraction pressure

The results showed that pressure had a direct influence on the distribution of the load of extracted material collected in each separator (S1 and S2). When 12 MPa were applied, almost 90% of the total extracted material was recovered in S1. Higher pressures (30 MPa) enhanced the extraction capacity yielding in S2. A previous study in mushroom SFE extraction obtained similar yield results testing similar pressure (Morales et al., 2018).

3.2.2 Influence of extraction time

Time also had a significant effect on the extraction with supercritical fluids modifying the distribution in the separators. Although the results for extracted dry matter were similar regardless of extraction time (Table 2), the extraction yield in separator 1 was gradually decreased as time increased; the trend was the opposite in the separator 2. After 3h extraction time 9.4 % of the total extracted material were recovered in S1 while after 5h extraction less than 3% were found in S2. However, from 3h the sum of extraction yield from separators were similar comparing with higher extraction times. For that, 3 h was selected as the optimal extraction time. Regardless of pressure, 3h extraction time, showed similar amount of material in both separators. For that, extraction time had a higher impact on the extraction time than the pressure. Similar behavior with time and pressure resulted for different plant and species (Fornari et al., 2012).

3.2.3 Influence of oil-trap

Grapeseed oil addition produced no meaningful differences in extraction yield compared to extractions carried out in its absence at the same pressure and time. Although truffles contain a higher lipid content than edible mushrooms, extraction yields were in the range of those obtained for instance for *Agaricus bisporus* (showing 1.4 - 2.1% (w/w) (Gil-Ramírez et al., 2013) or *Lentinula edodes* (1.1 - 1.7% w/w) (Morales et al., 2018). This result might suggest that under the selected parameters, SFE showed certain preference to specifically extract similar type of fungi compounds, *i.e.* truffles contain similar sterols amounts than mushrooms (4 - 6 mg/g) (Tejedor-Calvo et al., 2020a).

3.3 VOC's profile of obtained SFE extracts

The developed HS-GC-MS method allowed the identification of a higher number of compounds (Table 2) than previously reported (Caboni et al., 2020). Ninety-one volatile compounds were identified in fresh truffle, and almost half (54 compounds) were still detected after the truffle was freeze-dried. The compounds identified were acids, alcohols, aldehydes, aromatic compounds, esters, heterocycles, hydrocarbons, ketones, salts, and sulfur-containing molecules. These molecules were grouped according to their chemical characteristics and compared to those noticed in the SFE extracts and residues (Figure 1). The TP showed mainly ketones and aldehydes, followed by acids, alcohols, esters, and sulfur-containing compounds in lower quantities. A similar composition was found in the residues remaining (RM) after SFE extraction, indicating that not all the volatiles were extracted with the supercritical CO_2 in the selected conditions (Figure 1-A).

3.3.1 Influence of extraction pressure

The application of HP mainly recovered acid, aldehyde, ketone, and heterocyclic compounds in ES1, and salt, hydrocarbon, sulfur-containing, and aromatic compounds among others in ES2. However, some of these compounds were only extracted in ES2 when LP was applied. That was because pressure in both separators (LP condition) was the same (6MPa). But, when LP was applied, aromatic and sulfur-containing compounds were not extracted, probably because they might be extracted between 12 MPa (LP) and 30 MPa (HP).

3.3.2 Influence of extraction time

In the ES1 samples, aldehydes were the major group followed by ketones and acids (Figure 1-B). The amount of these groups, together with alcohols and heterocyclic compounds, were increasing with time extraction. In contrast, aromatic compounds and salts were only extracted in ES2 (Figure 1-C). The latter extracts showed a more heterogeneous composition being acids and aldehydes their major constituents, regardless the extraction time applied.

3.3.3 Influence of oil-trap

The SFE extraction applying HP for 3h was also carried out with oil in the separators to test whether it could trap the volatiles in its matrix during depressurization. After subtracting the VOCs specific from grapeseed oil, the extracts collected in ES1 contained lower compound levels than without oil; mainly esters were detected suggesting that depressurization induced esterification of the extracted acids (detected in ES1-HP 3h without oil). Also, heterocyclic and hydrocarbon compounds have not being retained within the oil-trap. However, in the ES2-oil, a higher level of alcohols (displacing the aldehydes, esters and hydrocarbon noticed in ES2) was observed.

3.3.4 Multivariate data analysis of volatile composition

To explore the possible correlations of the SFE conditions and fractionation with the volatile components of black truffle aroma, a principal components analysis (PCA) was performed (Figure 2). The first five principal components of the standardized VOCs concentration explained a combined 75.9% of the total variability. The first two components only explained 47.5% of the variability, indicating the complexity of the relationships between SFE conditions, fractionation, and volatile profiles. The rest of principal components were included in supplementary material (Table S1 and Figure S1). The compounds that showed the more positive loadings with the first PCA component were 3-octanol and hexanoic acid, whereas those showing the more negative loadings were 2,3-butanedione, carbon disulfide, DMDS, 2-heptanone, 3-methylanisole, and 4-(2-butyl) phenol (Figure 2-A). The compound that showed the more positive loading with the second PCA component was 2-butanone, whereas those showing the more negative loadings were benzeneacetic acid methyl ester, hydroxypropanone, methyl propanal, methyl acetate, methyl-caproate, methyl 2-hydroxypropanoate, methyl 3-hydroxybutanoate and octane. However, these two PCA components (PC1 and PC2) allowed to clearly separate the aroma profiles in four well-differenced groups: TP and RM samples (group 1), ES1-HP samples (group 2), ES2-HP samples (group 3) and extracts from oil added samples (group 4) (Figure 2-B).

The first group was characterized by a relatively higher contribution to the aroma of anisole, butanal, 2,3-butanedione, 4-(2-butyl)phenol, carbon disulfide, 3,4-dimethoxytoluene, DMDS, DMS, 2-heptanone, isoamyl isobutanoate, 2-methylpropanol, 3-methylanisole, 1-penten-3-ol, octanal and 2-octanone.

The second group, including ES1-HP samples, was relatively characterized by an increased content of aldehydes (acetaldehyde, hexanal, heptenal, (E)-2-heptenal, nonanal, propanal, pentanal, (E)-2-octenal,) and heterocyclic compounds (2-ethylfurane, 2-pentylfuran), but also by some alcohols (1-dodecanol, 1-heptenol, 3-methylhexanol, 1-octen-3-ol, 1-octanol). The PCA pointed out that the longer the extraction time, the higher content of these compounds is obtained (Figure 2b).

The third group (ES2-HP samples) is characterized by a relatively higher content of methyl-caproate, benzeneacetic acid methyl ester, hydroxypropanone, methyl propanal, methyl acetate, octane, methyl 2-hydroxypropanoate, methyl 3-hydroxybutanoate, and ethyl 3-methylbutanoate. Most compounds appear to be associated with one of these three groups, although a few are in intermediate situations: 2-butanone, 2,3,6-trimethyl-4-octene, and 2-butanol between groups 1 and 2; 2,3-pentadione between groups 1 and 3; and 3-octanol between groups 2 and 3. These compounds did not seem to be completely extracted, so that, they may be found in similar quantities in different groups.

The fourth group included the extracts obtained with oil-trap. This group is characterized by a relatively poor aromatic composition, indicating that adding oil did not extract higher amounts of aromatic compounds (Table 3). Finally, the performance of the SFE-LP samples was not homogeneous. The PCA grouped sample ES1-LP-3h with oil-trap samples, and ES2-LP-3h with ES1-HP samples, thus indicating that higher pressure is needed to extract the aromatic components from TP.

3.3 Olfactometric profile of obtained SFE extracts

In order to detect these odorants attending to their importance in the black truffle aroma, a GC-O study was performed. In the analyses carried out, 36 odor compounds were detected and identified (Table 3). Olfactometric scores (MF %) of the detected odorants

were included in supplementary material (Table S2), and values of <25 were discarded of the analysis. Also, grapeseed oil was analysed by GC-O showing the compounds with values below to the MF limit.

The TP sample was mainly composed by DMS (truffle), 3-methyl-butanal (rancid) and ethyl-2-methylbutanoate (strawberry) (Table S2). However, 2-acetil-2-pirroline, also present in TP, was high valued in RM sample. Ethyl pentanoate (fruity 2) and 1-hexen-3-one (metallic) shower high MF values in all ES1 samples. And ES2 samples contained DMDS (truffle1) and ethyl hexanoate (fruity 3) as the main odor components.

A PCA was used to explore the possible correlations of the SFE conditions and fractionation with the odor compounds of black truffle aroma. The PCA analysis explained 31.5 % of the data variability with the two first components. The compound that showed the more positive loadings with the first PCA component was 3-isobutyl-2-methoxypirazine (toasted almond) whereas those showing the more negative loadings was 1-butanol (green1) (Figure 3). The compound that showed the more positive loading with the second PCA component was ethyl-3-methyl butanoate (strawberry, pineapple), whereas those showing the more negative loadings was ethyl pentanoate (fruity 2).

The application of PCA analysis clearly separate the aroma profiles between TP, RM and extracts samples (Figure 3), as well as HS-GC-MS technique. At the top, TP sample was characterized by high MF in DMS and 3-methyl-butanal (truffle and rancid odor descriptor respectively). Also, ethyl-2-methyl-butanoate, 2-acetyl-pyroline and methional, related to strawberry, toasted almond and baked potato as odor descriptors, were only detected in TP sample. RM samples were located on the left of PCA graph, corresponding to negative loading of first PCA components. Almost all fruity descriptors were located on below right of the PCA, as ES1 samples. However, ES1-OIL-3h sample, which contained 2-methyl-butanoic acid (cheese) as mainly odor descriptor, was

389 positioned in RM samples area. That could be explained because 3h-HP extraction ratio
390 was poorer comparing to oil-trap extraction. As not all compounds were extracted, TP
391 were closely to RM samples (Figure 3). However, ES1-OIL-3h sample position indicated
392 that compounds remaining in RM samples without oil-trap, were collected in ES1 when
393 oil-trap is used. Conversely, ES2 samples were situated on the right part, except ES2-
394 OIL-3h which is closely to TP samples. This could indicate that most of the aromatic
395 compounds detected were extracted. In that sense, the use of oil-trap in separators allows
396 trapping some compounds better, obtaining similar profiles than TP aroma (see Figure 3).
397
398 *3.4 Composition of the remaining cake after SFE extraction*
399 In order to revalue the remaining material after the SFE extraction, accordingly to circular
400 bioeconomy goals, different chemical composition analyses were carried out.
401 Carbohydrates were the main truffle constituents (particularly, β-glucans and chitins),
402 followed by a high protein content (Table 4). These values were in agreement with
403 previous results (Tejedor-Calvo et al., 2019), although the content of all these compounds
404 might change depending on environmental conditions, developmental stage, etc. (Harki,
405 Bouya, & Dargent, 2006). After SFE, the remaining cakes showed a slightly lower
406 carbohydrate concentration than TP sample. It might be due to a β-glucan reduction since
407 no significant variation were noticed in chitins levels. Moreover, no significant
408 differences were noticed within the different extraction times suggesting that only 2h in
409 contact with CO_2 were sufficient to induce their modification; perhaps the acidic
410 environment generated could induce a partial degradation. However, CO_2 at the
411 conditions tested did not influence proteins levels, as expected, they were not extracted
412 by SFE, and their concentrations were analogous to the initial material. Correspondingly,
413 most of the phenolic compounds also remained in the cake and only a few were extracted

with longer extraction times, probably nonpolar phenols. Therefore, the remaining material after SFE showed high bioactive compounds levels, maintaining its potential bioactivity capacity as recent studies revealed (Morales et al., 2019; Tejedor-Calvo et al. (2020a).

4 Conclusions

The use of supercritical fluids with CO_2 results a promising methodology for truffle aroma extraction. Among the tested conditions, three hours at high pressure produced the best extraction yields. Also, the addition of grapeseed oil helped to trap key truffle aromatic compounds such as 2,3-butanodione, 2-methyl-1-butanol, octanal and DMDS. Thus, the optimized method (3h-30MPa) could be applied to other truffle species to obtain enriched aromatic fractions. However, a few odor compounds in black truffle (ethyl-2-methyl-butanoate, 2-acetyl-pyroline and methional) were not extracted. So, further research should be carried out to improve the extraction method and increase the content of truffles aromatic compounds. In addition, remaining material after SFE might also be a potential source of interesting bioactive compounds.

Conflict of interest

None

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580

581 **Tables**

582 Table 1. List of volatile compounds identified by HS-GC-MS in truffles species. Values

583 are given in mg/100g truffle.

Code	RRT	Name	CAS nº	RI $_{exp}$	RI $_{lit}$	*Terfezia claveryi*	*Tuber aestivum*	*Tuber melanosporum*
Acid								
67	2.69	Acetic acid	64-19-7	1449*	1452	0.60	-	-
74	2.98	Propanoic acid	79-09-4	1534	1540	-	-	-
77	3.09	2-Methylpropanoic acid	79-31-2	1566	1570	-	-	3.35
79	3.29	Butanoic acid	107-92-6	1625	1628	-	-	0.75
80	3.36	4-Hydroxybutanoic acid	591-811	1645	ND	-	-	-
83	3.44	2-Methylbutanoic acid	116-53-0	1669	1674	-	-	1.01
88	4.00	Hexanoic acid	142-62-1	1846*	1851	-	-	-
Alcohol								
19	0.89	Ethanol	64-17-5	945	935	-	0.23	0.07
24	1.11	2-Butanol	78-92-2	1026	1022	-	0.27	0.41
25	1.12	1-Propanol	71-23-8	1031	1032	0.11	-	-
32	1.33	2-Methylpropanol	78-83-1	1098*	1092	-	-	2.80
34	1.39	2-Pentanol	6032-29-7	1111	1117	-	-	-
36	1.47	1-Butanol	71-36-3	1132*	1148	-	-	0.08
38	1.64	1-Penten-3-ol	616-25-1	1177	1158	-	-	-
43	1.75	2-Methyl-1-butanol	137-32-6	1208	1208	-	3.23	2.06
44	1.78	3-Methyl-1-butanol	123-51-3	1212	1212	-	0.53	-
46	1.93	2-Hexanol	626-93-7	1253	1245	-	-	-
47	1.93	1-Pentanol	71-41-0	1259	1255	-	-	-
59	2.34	1-Hexanol	111-27-3	1359	1359	-	-	-
61	2.52	3-Octanol	589-98-0	1406	1397	-	-	-
62	2.54	3-Methylhexanol	13231-81-7	1413	1413	-	-	0.81
66	2.67	1-Octen-3-ol	3391-86-4	1449*	1450	0.42	-	2.14
68	2.71	1-Heptenol	111-70-6	1459	1461	-	-	-
76	3.07	1-Octanol	111-87-5	1560	1560	-	-	-
89	4.37	1-Dodecanol	112-53-8	1972	1974	-	-	-
Aldehyde								
5	0.51	Acetaldehyde	75-07-0	742	714	0.08	-	1.85
7	0.60	Propanal	123-38-6	797	799	1.21	-	0.36
9	0.62	Methyl propanal	78-84-2	806	818	-	-	-
12	0.63	Butanal	123-72-8	815	837	1.63	0.51	4.44
15	0.80	2-Methyl-1-butanal	96-17-3	911	910	11.98	3.59	41.90
16	0.82	3-Methyl-1-butanal	590-86-3	920	913	6.35	1.73	67.28
22	0.98	Pentanal	110-62-3	983*	982	1.59	-	4.35
31	1.30	Hexanal	66-25-1	1084*	1072	2.39	-	-
41	1.68	Heptanal	111-71-7	1182	1180	-	-	0.86
49	2.06	Octanal	124-13-0	1286	1289	-	-	-
55	2.23	(E)-2-Heptenal	18829-55-5	1329	1321	-	-	-
60	2.48	Nonanal	124-19-6	1397	1384	-	-	-
63	2.62	(E)-2-Octenal	2548-87-0	1434	1434	0.20	-	12.67
73	2.92	2-Nonenal	2463-53-8	1518	1537	-	-	0.04
85	3.73	2,4-Decadienal	2363-88-4	1760	1771	-	-	-
91	4.82	Tetradecanal	124-25-4	-	1927	-	-	0.14
Aromatic compounds								
58	2.29	Anisole	100-66-3	1347*	1340	0.06	-	-
65	2.66	3-Methylanisole	100-84-5	1446	1441	-	-	-
75	2.99	Benzaldheyde	100-52-7	1537	1550	-	-	-
78	3.25	4-(2-Butyl)phenol	99-71-8	1612	ND	-	-	0.07
81	3.38	Benzeneacetaldehyde	122-78-1	1650	1650	0.06	-	-

84	3.72	3-Methoxyanisole	151-10-0	1756	1737	-	-	-
86	3.76	Benzeneacetic acid, methyl ester	101-41-7	1768	ND	-	-	-
87	3.89	3,4-Dimethoxytoluene	494-99-5	1810	1806	-	-	-
90	4.72	3,4,5-Trimethoxytoluene	6443-69-2	-	ND	-	-	-
Ester								
17	0.84	Methyl isobutirate	547-63-7	928	924	-	-	3.82
18	0.85	1-Methylpropyl formate	589-40-2	933	ND	-	17.08	3.36
27	1.17	Ethyl 2-methylbutanoate	7452-79-1	1044	1052	-	-	-
28	1.21	Ethyl 3-methylbutanoate	108-64-5	1056	1053	-	-	-
33	1.35	Isobutyl isobutyrate	97-85-8	1094	1095	-	-	0.50
37	1.58	Isoamyl isobutanoate	2050-01-3	1162	ND	-	-	0.05
39	1.65	Isobutyl 2-methylbutanoate	2445-67-2	1179	ND	-	-	-
42	1.69	Methyl caproate	106-70-7	1192	1189	-	-	0.04
53	2.19	Methyl 2-hydroxypropanoate	2155-308	1320	1335	-	-	0.18
69	2.81	Methyl 3-hydroxybutanoate	1487-49-6	1486	1475	-	-	-
Heterocyclic								
20	0.91	2-Ethylfurane	3208-16-0	956	960	-	-	-
45	1.85	2-Pentylfuran	3777-69-3	1232	1228	-	-	-
48	2.01	2-Methylpyrazine	109-08-0	1273	1274	-	-	-
54	2.20	2-Hexylfuran	3777-70-6	1323	1323	-	-	-
82	3.43	2-Furanmethanol	98-00-0	1666	1668	-	-	-
Hydrocarbon								
1	0.37	Hexane	110-54-3	*	-	-	0.01	0.73
2	0.41	Heptane	142-82-5	*	-	-	-	-
8	0.61	Octane	111-65-9	*	-	0.43	0.04	-
23	1.04	2,3-Dimetil, 2-butene	563-79-1	1005	ND	-	-	0.27
56	2.26	4-Methyl-1-pentene	691-37-2	1338	ND	-	-	1.12
57	2.27	2,3,6-Trimethyl-4- octene	63830-65-9	1341	ND	-	-	0.25
70	2.83	2,6,11-Trimethyldodecane	31295-56-4	1492	ND	-	-	0.06
Ketone								
11	0.63	Propanone	67-64-1	812	820	68.14	12.18	82.06
14	0.78	2-Butanone	78-93-3	902	908	3.89	1.46	7.33
21	0.96	2,3-Butanedione	431-03-8	974*	975	4.85	0.54	1.18
26	1.14	2-Pentanone	107-87-9	1034	1025	0.05	-	0.07
29	1.22	2,3-Pentanedione	600-14-6	1058	1055	0.97	0.13	19.31
35	1.47	3-Penten-2-one	625-33-2	1132	1138	-	-	-
40	1.66	2-Heptanone	110-43-0	1180	1169	-	-	0.30
50	2.07	2-Octanone	111-13-7	1279	1278	-	-	5.80
51	2.14	2-Hydroxy-3-butanone	513-86-0	1305	1280	-	-	0.56
52	2.18	Hydroxypropanone	116-09-6	1317	1298	-	-	-
64	2.63	2-Nonen-4-one	32064-72-5	1437	1466	-	-	-
71	2.87	2-Octen-4-one	4643-27-0	1502	ND	-	-	-
72	2.88	2-Decanone	693-54-9	1505	1493	-	-	-
Salt								
10	0.62	1-Propen-2-ol, acetate	108-22-5	808	ND	-	-	-
13	0.65	Methyl acetate	79-20-9	825	822	-	-	0.19
Sulfur-containing								
3	0.45	Carbon disulfide	75-15-0	710	696	1.23	-	6.01
4	0.45	Methanethiol	74-93-1	720	710	11.07	0.18	32.15
6	0.56	Dimethyl sulfide	75-18-3	776*	757	0.08	1.03	1.29
30	1.27	Dimethyl disulfide	624-92-0	1075*	1069	-	-	4.34

584 RRT= Relative Retention Time with respect to the standard Fluorobenzene

585 RI exp= Retention Index experimental

586 RI lit= Retention Index Literature database NIST (NIST, 2020)

587 *=Standard compound in the condition of the method

588 - not detected or below 0.5 mg/100 g truffle.

589 Table 2. Extraction yields obtained in separators 1 (ES1) and 2 (ES2) after SFE of *T.*
590 *melanosporum* at differents pressure and time conditions. HP: high pressure; LP: low
591 pressure, O: oil addition into the separator.

Extraction	**Parameters**			**Yields (%, w/w)**	
	Pressure extraction (MPa)	Extraction time (h)	Added oil (mL)	ES1 (%)	ES2 (%)
HP-2h	30	2	-	0.14 ± 0.03^{b}	1.45 ± 0.15^{b}
HP-3h	30	3	-	0.20 ± 0.01^{b}	1.93 ± 0.11^{a}
HP-4h	30	4	-	0.09 ± 0.02^{c}	2.06 ± 0.12^{a}
HP-5h	30	5	-	0.05 ± 0.01^{c}	2.17 ± 0.13^{a}
LP-3h	12	3	-	1.70 ± 0.13^{a}	0.20 ± 0.03^{c}
HP-OIL-3h	30	3	4	0.20 ± 0.02^{b}	1.88 ± 0.12^{a}

592 Different letters (a–c) showed statistical significance ($P \geq 0.05$) between different
593 extractions

Table 3. List of odor compounds identified by GC-O analysis: retention time (RT), chemical identity, CAS number, odor descriptor and linear retention index (LRI).

Number	RT (min)	Identity	CAS number	Odor descriptor	LRI BD-WAX
1	3.53	Dimetilsulfide (DMS)[a]	78-18-3	Truffle	<1000
2	5.59	Dimethyldisulphide (DMDS)[a]	624-92-0	Truffle1	915
3	6.04	3-methyl-butanal[a]	96-17-3	Rancid	967
4	6.36	Pentanal[b]	110-62-3	Almond	972
5	7.50	ni	-	Fruity	-
6	8.16	2,3-butanodione[a]	431-03-8	Buttery	989
7	8.32	ni	-	Fruity1	-
8	8.50	ni	-	Green	-
9	9.24	Methyl 2-methylbutanoate[b]	868-57-5	Apple	1008
10	10.38	Ethyl 2-methylbutanoate[a]	7452-79-1	Strawberry	1052
11	11.12	Ethyl 3-methylbutanoate [a]	108-64-5	Strawberry, pineapple	1066
12	11.46	1-hexen-3-one[b]	1629-60-3	Metallic	1085
13	12.51	3-Methylbutyl acetate[b]	123-92-2	Banana	1117
14	13.21	Ethyl pentanoate[b]	539-82-2	Fruity2	1132
15	13.48	1-butanol[b]	71-36-3	Green1	1150
16	14.06	Myrcene[b]	123-35-3	Metallic1	1160
17	15.32	ni	-	Strawberry1	-
18	17.08	Ethyl hexanoate[b]	123-66-0	Fruity3	1243
19	17.33	Z-4-heptenal[a]	6728-31-0	Fish	1255
20	17.51	hexyl acetate[b]	142-92-7	Fruity4	1265
21	19.29	1-octen-3-one[a]	4312-99-6	Mushroom	1315
22	20.45	2-acetyl-1-pirroline[a]	99583-29-6	Toasted almond	1356
23	22.17	(Z)-3-Hexen-1-ol[b]	928-96-1	Green2	1406
24	22.33	2-Propanoyl-1-pyrroline[b]	133447-37-7	Roasty	1415
25	23.32	3-Isobutyl-2-methoxipyrazine[a]	24683-00-9	Bell pepper	1450
26	24.01	Acetic acid[a]	64-19-7	Vinegar	1470
27	24.27	Methional[a]	3268-49-3	Baked potato	1482
28	25.25	1-Octen-3-ol[a]	3391-86-4	Mushroom1	1516
29	26.42	3-Isobutyl-2-methoxipyrazine[b]	27300-27-2	Toasted almond1	1563
30	27.27	ni	-	Humidity	-
31	27.5	ni	-	Garlic	-
32	29.16	3-Methylbutanoic acid[b]	503-74-2	Sweaty	1660
33	29.59	2-Phenylethanal[b]	60-12-8	Honey	1677
34	30.09	E,E-2,4-nonadienal[a]	5910-87-2	Rancid1	1694
35	30.31	2-Methylbutanoic acid[b]	116-53-0	Cheese	1709
36	32.22	3-Methylbutanoic acid[b]	503-74-2	Cheese1	1784

ni= not identified

[a] Identification based on coincidence of gas chromatographic retention with those of the pure compounds available in the laboratory.

[b] Tentative identification based on comparison with LRI databases published in the literature

601 Table 4. Levels of total carbohydrates (CH), β-glucans, chitins, total proteins, and total
602 phenolic compounds (PC) in TP and remaining cakes (RM) after supercritical extractions.

Sample	CH (g/100g)	β-glucans g (g/100g)	Chitin (g/100g)	Proteins (g/100g)	PC (mg/g)
TP	30.55 ± 3.40^{a}	16.04 ± 1.20^{a}	11.68 ± 0.50^{a}	8.58 ± 0.50^{a}	1.13 ± 0.03^{a}
RM-2h	21.69 ± 2.96^{b}	11.86 ± 0.83^{b}	11.48 ± 0.26^{ab}	10.00 ± 0.90^{a}	0.89 ± 0.09^{b}
RM-3h	20.97 ± 1.50^{b}	12.47 ± 0.95^{b}	12.35 ± 0.09^{a}	10.62 ± 0.47^{a}	1.02 ± 0.04^{ab}
RM-4h	21.10 ± 3.24^{b}	10.77 ± 0.92^{b}	11.17 ± 0.24^{ab}	9.00 ± 0.03^{a}	1.08 ± 0.07^{ab}
RM-5h	25.93 ± 2.58^{ab}	10.61 ± 0.96^{b}	11.48 ± 0.82^{ab}	8.44 ± 0.91^{a}	0.90 ± 0.04^{b}
RM-LP-3h	20.08 ± 4.37^{b}	9.23 ± 0.48^{b}	10.86 ± 1.47^{ab}	8.40 ± 0.34^{a}	0.96 ± 0.04^{ab}
RM-OIL-3h	19.33 ± 2.79^{b}	10.28 ± 0.80^{b}	8.67 ± 0.58^{b}	9.52 ± 1.16^{a}	0.97 ± 0.05^{ab}

603 Different letters (a, ab, b) showed statistical significance ($P \geq 0.05$) between different
604 extractions.

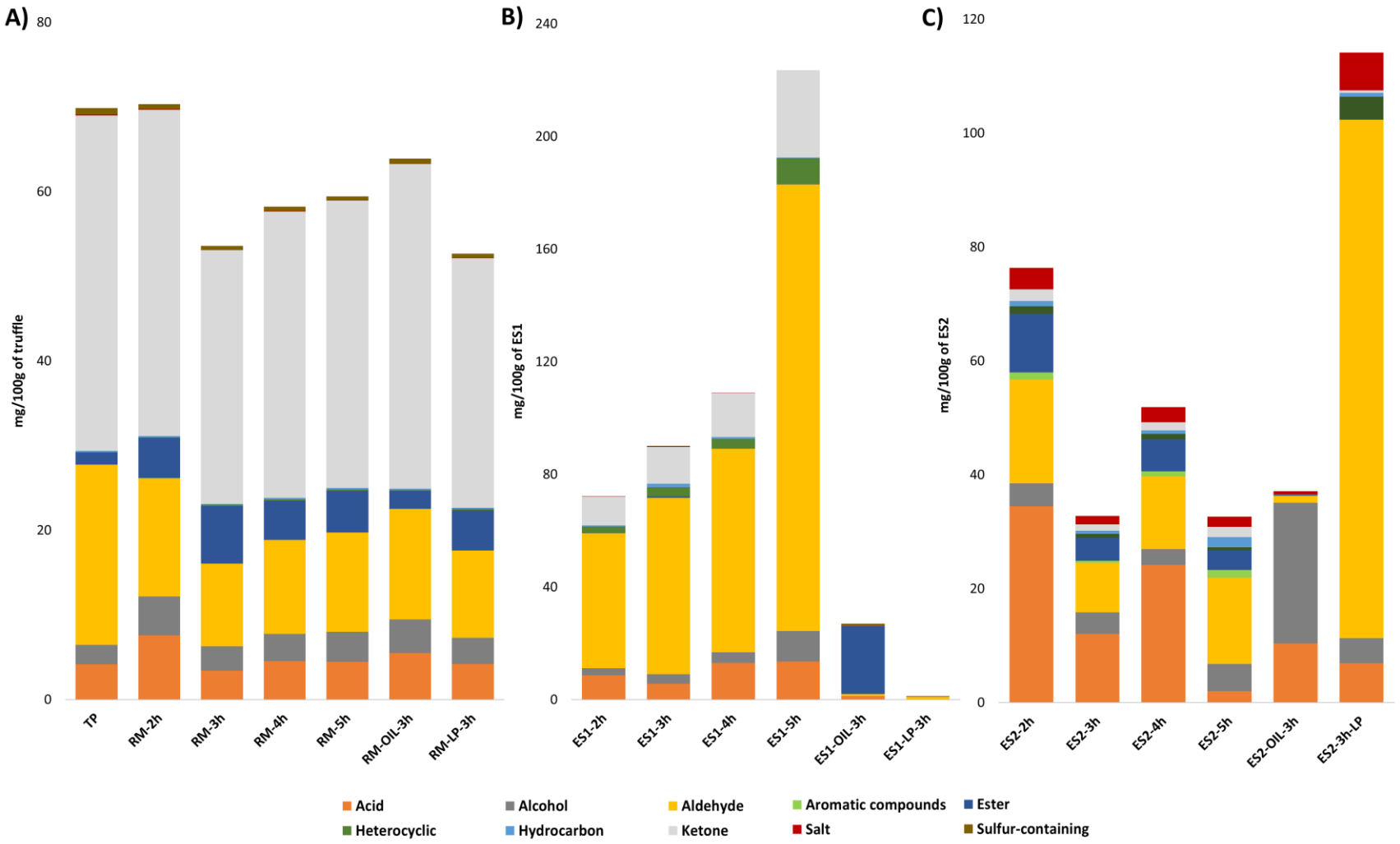
Figures

Figure 1. Distribution by chemical groups of the different volatile compounds identified by HS-GC-MS in A) dry truffle powder (TP) and SFE residues (RM), and in extracts obtained from B) separator 1 (ES1) and C) separator 2 (ES2).

Figure 2. PCA a) loading plot for volatile compounds detected by HS-GC-MS and b) score plot for aroma variation among SFE samples. Samples names were those indicated in Table 1 and compound numbers were those listed in Table 2. Arrow color indicates the contribution of a compound to the PCA components (contrib) and sample color indicates the quality of representation for the sample (cos2).

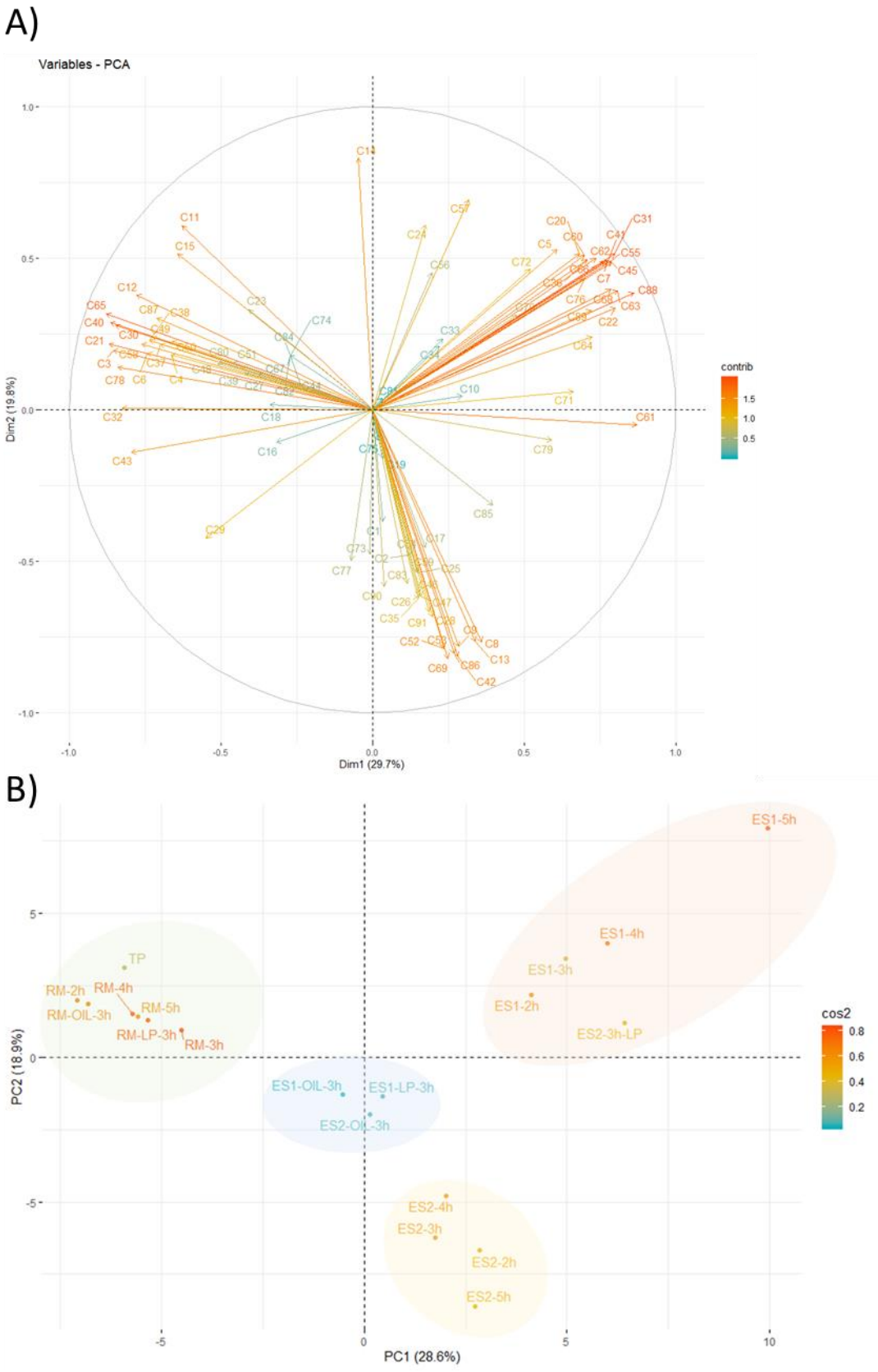
Figure 3. PCA plot corresponding to odorous attributes detected by CG-O. Odors descriptors were those listed in Table 4. Arrow color indicates the contribution of a compound to the PCA components (contrib) and sample color indicates the quality of representation for the sample (cos2).

618 Figure 1



619

620 Figure 2



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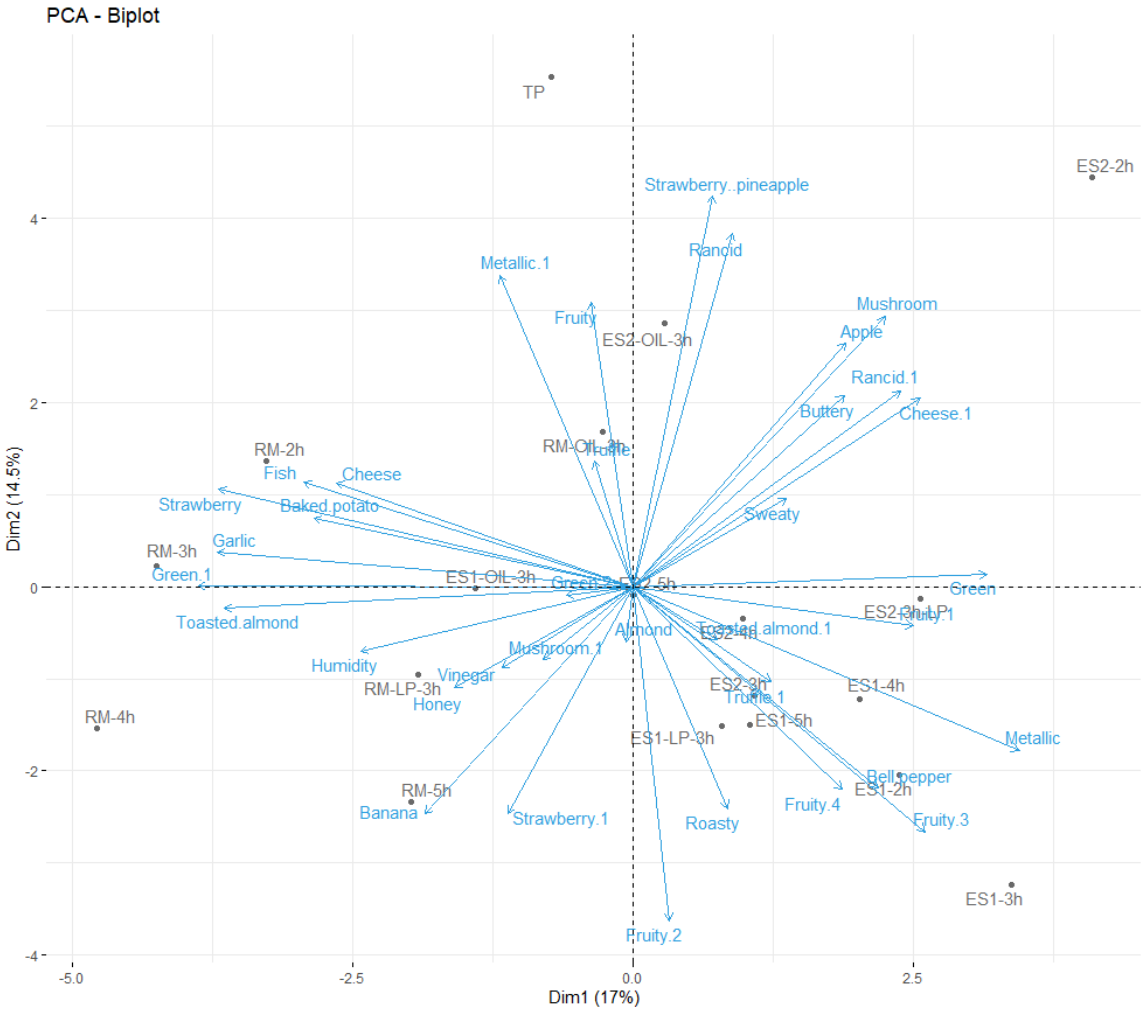
622

623 Figure 3



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