Chemical and sensory effects of the freezing process on the aroma profile of black truffles (*Tuber melanosporum)*

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ABSTRACT

The aim of this work is to evaluate the effect of freezing black truffles (*Tuber melanosporum*) on their aroma both in sensory and chemical terms. The truffles were frozen at temperatures of -20ºC to -80ºC. Sensory and chemical analyses were carried out after 24 hours and after 20, 40 and 60 days. The sensory analysis was both descriptive and discriminative. Chemical analysis was done by headspace solid phase microextraction followed by gas chromatography mass spectrometry analysis (HS-SPME-GC-MS). Fifteen compounds with high aromatic potential in truffles were determined. Their selective ion peak areas were calculated, summed and expressed as percentage of active odor compound, in order to monitor changes in odor profile.

The sensory study concluded that the aroma of frozen truffles differed significantly from the aroma of fresh truffles. This result was fully confirmed by volatile composition data, which reveal that *T.melanosporum* aromatic profile is deeply modified as a consequence of a freezing process. Odor profiles become enriched in compounds such as diacetyl, 1-octen-3-one, 1-octen-3-ol, 2-methylisoborneol and dimethyltrisufide after the freezing period, while isoamyl alcohol, methanethiol and ethyl 3-methylbutyrate decreased. These aromatic changes could explain the loss of freshness observed in all frozen truffles.

On the other hand, methional and some phenols such as 3-ethyl-5-methylphenol increased after 40 days which suggest that these compounds may be used as markers of freezing time. Interestingly, the percentage area of 1-octen-3-one was constant in frozen samples and much higher than in fresh ones, which suggests that it is a general marker of freezing process.

Keywords: freezing storage; freezing markers; freshness; aroma; *Tuber melanosporum;* 1-octen-3-one; black truffle

Introduction

The black truffle or *Tuber melanosporum* is highly appreciated due to its intense and distinctive aroma. It has been described as the queen of truffles and is one of the most prized foods worldwide. Truffles have their highest organoleptic value when fresh. However, like many other vegetable commodities, they are highly perishable mainly due to bacterial and mold growth and dehydration which contribute to the rapid loss of organoleptic properties such as texture, aroma, and taste (Rivera, Blanco, Salvador & Venturini, 2010). Moreover, truffles are seasonal so long term storage methods are used to ensure their availability throughout the year. Freezing is one of the most important methods for retaining food quality during long-term storage. Factors such as variety, maturity, growing area and seasonal variations influence the vegetable freezing process to an extent that may override the positive effect of a high freezing rate (Skrede, 1996). For many processes it is important to know the typical pigment and volatile compound profile of a fresh product in order to identify the color and aroma changes produced during treatment (de Ancos, Cano & González, 1999; Ibañez, López-Sebastian, Ramos, Tabera & Reglero, 1998). Some studies have been carried out in order to examine the effect of the freezing process on the volatile composition of several products. For example, de Ancos, Ibañez, Reglero and Cano (2000) process and long-term frozen storage conditions do not significantly affect the aroma of the raspberry cultivars they studied. However, aroma is usually affected by freezing. Some changes in the aromatic profile of many other products after frozen storage have been observed. For example, Kjeldsen, Christensen and Edelenbos (2003) detected an off-flavor, described as soapy and paraffin-like, in unblanched carrots during long-term freezing. Other authors concluded that the aroma in unblanched leek slices changed after frozen storage for 12 months as the amount of sulfur compounds decreased (Nielsen & Poll, 2004). Some

possible markers were proposed for the differentiation between fresh and frozen-thawed fish, as for example 1-octen-3-ol or 1-penten-3-ol (Iglesias, Medina, Bianchi, Careri, Mangia & Musci, 2009). Other examples of changes between fresh and frozen products have been evaluated in lamb meat (Bueno, Resconi, Campo, Cacho, Ferreira & Escudero, 2011), Turkish Motal cheese (Andic, Tuncturk & Javidipour, 2011) or in cantaloupe melon (Ma et al., 2007). The latter paper demonstrated that with the prolongation of the freezing time, an increasing number of unsaturated alcohols and aldehydes with 6 and 9 carbons were produced, the green notes of frozen melon became more and more intense, and the ester fragrance became increasingly less apparent.

It is therefore important to evaluate the effects on aroma of the different methods of preservation. Very few previous works (Al-Ruqaie, 2006; Jaworska & Bernás, 2009) have evaluated the effect of frozen storage on truffles and mushrooms. Al-Ruqaie (2006) revealed that the freezing process seems to be more effective than drying, at least in the two species of truffles studied (*T. claveryi* and *T. hakizi*), and concluded that blanching in a 4 % NaCl solution and storage at -18 ºC proved to be the best preservation method in terms of quality of the truffles. The other paper cited (Jaworska et al., 2009) proposed a maximum storage period of four months for the frozen product in the case of unblanched mushrooms.

The goal of the present work is to study the influence of freezing and long-term frozen storage on the aroma composition of *T. melanosporum*, using sensory analyses and analytical techniques (HS-SPME coupled with GC-MS).

2. Materials and Methods

2.1. Truffle samples

The ascocarps of *T. melanosporum* were collected in cultivated truffle-grounds under holm oak trees (*Quercus ilex* subsp. *balllota*) in Sarrión (Teruel, Spain), with the help of a hunter dog. The truffles were harvested in January-February and were in stage VI of maturation (fully mature). Their organoleptic properties were at their maximum, according to the description of maturation stages given in (Montant, Kulifak & Gleyze, 1983). The truffles were transported to the laboratory with covering soil in insulated boxes with ice packs. The samples were brushed with a wet soft brush, rinsed with tap water and forced-air dried in a laminar cabinet. Qualitative selection of the carpophores was made by discarding truffles with soft texture as well as those parasitized or damaged during harvest.

2.2. Standards

Fifteen compounds were chosen for this study due to their potential aromatic importance in *T.melanosporum*. Table 1 provides useful information relating to their gas chromatographic retention data, the masses employed for the study and their aromatic descriptors.

The standards used for identifications were supplied by Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland), PolyScience (Niles, USA), Lancaster (Strasbourg, France), and Alfa Aesar (Karlsruhe, Germany).

2.3. Experimental design

Figure 1 illustrates the experimental design of this work which evaluates possible changes in *T. melanosporum* aroma produced by freezing. Two freezing temperatures (-20ºC and -80ºC) and different time periods (1, 20, 40 and 60 days) have been assessed. The scheme shown in the figure was followed in order that to reduce the effects of the

118 study should not be affected by the intrinsic individual variations of the truffles. Four
119 spherical fresh truffles of approximately 20 grams each were used. A different truffle
120 was used for each time period. The four truffles were divided into three pieces of almost
121 identical size. One piece acted as the control sample. The other two were vacuum
122 packed in polyethylene bags (Oriented Polyamide/Polypropylene, 15/65, 80 μm (Orved,
123 Musile di Piave, Italy)) with a VM-12 vacuum sealer (Orved). Then, one piece was
124 frozen at -20ºC in a Templow-S freezer (J.P. Selecta, Barcelona, Spain) and the other at
125 -80ºC in MDF-U3286S freezer (Sanyo Electric Co., Japan). The temperature of both
126 freezers was monitored throughout the experiment using a Testo 177-14 Data Logger
127 with a J type probe (Testo Instruments, Barcelona, Spain). The temperatures were
128 maintained at - 20 ± 1 ºC and at -80 ± 1 ºC.

129 **2.4. Sensory analysis**

130 The frozen truffles were unpackaged, sliced (~1 mm) and left at room temperature for 2
131 h to warm the product. The samples were then presented to the panelists in closed
132 opaque containers in random order.

133 **2.4.1. Sensory descriptive analysis**

134 Prior to the freezing experiment, a panel of five trained judges evaluated the quality
135 characteristics of black truffles. Two specific 1 h training sessions were carried out in
136 which the judges identified descriptive terms to define the different *T. melanosporum*
137 samples used in this study. As a result of these discussions, seven aroma attributes were
138 chosen as the most appropriate to describe the samples. This group of descriptors
139 comprised: sulfur, mushroom, mould, animal, boiled potatoes, butter and finally cheese.
140 In the third training session, the panelists scored the intensity of each attribute using a
141 five-point scale (5 = very intense; 4 = intense; 3 = moderate; 2 = slight and 1 = no
142 smell) and in the fourth session they were asked to score general parameters such as

intensity of characteristic aroma using a nine point rating scale (9 = full typical aroma; 7 = moderately full aroma; 5 = moderate aroma; 3 = slight aroma and 1 =no typical smell).

2.4.2. Sensory discriminative analysis (triangular tests)

The test panel that carried out the sensory discriminative analysis was composed of 8 members of the laboratory staff (three women and five men, ranging from 23 to 50 years of age). All of them participate regularly in sensory tests.

To assess whether the different freezing conditions have a significant effect on the aroma of *T.melanosporum*, triangular tests were carried out. The samples examined in each test were fresh (control) and frozen (both from the same truffle). The fresh control samples used on the different analysis days were obtained from the same cultivated truffle-grounds and were at the same stage of maturation as those previously subjected to the freezing process. Three closed containers were presented to each judge, who had to decide which sample was different from the other two. The number of correct answers was compared with tabulated values to decide if significant differences existed. In the case of a difference being detected, the judges were asked to note the descriptors which caused the difference.

2.5. Headspace Solid-Phase Micro Extraction (HS-SPME)

An SPME holder (Supelco, Bellefonte, PA, USA) was used to perform these experiments. A fiber of medium polarity was needed to avoid discrimination towards very non polar and polar volatile compounds. A fused silica fiber coated with a 50/30 µm layer of divinylbenzene/carboxen/polydimethylsiloxane (Supelco) was chosen to extract the fifteen aromatic compounds.

The method applied for this analysis was described in (Culleré, Ferreira, Venturini, Marco & Blanco, 2012). This is based on a methodology designed and published in

different papers (Díaz, Ibáñez, Señoráns & Reglero, 2003; Diaz, Señorans, Reglero & Ibañez, 2002), but with some changes, as for example the mass of truffle analyzed. Approximately 2 grams of sample was placed in a 20 mL vial closed with a plastic film. Once the desired temperature (53ºC) was reached, the vial was allowed to condition for the equilibrium time (5 min). After this time, the fiber was introduced into the vial and exposed to the headspace of the sample during 13.6 minutes. These extraction conditions were optimized in a previous work (Diaz et al., 2002).

Three units of truffles for each treatment were cut into thin slices using a sharp knife. A total of three replicates of each truffle species were analyzed.

2.6. Gas Chromatography-Mass Spectrometry (GC-MS)

The analyses were performed with a CP-3800 chromatograph coupled to a Saturn 2200 ion trap mass spectrometric detection system supplied by Varian (Sunnyvale, CA, USA). A DB-WAXETR capillary column (J&W Scientific, Folsom, CA, USA) of 60 m x 0.25 mm I.D., a film thickness of 0.25 µm, and preceded by a 3 m x 0.25 mm uncoated (deactivate, intermediate polarity) precolumn from Supelco (Bellefonte, PA, USA) was used. Helium was the carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature was initially 40 ºC during 5 minutes, then raised by 4 ºC min^{-1} to 140 ºC, followed by a rate of 10 ºC min^{-1} to 220 ºC and finally held at this temperature for 10 minutes. The MS parameters were an MS transfer line and chamber ionization temperature of 200 ºC, and a trap emission current of 80 µA. The global run time was recorded in full scan mode (45-250 m/z mass range). The injection was in Splitless mode (splitless time 5 min) at a temperature of 200 ºC. A desorption time of 15 minutes was used. The chromatographic data were analyzed by Varian Saturn GC-MS Version 5.2 software. The identity of the odorants was determined by a comparison of their

chromatographic retention index and MS spectra with those of pure reference compounds.

The data is expressed in area percentages. However, given that some of the selected analytes do not give a signal in TIC or else appear together with peaks of interference that make it difficult to integrate them (as is the case with 2,3-butanedione and 3-ethyl-5-methylphenol), it was decided to work with area percentages but also considering selective mass. In this way, 100 % constituted the sum of the selective ion peak areas of all the compounds of interest.

2.7. Statistical analyses

For the data obtained from the sensory descriptive analyses, one-factor (time) and two-factor (time and temperature) analyses of variance (ANOVA) were carried out. A t-test analysis was also employed in order to check possible changes between fresh and 24 hour-frozen samples.

Similar one-factor and two-factor ANOVA tests were also carried out in the case of the chemical quantitative data in order to evaluate the existence of significant differences in the volatile composition between the control samples (fresh truffles) and the samples subjected to the different freezing conditions. Differences between the averages in both sets of data were studied with Duncan's test and significant differences were established at $p < 0.05$.

All these analyses were performed using SPSS software (version 15.0) from SPSS Inc. (Chicago. IL).

3. Results and discussion

3.1. Sensory analysis

Eight triangular tests were carried out in order to check the possible effects provoked by the freezing process on the aroma of *T.melanosporum*. Differences with a significance level of ≥ 99.9% were found in all cases. That is to say, significant changes in the final aroma of black truffle were caused even by the mildest conditions, freezing at -20ºC for only 24 hours.

Furthermore, a panel of five truffle experts carried out a sensory descriptive analysis of each of the samples (both the fresh samples and those frozen for different time periods under different conditions). The results are shown in table 2. One of the most relevant conclusions, consistent with the triangular tests, was that after only 24 hours of freezing there was a significant reduction in the term "characteristic aroma", although the intensity of the aroma did not seem to be affected. However, the descriptive term "sulfur", usually attributed to the characteristic aroma of truffles, did not appear to be affected by freezing (irrespective of the duration or intensity, -20ºC or -80ºC). Therefore, it can be deduced that in addition to the sulfur attribute, other notes contribute substantially to the typical truffle aroma. Moreover, the panellists all agreed that none of the frozen samples retained the aroma of fresh truffle.

As regards the other attributes, the descriptors "animal", "butter" and "cheese" had very low intensities and were very similar in all cases, irrespective of whether the sample was fresh or frozen, or even of the freezing temperature and time period. However, the results for other descriptors such as "mushroom", "mould" and "boiled potatoes" were more significant. The term "mushroom" appeared as a significant distinguishing characteristic after 20 days of freezing but only at the higher freezing temperature of -20 ºC. The "mould" descriptor followed a more logical path. After freezing at -80 ºC the

242 difference was noticeable after only one day, while for a temperature of -20ºC twenty
243 days were required for any aromatic repercussion to be detected in this note. It was
244 curious that after 20 days of freezing, maximum intensities of “mushroom” and
245 “mould” were detected. For the “boiled potatoes” attribute, on comparing the fresh with
246 the frozen samples significant changes were observed for the -20 ºC temperature after
247 60 days, while for -80 ºC these changes occurred after only 20 days.

248 **3.2. Quantitative analysis**

249 Table 3 shows all the quantitative results expressed as area percentages for the four sets
250 of samples evaluated. Each set comprises data from the same truffle fresh and stored a
251 certain time, at -20ºC or -80ºC, making it possible to assess directly the effect of the
252 freezing process.

253 As can be seen, fifteen aromatic compounds have been quantified. Some of them had
254 already been identified in a recently published olfactometric study (GC-O), (Culleré,
255 Ferreira, Chevret, Venturini, Sánchez-Gimeno & Blanco, 2010), as playing a relevant
256 role in the aroma of *T.melanosporum.* Other odorants were chosen as being likely
257 candidates for producing the aromatic attributes described by the sensory panel. This is
258 the case of 1-octen-3-one (a very potent aroma directly related to mushroom notes), 1-
259 octen-3-ol (possibly responsible for both mushroom and mould notes (Schieberle &
260 Buettner, 2001), 2-methylisoborneol (described as mouldy or earthy) (Darriet, Pons,
261 Lamy & Dubourdieu, 2000; La Guerche, Dauphin, Pons, Blancard & Darriet, 2006),
262 and finally methional (characterized by a distinct aroma of boiled potatoes) (Escudero,
263 Cacho & Ferreira, 2000). Furthermore, it was considered interesting to include some
264 aromatic sulfur compounds such as dimethyltrisulfide, dimethylsulfoxide and
265 methanethiol, which may contribute to the characteristic truffle aroma.

Regarding the results obtained, it can be observed that several compounds suffered an important increase in their levels as a consequence of a freezing step. This was the case of diacetyl, 1-octen-3-ol y dimethyltrisulfide. Another interesting finding was that levels of methional and 3-ethyl-5-methylphenol were also increased, but especially from day 40, suggesting that these odorants may be related to degradation processes in truffles as a result of long periods of freezing. On the contrary, isoamyl alcohol exhibited an important decrease over time.

It is worth nothing the high values of 1-octen-3-ol and 2-methylisoborneol after 20 days and of methional after 40 days, which was consistent with the sensory data. Therefore, it could be said that the aromatic profile of *T.melanosporum* was modified in a major way as a consequence of freezing process.

In order to complete this study, a principal component analysis (PCA) was carried out on quantitative data of all the compounds analysed. Figure 2 shows the projection of the samples on the plane formed by the first two dimensions. As can be seen, this plot explains 60 % of the total variance and reveals clearly that freezing deeply changes aroma profile, since fresh unfrozen samples were clearly separated from the frozen ones along the first component (PC1), which explains a 34 % of variance: fresh samples had the most negative values on this axis (-1.0, -0.8) and all the frozen truffles presented positive values. Considering the group of frozen truffles, it can be appreciated that the importance of the temperature factor increases, as expected, with the freezing time: Samples stored 1 day are plotted together with each others, those stared 20 days are relatively close, while those stored 40 or 60 days are more far apart.

Attending to variable loadings, the aroma profiles of fresh samples were characterized by higher levels of isoamyl alcohol (c6), ethyl 3-methylbutyrate (c4) and methanethiol

290 (c1) and smaller levels of diacetyl (c3), 1-octen-3-one (c7), 1-octen-3-ol (c9), 2-
291 methylisoborneol (c12) and dimethyltrisulfide (c8).
292 It is remarkable that some relevant truffle aroma such as dimethylsulfide (c2),
293 dimethyldisulfide (c5) and dimethylsulfoxide (c13) were not related to the freezing step,
294 which coincides with the sensory study, in which the sulfur odor note did not suffer any
295 significant changes during the freezing.

296 A second PCA containing only data from frozen samples was carried out in order to
297 evaluate the existence of different patterns depending on the conditions of the freezing
298 process. As shown in figure 3, samples were classified attending to storage time in the
299 second component (30% of the total variance) while the first component suggested that
300 the two most different samples were those stored 20 days. This difference could be
301 attributed to a sample effect, however the fact that control samples were plotted together
302 (see figure 2), suggest that the difference is caused by real changes in the aroma profile
303 during freezing time. According to the figure 3, the aroma profile of truffles changed
304 continuously during the storage time, until the 40^{th} day. The higher the freezing time the
305 richer the aroma profile beacomes in 3-ethyl-5-methylphenol (c11), methional (c10), 3-
306 ethylphenol (c15) and the poorer in methanethiol (c1), dimethylsulfide (c2),
307 dimethylsulfoxide (c13). At intermediate storage times, profiles become richer in ethyl
308 3-methylbutyrate (c4), dimethylsulfide (c5), dimethyltrisulfide (c8), 1-octen-3-ol (c9),
309 2-methylisoborneol (c12) and poorer in dimethylsulfide (c2).

310 Table 4 shows the results of a two factors ANOVA (time and temperature). The
311 variations with respect to the corresponding control samples have been estimated in
312 percentage terms. It can be seen that all the compounds analysed, except 1-octen-3-one,
313 were significantly affected by the time factor. Furthermore, seven compounds (sulfurs
314 as dimethyldisulfide and dimethyltrisulfide, phenols as p-cresol, 3-ethyl-5-methylphenol

315 and 3-ethylphenol, isoamyl alcohol and ethyl 3-methylbutyrate) varied significantly
316 with the freezing temperature. This influence was affected by the time period in mostly
317 of the cases (with the exception of isoamyl alcohol). That is to say, there was an
318 interaction between both effects in these compounds.
319 A t-test was carried out to evaluate if there were any significant differences between
320 samples frozen for 24 hours (irrespective of the temperature) and the fresh samples.
321 This test was possible for all the compounds except methanethiol, for which significant
322 differences were indeed observed between the samples frozen at -20ºC and at -80ºC
323 after 24 hours. The test indicated that after 24 hours of freezing, there were already
324 marked changes in the levels of some important odorants such as diacetyl, isoamyl
325 alcohol, 3-ethyl-5-methylphenol, 1-octen-3-one, 1-octen-3-ol, 2-methylisoborneol and
326 methional. Finally, it should be noted that the table shows the relevant role of 1-octen-3-
327 one as a general marker for the freezing process (irrespectively of the time and the
328 temperature of storage). Figure 4 shows the average levels of this compound (1-octen-3-
329 one) for all the samples analysed (four fresh and eight frozen).

330 **4. Conclusions**

331 The sensory study reveals significant differences in the aroma of *T.melanosporum* after
332 only 24 hours of freezing (independently of the temperature), with a substantial
333 reduction in the characteristic fresh truffle aroma, although sulfur odor nuances does not
334 exhibit significant change. This lost of freshness could be explained by the quantitative
335 data, which reveal that frozen samples are richer in compounds such as diacetyl, 1-
336 octen-3-one, 1-octen-3-ol, 2-methylisoborneol and dimethyltrisulfide, and poorer in
337 others as isoamyl alcohol, ethyl 3-methylbutyrate and methanethiol. Therefore, the odor
338 profile of *T.melanosporum* is deeply modified as result of the freezing process.

Both quantitative and sensory results suggest that some relevant truffle aroma such as dimethylsulfide, dimethyldisulfide are not related to the freezing step,

A further relevant conclusions of this study are: 1) the importance of the temperature factor increases with the freezing time (from 40 days), 2) the aroma profile of *T.melanosporum* changed continuously during the storage time, until the 40^{th} day, 3) the role of 1-octen-3-one as a possible marker for freezing processes, since it was the only odorant which experienced a significant increase in concentration after the truffles were frozen, irrespective of the freezing temperature and time period, and 4) the possibility of using methional and some phenols such as 3-ethyl-5-methylphenol as markers of freezing time.

Acknowledgements

The authors thank the Spanish Ministry for Education and Science (project RTA2010-00070-C02-02) for financial support.

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FIGURE CAPTION

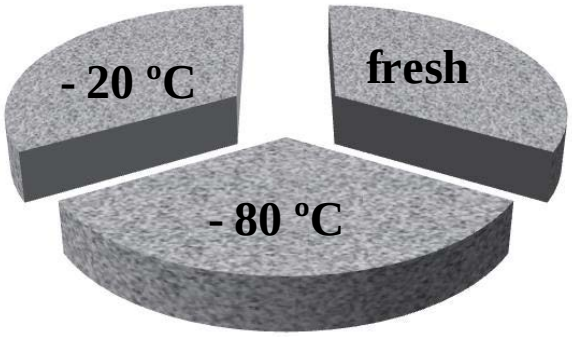
Figure 1. Experimental design

Figure 2. Score plot of Principal Component Analysis (PCA) applied to quantitative data from fresh and frozen truffles (at two different temperatures: -20ºC, -80ºC and at different times: 1, 20, 40 and 60 days).

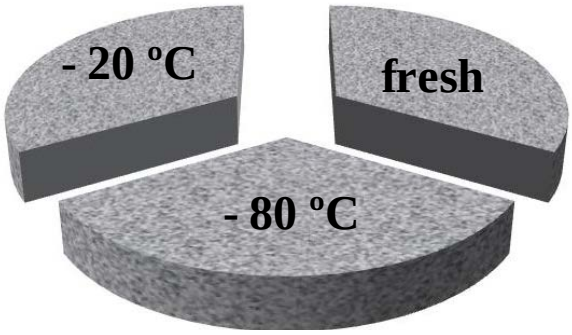
Figure 3. Score plot of Principal Component Analysis (PCA) applied to quantitative data from only frozen truffles

Figure 4. Average levels of 1-octen-3-one found in all truffles analysed

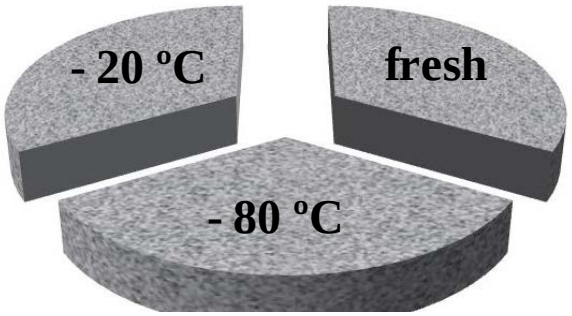
Truffle 1
(frozen period = 1 day)



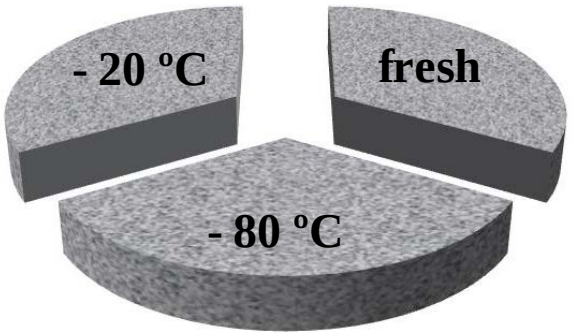
Truffle 2
(frozen period = 20 days)

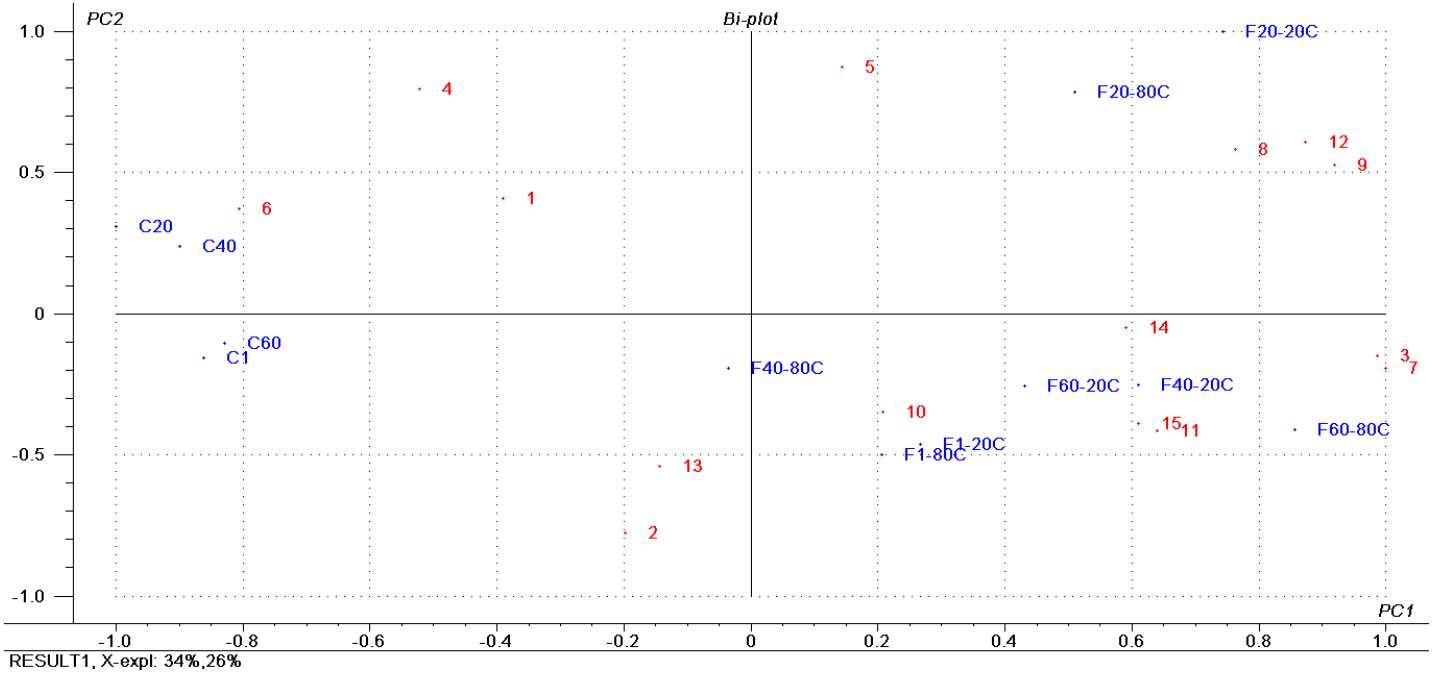


Truffle 3
(frozen period = 40 days)

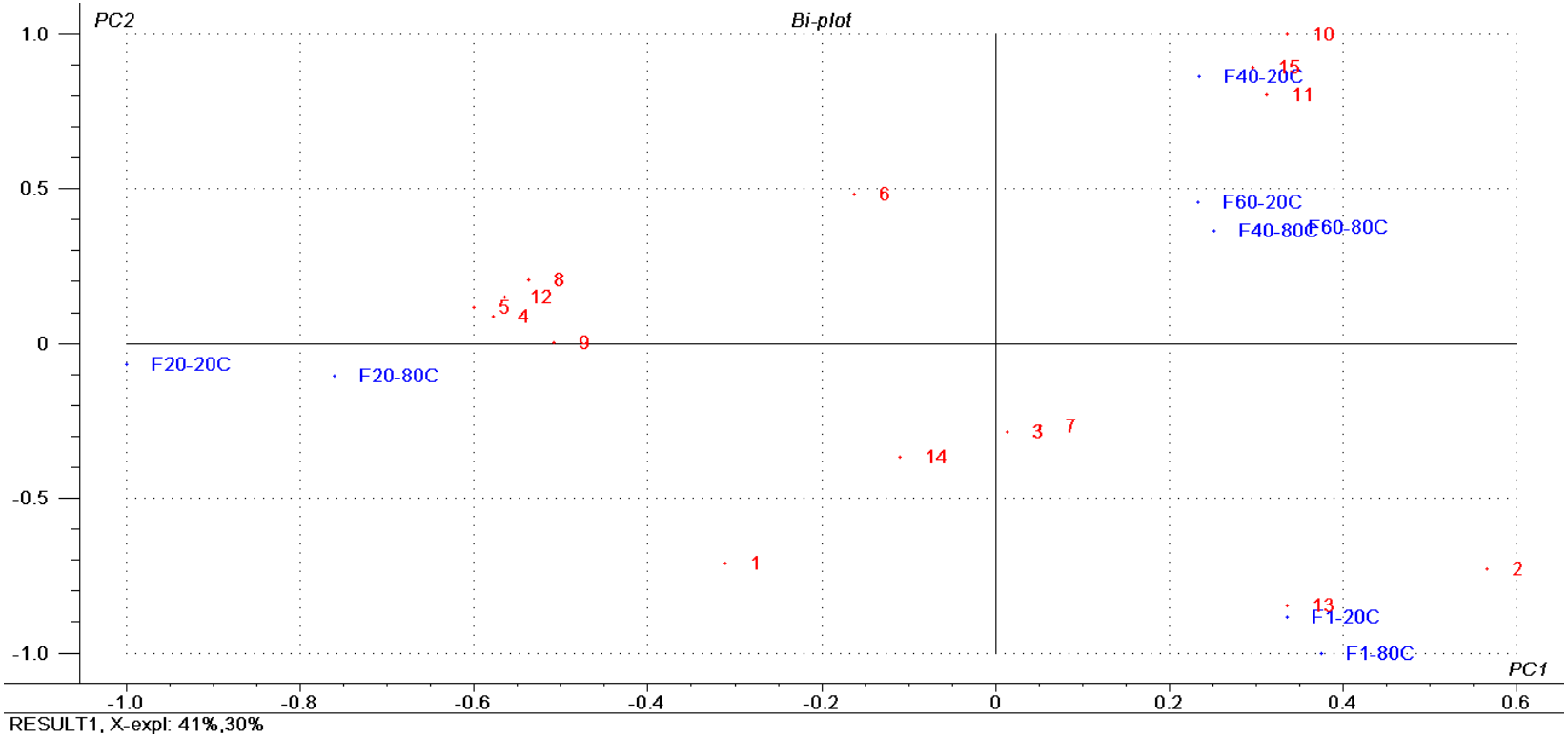


Truffle 4
(frozen period = 60 days)





Compounds: 1=methanethiol, 2=dimethylsulfide, 3=diacetyl, 4=ethyl 3-methylbutyrate, 5=dimethyldisulfide, 6=isoamyl alcohol, 7=1-octen-3-one, 8=dimethyltrisulfide, 9=1-octen-3-ol, 10=methional, 11=3-ethyl-5-methylphenol, 12=2-methylisoborneol, 13=dimethylsulfoxide, 14=p-cresol, 15=3-ethylphenol. C1, C20, C40, C60: Fresh samples (control), F1-20C: Frozen samples at -20ºC during 1 day, F1-80C: frozen samples at -80ºC during 1 day; F20-20C: frozen samples at -20ºC during 20 days, F20-80C: frozen samples at -80ºC during 20 days, F40-20C: frozen samples at -20ºC during 40 days, F40-80C: frozen samples at -80ºC during 40 days, F60-20C: frozen samples at -20ºC during 60 days and F60-80C: frozen samples at -80ºC during 60 days.



Compounds: 1=methanethiol, 2=dimethylsulfide, 3=diacetyl, 4=ethyl 3-methylbutyrate, 5=dimethyldisulfide, 6=isoamyl alcohol, 7=1-octen-3-one, 8=dimethyltrisulfide, 9=1-octen-3-ol, 10=methional, 11=3-ethyl-5-methylphenol, 12=2-methylisoborneol, 13=dimethylsulfoxide, 14=p-cresol, 15=3-ethylphenol. C1, C20, C40, C60: Fresh samples (control), F1-20C: Frozen samples at -20ºC during 1 day, F1-80C: frozen samples at -80ºC during 1 day; F20-20C: frozen samples at -20ºC during 20 days, F20-80C: frozen amples at -80ºC during 20 days, F40-20C: frozen samples at -20ºC during 40 days, F40-80C: frozen samples at -80ºC during 40 days, F60-20C: frozen samples at -20ºC during 60 days and F60-80C: frozen samples at -80ºC during 60 days.

1

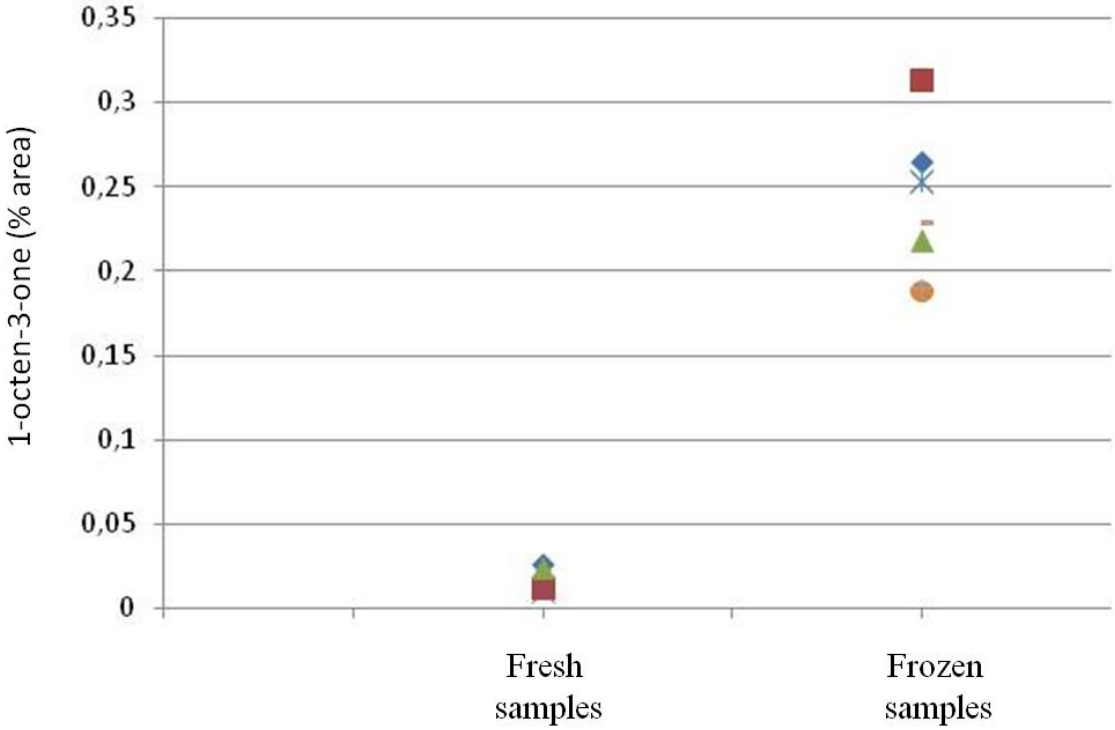

0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
1-octen-3-one (% area)
Fresh samples
Frozen samples

Table 1. Retention time, aromatic descriptor and selective masses corresponding to each of the fifteen compounds studied.

	m/z*	retention time (min)	aromatic descriptor
Relevant odorants (by GC-O)			
Dimethylsulfide	62	4.15	truffle, sulfur
Diacetyl	43	10.14	butter, cream
Ethyl 3-methylbutyrate	88	13.76	fruit, anise
Dimethyldisulfide	94	14.08	truffle, sulfur
Isoamyl alcohol	70	19.49	cheese
3-Ethyl-5-methylphenol	121	30.96	phenolic/leather
p-Cresol	107	40.56	phenolic/leather
3-Ethylphenol	122	41.98	phenolic/leather
Mushroom- like notes			
1-Octen-3-one	70	22.93	mushroom
1-Octen-3-ol	57	27.99	mushroom, earth, mould
Mouldy/earthy- like notes			
2-Methylisoborneol	95	31.48	mould, earth
Boiled potatoes- like notes			
Methional	104	28.73	boiled potatoes
Other sulfuric compounds			
Methanethiol	47	3.70	cooked cabbage, vegetal
Dimethyltrisulfide	126	24.43	rotten food
Dimethylsulfoxide	78	32.34	garlic-like note

*m/z: selective masses chosen for identifying each compound

Table 2. Sensory descriptive analysis of *T. melanosporum* (black truffle) frozen at -20 ºC and at -80 ºC

Parameter	**Day**	**Fresh**	**Frozen***	
			at -20 ºC	at -80 ºC
Aroma intensity	1	7.7 ± 1.3a	6.8 ± 1.7a,A	7.0 ± 0.7a,A
	20		7.2 ± 1.5a,A	6.9 ± 0.7a,A
	40		6.6 ± 0.9a,A	6.3 ± 0.8a,A
	60		6.4 ± 1.9a,A	6.6 ± 1.1a,A
Characteristic fresh truffle aroma	1	7.8 ± 1.3a	5.9 ± 1.8b,A	5.6 ± 1.4b,A
	20		5.5 ± 0.7b,A	6.0 ± 1.0b,A
	40		6.2 ± 1.1ab,A	5.2 ± 0.9b,A
	60		4.8 ± 1.4b,A	5.1 ± 1.3b,A
Sulfur	1	1.7± 0.8a	2.1 ± 0.5a,A	2.6 ± 0.7a,A
	20		2.3 ± 0.6a,A	2.3 ± 0.4a,A
	40		2.4 ± 0.6a,A	2.2 ± 0.5a,A
	60		1.6 ± 0.8a,A	2.0 ± 0.9a,A
Mushroom	1	2.4 ± 1.1a	1.8 ± 0.4a,A	1.8 ± 0.6a,A
	20		3.7 ± 1.0b,B	2.7 ± 0.9a,A
	40		2.6 ± 1.1a,AB	2.4 ± 0.7a,A
	60		2.0 ± 0.7a,A	2.2 ± 0.9a,A
Mould	1	1.0 ± 0.0a	1.0 ± 0.0a,A	2.7 ± 1.1b,A
	20		4.0 ± 0.7b,B	1.0 ± 0.0ac,B
	40		1.0 ± 0.0a,A	3.8 ± 0.4b,C
	60		1.0 ± 0.0a,A	1.0 ± 0.0a,B
Animal	1	1.9 ± 0.9a	1.9 ± 0.5a,A	2.1 ± 0.7a,A
	20		2.4 ± 1.0a,A	2.2 ± 0.9a,A
	40		2.0 ± 1.0a,A	1.9 ± 1.2a,A
	60		1.8 ± 1.0a,A	1.8 ± 1.2a,A
Boiled Potatoes	1	1.4 ± 0.5a	2.0 ± 1.1a,A	1.7 ± 0.7a,A
	20		2.2 ± 0.8ab,A	2.9 ± 0.5b,B
	40		2.4 ± 0.9a,AB	2.4 ± 0.5a,AB
	60		3.5 ± 0.7b,B	2.8 ± 0.6b,AB
Butter	1	1.5 ± 0.5a	1.2 ± 0.3a,A	1.4 ± 0.7a,A
	20		1.3 ± 0.4a,A	1.4 ± 0.4a,A
	40		1.4 ± 0.4a,A	1.4 ± 0.5a,A
	60		1.4 ± 0.8a,A	1.4 ± 0.8a,A
Cheese	1	1.3 ± 0.7a	1.3 ± 0.4a,A	1.1. ±0.2a,A
	20		1.3 ± 0.4a,A	1.6 ± 0.6a,A
	40		1.4 ± 0.6a,A	1.3 ± 0.5a,A
	60		1.0 ± 0.0a,A	1.0 ± 0.0a,A

*Values in the same line followed by different lowercase letters show significant differences between batches for the same day ($p<0.05$). In this case frozen samples were always compared with the initial value for the fresh sample (day 0). Values in the same column followed by different capital letters are significantly different ($p<0.05$).

Table 3. Data expressed as area percentages, the sum of the areas of the 15 analytes being 100%.

	Set 1 day (n=3)						Set 20 days (n=3)						Set 40 days (n=3)						Set 60 days (n=3)					
	Control		- 20ºC		- 80ºC		Control		- 20ºC		-80ºC		Control		-20ºC		-80ºC		Control		-20ºC		-80ºC	
Relevant odorants (by GC-O)	av	SD	av	SD	av	SD	av	SD	av	SD	av	SD	av	SD	av	SD	av	SD	av	SD	av	SD	av	SD
Dimethylsulfide	57.0	1.84	57.3	3.35	62.7	5.62	48.7	3.97	35.3	6.74	33.1	11.0	39.9	3.33	40.8	5.90	49.2	5.51	44.0	4.57	41.7	3.52	50.0	4.71
Diacetyl	0.14	0.01	0.69	0.06	0.49	0.12	0.16	0.00	0.51	0.07	0.52	0.13	0.13	0.00	0.36	0.03	0.34	0.05	0.16	0.01	0.62	0.02	0.59	0.04
Ethyl 3-methylbutyrate	0.03	0.00	0.01	0.00	0.01	0.00	0.03	0.00	0.03	0.01	0.03	0.01	0.02	0.00	0.01	0.00	0.01	0.00	0.02	0.00	0.02	0.00	0.00	0.00
Dimethyldisulfide	0.17	0.03	0.18	0.03	0.21	0.02	0.55	0.06	0.57	0.03	0.54	0.06	0.38	0.03	0.24	0.01	0.25	0.01	0.22	0.01	0.22	0.03	0.39	0.05
Isoamyl alcohol	41.9	1.87	30.3	2.65	25.8	3.03	49.9	4.13	37.3	4.88	39.7	7.23	59.0	3.40	41.4	3.75	37.3	3.29	53.8	0.71	40.4	1.42	30.0	3.48
3-Ethyl-5-methylphenol	0.02	0.00	0.36	0.10	0.11	0.02	0.01	0.00	0.08	0.01	0.04	0.01	0.07	0.01	4.39	0.63	0.03	0.00	0.04	0.00	4.5	0.05	5.8	1.98
p-Cresol	0.02	0.00	0.03	0.01	0.03	0.00	n.d.		0.03	0.00	0.02	0.00	0.01	0.00	0.04	0.01	n.d.		0.01	0.00	0.01	0.00	0.01	0.11
3-Ethylphenol	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.01	0.00	n.d.		n.d.		0.01	0.00	0.01	0.00
Mushroom- like notes																								
1-Octen-3-one	0.03	0.01	0.29	0.10	0.34	0.05	0.01	0.00	0.23	0.06	0.26	0.08	0.02	0.00	0.28	0.03	0.21	0.07	0.01	0.00	0.20	0.04	0.24	0.09
1-Octen-3-ol	0.01	0.00	10.2	2.06	9.73	2.48	0.02	0.00	24.1	1.96	25.0	3.49	2.20	0.80	11.0	1.14	11.3	2.09	1.26	0.12	11.3	0.66	11.6	1.92
Mouldy/earthy-like notes																								
2-Methylisoborneol	0.02	0.00	0.01	0.00	0.01	0.01	0.01	0.00	0.05	0.01	0.05	0.02	n.d.		0.01	0.00	0.01	0.01	n.d.		0.02	0.02	0.03	0.02
Boiled potatoes-like notes																								
Methional	0.12	0.02	n.d.		nd.		0.17	0.01	n.d.		n.d.		0.18	0.03	0.91	0.32	0.97	0.19	0.14	0.04	0.37	0.02	0.40	0.06
Other sulfuric compounds																								
Methanethiol	0.17	0.01	0.12	0.01	0.14	0.00	0.17	0.00	0.14	0.01	0.14	0.03	0.11	0.01	0.08	0.01	0.08	0.01	0.07	0.00	0.07	0.01	0.07	0.00
Dimethyltrisulfide	0.01	0.00	0.11	0.06	0.12	0.09	0.02	0.00	1.42	0.26	0.41	0.12	0.03	0.00	0.37	0.07	0.13	0.03	0.02	0.00	0.29	0.00	0.58	0.10
Dimethylsulfoxide	0.33	0.01	0.35	0.01	0.34	0.07	0.23	0.08	0.14	0.00	0.16	0.01	0.16	0.08	0.09	0.01	0.10	0.01	0.25	0.01	0.27	0.01	0.28	0.01

Table 4. Significance (p value) of "time" and "temperature" factors measured by ANOVA

	time effect	temperature effect	time x temperature
Relevant odorants (by GC-O)			
Dimethylsulfide	0.001	0.094	0.519
Diacetyl*	0.001	0.067	0.121
Ethyl 3-methylbutyrate*	0.001	0.003	0.022
Dimethyldisulfide	0.001	0.001	0.001
Isoamyl alcohol*	0.005	0.018	0.078
3-Ethyl-5-methylphenol*	0.001	0.000	0.000
p-Cresol	0.001	0.000	0.000
3-Ethylphenol	0.001	0.004	0.001
Mushroom-like notes			
1-Octen-3-one*	0.174	0.580	0.372
1-Octen-3-ol*	0.001	0.774	0.950
Mouldy/earthy-like notes			
2-Methylisoborneol**	0.001	0.737	0.762
Boiled potatoes-like notes			
Methional*	0.001	0.704	0.981
Other sulfuric compounds			
Dimethyltrisulfide	0.001	0.001	0.001
Dimethylsulfoxide	0.001	0.512	0.971
Methanethiol[a]	0.001	0.643	0.710

*Compounds that differ at a significant level (> 95%) between samples frozen during 24 hours (irrespective of temperature of -20ºC or -80ºC) and fresh samples by a two-tailed t distribution. ** Compound that differs at a significant level (> 90%) between samples frozen during 24 hours (irrespective of temperature of -20ºC or -80ºC) and fresh samples by a two-tailed t distribution. [a] Compound that differs at a significant level (>95%) between samples frozen during 24 hours at -20ºC and samples frozen during this time at -80ºC.