Authentication of prized white and black truffles in processed products using quantitative real-time PCR

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Abstract

Truffles, such as *Tuber magnatum* and *Tuber melanosporum*, are greatly appreciated throughout the world, both as fresh fruiting bodies and as ingredients in processed products. Diagnostic methods are therefore required to check the identity of truffles in this kind of food. The present paper reports the application of microscopic and molecular techniques to authenticate truffle species in commercial products. Through the application of microscopic techniques, it has been possible to distinguish spores in a cream that could be ascribed to the truffle species (*T. magnatum*, the highest priced truffle) declared on the label and also spores of a less prized and aromatic truffle that was not indicated on the label. Good quality DNA was quickly obtained in a few hours using a kit generally employed for DNA extraction from soil. A new primer pair was developed to authenticate *T. magnatum* in commercial products and it was employed in a quantitative PCR assay (qPCR). *T. melanosporum*, which was neither indicated on the label nor recognized in the processed products containing truffles, was also detected in a cream and its amount was quantified by qPCR. This method can therefore be used to detect fraudulent practices and to protect the consumer of truffle delicacies.

1. Introduction

Truffles (*Tuber* spp.) are hypogeous edible fungi that undergo a complex life cycle during which the mycelium establishes a symbiotic interaction with plant roots. From this step hyphae aggregate and develop what we call a truffle, an aromatic fruiting body, which produces hundreds of volatiles (Splivallo, Ottonello, Mello, & Karlovsky, 2011). These volatiles attract insects and mammals, which, in turn, spread truffle ascospores, and in addition seduce humans, once the truffles have been found by trained dogs (Trappe & Claridge, 2010). Some species, such as *Tuber magnatum* Pico, "the Alba white truffle", and*Tuber melanosporum* Vittad., "the Périgord black truffle", are highly appreciated in many countries because of their special taste and smell. While French researchers have managed to cultivate *T. melanosporum* in orchards since the early 19th century, the white truffle has resisted domestication and commands the highest prices: at the Alba market, this fungus is routinely sold for $4000 per kilogram (Bohannon, 2009). Besides these two species, other morphologically similar but less aromatic species are sold with *T. magnatum* and *T. melanosporum* labels. Truffle fruiting bodies are usually identified on the basis of the features of their spores and asci, of the peridium (outer part) and gleba (inner part) (Ceruti et al., 2003 and Montecchi and Sarasini, 2000). Nevertheless, these features are generally only recognized by specialists and identification is sometimes unreliable. To solve these problems and to limit frauds, specific primers have been developed to unambiguously identify *Tuber* species (Mello, Murat, & Bonfante, 2006). In the framework of "gourmet" genome projects between French and Italian scientists, the *T. melanosporum* genome has just been sequenced and the secrets of the aroma have been revealed. Moreover, the possibility of developing numerous polymorphic markers to trace the geographic origin of truffles has emerged (Martin et al., 2010). This new opportunity is crucial as the need for labeled food is continuously increasing because consumers are more and more conscious of the importance of food traceability and fraud. But prized truffles are not only eaten as fresh fruiting bodies, they are also used in the production of a wide variety of food products. Therefore, diagnostic methods are required to check the identity of truffles in processed products.

The present paper reports the application of microscopic and molecular techniques to identify truffle species in commercial products such as cream- and butter-based products. In spite of the high number of papers on the biology and ecology of truffles, only a few of them have dealt with processed truffles, comparing different DNA extraction methods and conventional molecular approaches (Amicucci et al., 2002, Douet et al., 2004 and Mabru et al., 2004).

The aim of this work was to set up a protocol to authenticate *T. magnatum* in processed products, especially those subjected to intensive transformation processes. Since quantitative PCR (qPCR) is more reliable than the common PCR, in terms of sensitivity and specificity, it was chosen to identify and quantify this prized truffle. Microscopic techniques have been used in order to support molecular identification. A pair of primers, suitable for analyses by qPCR, was designed targeting the internal transcribed spacer-2 (ITS2). This tool could be used to check expensive truffle processed foods in order to protect the consumer, and it opens the way towards monitoring these products in the marketplace.

2. Materials and methods

2.1. Sample collection

The processed truffle products used in this study were two creams in tubes (C_RR and C_MV), a cream in a pot (C_MT) and a butter in a pot (BT). The labels stated that all the products contained the white truffle *T. magnatum* Pico and that there was also *Boletus edulis* Bull. (porcini) in the pot cream. The other non-fungal ingredients were listed in Table 1. Samples of these products were analyzed using both morphological and molecular approaches. The C_RR cream and the BT butter were patchy material, because they showed visible pieces of truffles unlike the other two creams, which were homogeneous.

Table 1.
Characterization of the analyzed processed products. "No Ct" means that the quantification was not possible. Values are means of three replicates +/− standard deviation.

Samples	Ingredients	*Tuber magnatum* DNA (ng/µl)	*Tuber magnatum* per product (mg/g)	*Tuber melanosporum* DNA (ng/µl)	*Tuber melanosporum* per product (mg/g)
C_RR	*Tuber magnatum*, corn oil, salt, aroma.	0.001 ± 0.001	0.003 ± 0.004	1.576 ± 1.11	3.526 ± 2.49
C_RRn		No Ct		0.09 ± 0.09	0.200 ± 0.19
C_MV	*Tuber magnatum*, whole milk, salt, antioxidants E300, E301, E330.	0.032 ± 0.02	0.116 ± 0.07	No Ct	
C_MT	*Boletus edulis* complex (45%), olive oil, salt, *Tuber magnatum* (1.2%), wine vinegar, milk, aroma.	No Ct		No Ct	
BT	*Tuber magnatum*, butter.	2.225 ± 1.339	8.021 ± 4.828	No Ct	

2.2. Morphological analysis

Portions of cream and butter were mounted on slides and observed under a light microscope (Primo Star Zeiss). Images were captured with a camera (Nikon eclipse E400) connected to a computer. The morphological characteristics of *Tuber* spores were compared with the description of Montecchi and

Sarasini (2000) and of Ceruti et al. (2003), while those of *B. edulis* were compared with the description ofMuñoz (2005).

The relative *Tuber* spore count was only made for one tube cream (C_RR) using three replicates (C_RRA, C_RRB, C_RRC) as follows: for each replicate 120 mg ca. of cream was homogenized in 30 μ l of water using a pestle; 4 mg of this homogenate was transferred to a new 1.5 ml tube containing 50 μ l of water and homogenized. Drops of ca 3–4 μ l were spotted on a slide with 10 circular wells having a 6 mm diameter (SPI supplies West Chester, PA 19381 USA) and observed under a light microscope to count the expected*T. magnatum* and the non-*T. magnatum* spores. The percentage of spores was calculated on 10 repetitions for each replicate. A new sample, C_RRn, containing only non-*T. magnatum* spores, was prepared from the C_RR tube cream by separating this type of spore from the others at the light microscope and putting it in a 1.5 ml tube. In this tube the matrix that envelops the spores of the truffle (gleba) was deliberately reduced to a minimum.

2.3. DNA extraction and amplification conditions

DNA was extracted using the Fast DNA Spin kit for soil (MP Biomedicals, LLC) adding 10 washes with 5.5 M guanidine thiocyanate (Luis, Walther, Kellner, Martin, & Buscot, 2004). The DNA quality and quantity were tested for each sample via a spectrophotometer (NanoDrop ND 1000, Thermo Fisher Scientific, Wilmington, Delaware). Three extractions were made for each product (C_RRA, C_RRB, C_RRC; C_MV1, C_MV2, C_MV3; C_MT1, C_MT2, C_MT3; BT1, BT2, BT3) as well as for the new sample C_RRn with non-*T. magnatum* spores (C_RRnF, C_RRnG and C_RRnH). DNA amplificability was tested with the fungal primers ITS1 and ITS2 (White, Bruns, Lee, & Taylor, 1990), using standard reaction conditions. In order to verify whether the non-*T. magnatum* spores belonged to *T. borchii*, a nested PCR was performed using the universal primers ITS1-F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990) in the first round and the *T. borchii* specific primers (Mello, Garnero, & Bonfante, 1999) in the second round. *T. melanosporum* specific primers (Bonito, 2009 and Paolocci et al., 1999) were used for the *T. melanosporum* analysis. *B. edulis*specific primers (Mello et al., 2006a) were used for the *B. edulis*. The primers used and their sequences are shown in Table 2.

Table 2
List of the primers employed in this work. The start and the end (indicated in the parenthesis) of primers (ITS1/ITS2, ITS4, ITSML/ITS4LNG and Tmel_for/Tmel_rev) were identified on the target sequence AF132501.1 of *Tuber melanosporum*.

Name	Sequence	Reference
ITS1 (8–10)	TCCGTAGGTGAACCTGCGG	White et al. (1990)
ITS2 (224–243)	GCTGCGTTCTTCATCGATGC	White et al. (1990)
ITS1-F (1–22)[a]	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993)
ITS4 (592–611)	TCCTCCGCTTATTGATATGC	White et al. (1990)
TBA (38–57)[b]	TGCCCTATCGGACTCCCAAG	Mello et al. (1999)
TBB (450–469)[b]	GCTCAGAACATGACTTGGAG	Mello et al. (1999)
ITSML (166–187)	TGGCCATGTGTCAGATTTAGTA	Paolocci et al. (1999)
ITS4LNG (578–599)	TGATATGCTTAAGTTCAGCGGG	Paolocci et al. (1999)
Tmel_for (84–104)	TTGCTTCCACAGGTTAAGTGA	Bonito (2009)
Tmel_rev (454–472)	TAAAGTCCGTCGTTCATGC	Bonito (2009)
Bedu1f (81–100)[c]	ATGGAGGAGTCAAGGCTGTC	Mello, Ghignone et al. (2006) and Mello, Murat et al. (2006)
Bedu2r (575–594)[c]	TAGATTAGAAGCGATTCACT	Mello, Ghignone et al. (2006) and Mello, Murat et al. (2006)
Tmag3 (393–415)[d]	TTAACTGTTTAAGTTTGTCAGGC	This work
Tmag4 (521–543)[d]	CCTGAATATCTCCTGTGTACCAT	This work

[a] Position in the sequence HM190013.1 of *Tuber puberulum*.
[b] Position in the sequence FJ554516 of *Tuber borchii*.
[c] Position in the sequence GU198978.1 of *Boletus edulis*.
[d] Position in the sequence FM205629 of *Tuber magnatum*.

2.4. Design and test of the specific primers for *T. magnatum*

ITS sequences of the following seven samples (*B. edulis*DQ131623, *Mattirolomyces terfezioides* (Mattir.) E. Fisch. GQ422438, *T. borchii* Vittad. FJ554516, *T. magnatum*FM205629, *T. oligospermum* (Tul. & C. Tul.) Trappe FM205504, *T. rufum* Pollini FM205636, *T. melanosporum*AF300826) were taken from the National Centre for Biotechnology Information (NCBI) and aligned using CLUSTALW (http://npsa-pbil.ibcp.fr/cgibin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html) to search for genomic regions which were different between *T. magnatum* and the other fungi. The forward primer Tmag3 and the reverse primer Tmag4 (Table 2) were designed within the ITS2 region using Perl Primer software, an open-source application that also designs primers for qPCR, by calculating the possible primer-dimers. The primers were tested *in silico* using the Primer3 software (http://frodo.wi.mit.edu/primer3/). The new primer pair was tested on the 33 fungal samples listed in Table 3 to check its specificity. Amplification reactions were carried out in a 25 μl final volume on a Mastercycler ep gradient (Eppendorf). The mix contained: 10 $\times$ buffer (2.5 μl), 2.5 mM dNTPs (1.5 μl) 10 μM of each primer (1 μl), sterile water (16.8 μl) Dream Taq (1 U/μl) (Fermentas) and a 2 μl template (10–20 ng/μl). The PCR cycles involved an initial denaturation at 94 $^\circ$ C for 3 min, followed by 35 cycles of 94 $^\circ$ C for 30 s, 56 $^\circ$ C for 30 s and 72 $^\circ$ C for 2 min. A final extension was carried out at 72 $^\circ$ C for 5 min.

Table 3
Collection of fungi used to test the new primer pair. Some species were kindly provided by Dr Zambonelli from Universitiy of Bologna (Italy) or by Dr Bonito from Duke Universitiy (USA) and deposited in their universities. The other species are deposited at Dipartimento di Scienze della Vita e Biologia dei Sistemi in Turin (Italy).

Fungal species	Voucher number or origin
1. *Tuber oregonense*	Kindly provided by Dr Bonito
2. *Tuber lyonii*	Kindly provided by Dr Bonito
3. *Tuber* spp.	Kindly provided by Dr Bonito
4. *Tuber canaliculatum*	Kindly provided by Dr Bonito
5. *Tuber gibbosum*	Kindly provided by Dr Bonito
6. *Tuber youngii*	Kindly provided by Dr Bonito
7. *Tuber quercicola*	Kindly provided by Dr Bonito
8. *Tuber candidum*	Kindly provided by Dr Bonito
9. *Tuber separans*	Kindly provided by Dr Bonito
10. *Tuber spinoreticulatum*	Kindly provided by Dr Bonito
11. *Tuber mansenii*	Kindly provided by Dr Bonito
12. *Tuber indicum*	080110-1
13. *Tuber mesentericum*	14
14. *Tuber panniferum*	161
15. *Tuber brumale*	080130-1
16. *Tuber oligospermum* 2416	Kindly provided by Dr Zambonelli
17. *Tuber foetidum*	175
18. *Tuber puberulum*	118
19. *Tuber melanosporum*	080107
20. *Tuber rufum*	2773
21. *Tuber dryophilum* 3006	Kindly provided by Dr Zambonelli
22. *Tuber borchii*	F9
23. *Tuber excavatum* 3438	Kindly provided by Dr Zambonelli
24. *Tuber magnatum*	F8
25. *Tuber macrosporum*	428
26. *Tuber aestivum*	10
27. *Tuber maculatum crixo*	200
28. *Choiromyces*	160
29. *Humaria hemisphaerica*	080710
30. *Hymenogaster*	159
31. *Terfezia* 1996	Kindly provided by Dr Zambonelli
32. *Genea*	158
33. *Boletus edulis*	070220

2.5. Sequence analysis

The amplified products were separated and visualized on 1–2% agarose gel. A subsample of them was then purified according to the Qiaquick PCR purification kit (Qiagen SA) protocol and sequenced by DiNAMYCODE S.R.L. (Torino, Italy). The sequences were compared using the NCBI online standard Basic Local Alignment Search Tool (BlastN) algorithm (Altschul et al., 1997). The accession numbers are: HE613436 for the C_MV1 cream amplified with Tmag3/Tmag4, HE613437 and HE613438 for the C_RRnF cream amplified with Tmelfor/Tmelrev and with ITS1/ITS2, respectively.

2.6. qPCR assay to quantify *T. magnatum*

Quantitative assays were performed using MX3000P (Stratagene). Each PCR reaction was conducted on a total volume of 20 μl, containing 1 μl DNA (from 4 to 11 ng/μl), 10 μl SsoFastTMEva Green®Supermix (BioRad) and 2 μl of each primer (Tmag3/Tmag4) (3 μM) as well as 5 μl of sterile Milli-Q water, using a 48-well plate. DNA-free controls were run for each experiment. The used PCR program was: 95 $^{\circ}$ C for 10 min, 40 cycles of 95 $^{\circ}$ C for 30 s, 56 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 30 s, followed by 95 $^{\circ}$ C for 1 min, 56 $^{\circ}$ C for 30 s, and 95 $^{\circ}$ C for 30 s for the calculation of a melting curve. Three technical replicates were performed for each reaction. Each standard curve was generated using 10-fold serial dilutions of up to

10^{-6} of *T. magnatum*fruiting body genomic DNA (37 ng/μl). The data were analyzed using the MXPro software packages (Stratagene). The new sample C_RRn, containing only non-*T. magnatum* spores, was used as negative control.

2.7. qPCR assay to quantify *T. melanosporum*

A quantitative assay was performed using the StepOne Real Time PCR System (Applied Biosystem). The mix and the PCR program were the same as those set up by Zampieri, Rizzello, Bonfante, and Mello (2012) for the quantification of *T. melanosporum* in soil. Each standard curve was generated using 10-fold serial dilutions of up to 10^{-5} of *T. melanosporum* fruiting body genomic DNA (59.6 ng/μl). Specific amplified product formation was confirmed through melting curve analysis. The data were analyzed using the StepOne v.1.0 software packages (Applied Biosystems). The samples C_MT, C_MV and BT were used as negative control, since the absence of *T. melanosporum* had been demonstrated through the morphological approach and the conventional PCR.

2.8. Data analysis

The threshold cycle (Ct) values of the unknown samples were converted to ng/μl of DNA by comparing these Ct values with those of the standards. Draft quantification (mg) of the truffles employed in each processed food (g) was determined by comparing the ratios (DNA/mg) of the fruiting body used for the DNA extraction (100 mg). The standard deviation was calculated for the three replicate data sets resulting from each processed food.

2.9. Patent application

The DNA extraction method from processed products as well as the *T. magnatum* specific primers and their use in conventional- and qPCR are under a patent application.

3. Results

3.1. Morphological analyses and relative spore count

As far as the cream tubes are concerned, two different conditions were shown under the light microscope. In the C_MV cream only spores resembling those of *T. magnatum* were present, while in the C_RR cream spores probably belonging to *T. borchii* (Fig. 1) were also present, but were not declared on the label. In manual counts the percentage of spores was estimated as 60% for *T. magnatum* and 40% for the non-*T. magnatum* species, respectively. Spores resembling those of *T. magnatum* and of the *B. edulis* complex (porcini) were found in the C_MT pot cream, in agreement with the stated label ingredients. In the BT

butter the microscopic features of the spores were attributable to a unique truffle species, likely *T. magnatum* (Fig. 1).

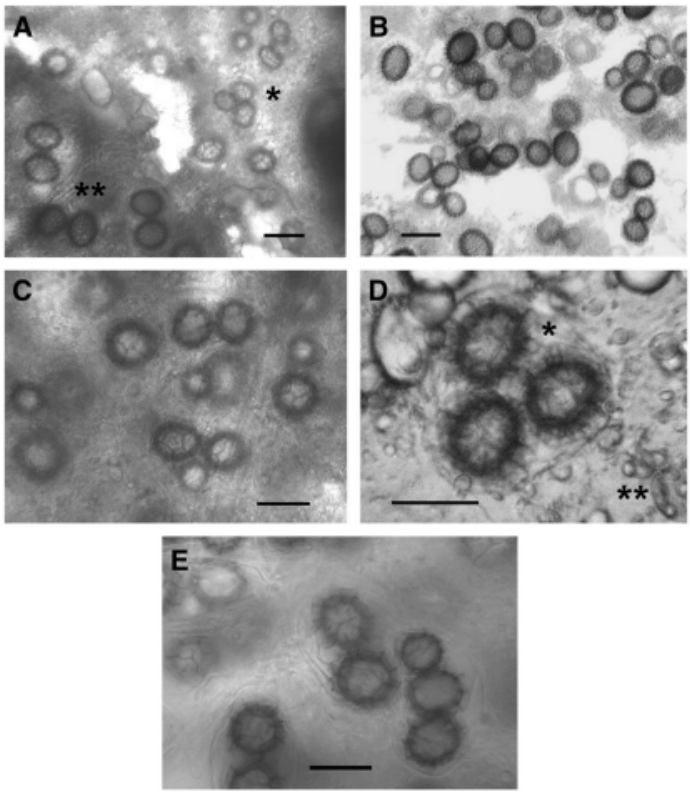

Fig. 1. Fungal spores present in the analyzed processed products. A: *T. magnatum* (*) and *T. borchii* (**) spores in the same sample of the C_RR cream; B: *T. borchii* spores of the C_RRn sample created from the C_RR cream; C: *T. magnatum* spores in the C_MV cream; D: *T. magnatum* (*) and *B. edulis* complex (**) spores in the same sample of the C_ MT cream; and E: *T. magnatum* spores in the BT butter. Bars = 50 μm.

3.2. Molecular analyses

All the samples, listed in Table 1, were successfully amplified with the universal fungal primers, revealing the amplificability of the extracted DNA. The specific primers for *T. borchii* (TBA/TBB) were used to verify the presence of *T. borchii* in the C_RR cream. The primers did not give any positive result, even in the samples containing only spores probably belonging to *T. borchii* (data not shown). The presence of *B. edulis* was checked by means of the specific Bedu1f/Bedu1r primers, which gave the expected band in the C_MT1, C_MT2, C_MT3 samples (data not shown).

3.3. *T. magnatum* quantification

The PCR carried out with the new primer pair Tmag3/Tmag4 resulted in specific amplification, and only showed the expected 151 bp amplicon in the *T. magnatum* sample (Fig. 2). These primers were first used in conventional PCR on the processed products and then tested in qPCR. In the conventional PCR, they showed the band in samples from the C_MV cream and the BT butter (Fig. 3), but not in the C_MT cream or both in the C_RR cream and the C_RRn sample. We then set up a qPCR with all the processed products listed in Table 1. As a result, the average quantity of *T. magnatum* DNA ranged from 0.001 ng (in the C_RR cream) to 2.225 ng (in the BT butter) (Table 1). Converting these values in mg of *T. magnatum*/g of processed products, we estimated from 0.003 to 8.021 mg (Table 1). The average efficiency of the system was 95.44%. It was not possible to quantify *T. magnatum* in the C_MT cream and also in the C_RRn sample as expected.

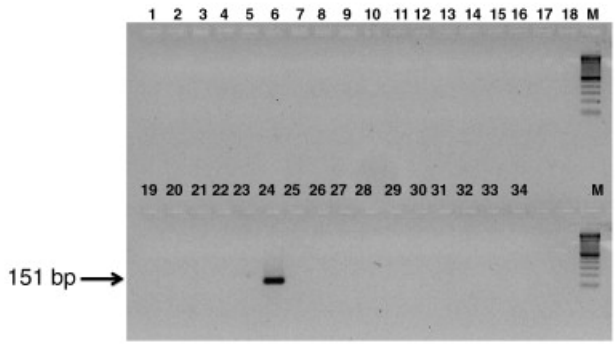

Fig. 2.

PCR amplification with Tmag3/Tmag4 of the 33 fungal samples.

Lane 1: *Tuber oregonense*; lane 2: *T. lyonii*; lane 3: *Tuber* sp.; lane 4: *T. canaliculatum*; lane 5: *T. gibbosum*; lane 6: *T. youngii*; lane 7: *T. quercicola*; lane 8: *T. candidum*; lane 9: *T. separans*; lane 10: *T. spinoreticulatum*; lane 11: *T. mansenii*; lane 12: *T. indicum*; lane 13: *T. mesentericum* 14; lane 14: *T. panniferum*; lane 15: *T. brumale*; lane 16: *T. oligospermum*; lane 17: *T. foetidum*; lane 18: *T. puberulum* 118; lane 19: *T. melanosporum*; lane 20: *T. rufum* 2773; lane 21: *T. dryophilum*; lane 22: *T. borchii* F9; lane 23: *T. excavatum* 3439; lane 24: *T. magnatum* F8; lane 25: *T. macrosporum* 428; lane 26: *T. aestivum*; lane 27: *T. maculatum* crixo; lane 28: *Choiromyces*; lane 29: *Humaria hemisphaerica*; lane 30: *Hymenogaster*; lane 31: *Terfezia* 1996; lane 32: *Genea*; lane 33: *Boletus edulis*; lane 34: negative control; and lane M: 100 bp (Invitrogen).

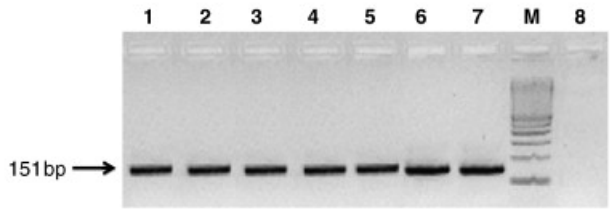

Fig. 3.

PCR amplification with Tmag3/Tmag4 of the C_MV cream and the BT butter.

Lane 1: C_MV1, lane 2: C_MV2, lane 3: C_MV3, lane 4: BT1, lane 5: BT2, lane 6: BT3, lane 7: *T. magnatum* F8 fruiting body, lane M: 100 bp (Invitrogen), and lane 8: negative control.

3.4. *T. melanosporum* quantification

In order to identify the *Tuber* species present in the C_RRn sample containing only spores of the non-*T. magnatum* species, we sequenced the band obtained with the universal primers ITS1/ITS2. The analysis of the obtained sequence surprisingly revealed the presence of *T. melanosporum* in this sample. This finding was confirmed by amplifications with the specific primers for *T. melanosporum* (data not shown). On the bases of these results we hypothesized that the *T. melanosporum* was more abundant than the non-*T. magnatum* species preventing, in this way, its molecular identification. In order to evaluate the quantity of *T. melanosporum*, we applied a protocol for qPCR, which was set up by Zampieri et al. (2012)for the quantification of *T. melanosporum* in soil. The same protocol was also applied to the C_MV, the C_MT and the BT products to verify whether *T. melanosporum* was present. The quantification was possible only in the C_RR and C_RRn samples. An average amount of DNA ranging from a minimum of 0.09 ng (in C_RRn) to a maximum of 1.576 ng (in C_RR) in 1 μ l of DNA was obtained (Table 1). Converting these values into mg

of *T. melanosporum*/g of cream, we estimated from 0.2 to 3.526 mg of this truffle species (Table 1). The average efficiency of the system was 90%.

4. Discussion

The present paper reports the detection and quantification of the prized white and black truffles in commercial products such as cream- and butter-based products. The most important result of this investigation was the development of sensitive methods, which led to detection of fraudulent mislabeling in processed products containing truffles.

On the basis of the application of microscopic techniques, it was possible to distinguish two types of spores in a cream, which could probably be ascribed to *T. magnatum*, the most costly truffle, as declared on the label, and to *T. borchii*, a less prized and aromatic truffle, which was not indicated on the label. Assuming that the two species presented the same level of maturation when used for the preparation of the cream, we counted their spores to see whether *T. magnatum* was at least the most abundant. In fact, it was more abundant than the non-*T. magnatum* species (60% vs 40%, where 100 is the total number of spores). From the molecular point of view, it was not possible to identify the non-*T. magnatum* species, but the ITS1 sequencing highlighted the unexpected presence of black truffle, which was also verified using *T. melanosporum* specific primers (Bonito, 2009 and Paolocci et al., 1999). An explanation for the presence of the black truffle could be that producers sometimes use unripe, and consequently not fully scented, fruiting bodies of *T. melanosporum* for the production of *T. magnatum* processed food because of their lower cost (Amicucci et al., 2002). When the fruiting body samples are immature, the asci containing the spores are empty and cannot be identified through a microscopic examination; molecular biology techniques are therefore necessary to detect frauds.

An important key result of our investigation was that good quality DNA could be obtained quickly in a few hours using a kit generally employed for DNA extraction from soil; in fact the matrix of processed food could be compared to a complex matrix such as soil. The Fast DNA Spin kit for soil has, therefore, been validated as a reliable tool for DNA extraction under our experimental conditions. Even though the lipids and salt present in the ingredients can negatively influence the DNA yield (Bleve, Rizzotti, Dellaglio, & Torriani, 2003), it was always possible to amplify the DNA extracted with this kit with universal primers (ITS1/ITS2) from all the samples.

In order to authenticate *T. magnatum* in the processed products, we set up a quantitative PCR protocol. The quantitative PCR technique is based on the detection of signals emitted during the exponential phase. This is one of the greatest advantages of qPCR over conventional PCR methods (Sharma et al., 2007). Quantitative PCR has already been used to detect and quantify poisonous mushrooms in different matrices (Epis et al., 2010) and *Gadus morhua* (the Atlantic cod) in fresh, frozen and processed food (Herrero, Madriñán, Vieites, & Espiñeira, 2010). For our purpose we decided not to use the available specific *T. magnatum* primers (Amicucci et al., 1998, Mello et al., 1999 and Rubini et al., 2001), because the size of the amplified fragment is longer than what is usually recommended in a quantitative analysis. The low level of genetic variability found in *T. magnatum* (Mello et al., 1999) and the high number of available sequences in GenBank, allowed us to design a specific primer pair (Tmag3/Tmag4) for this fungus. The primers were first employed in direct PCR on DNA obtained from our samples, and a signal was given for the C_MV tube cream and the BT butter but not for the C_RR tube cream, C_RRn sample or the C_MT pot cream. In order to check whether samples that did not show any amplification products could give a fluorescence signal, we set up a qPCR protocol. With this method, C_RR was positive for the presence of *T.*

magnatum. The *T. magnatum* average quantity in this cream was 0.001 ng/ μ l (standard deviation 0.001 among the three replicates), indicating a low quantity of truffles and low variability among the replicates. The *T. melanosporum* DNA was also quantified to verify whether the unripe *T. melanosporum* fruiting bodies were so abundant in the C_RR cream as to prevent molecular detection of the non-*T. magnatum* species (likely *T. borchii*). The quantity was 1.576 ng/ μ l (standard deviation 1.11 among the three replicates) and more than that found in the C_RRn sample (0.09 ng/ μ l). In fact, in the C_RRn sample there were only non-*T. magnatum* spores with a small quantity of gleba around them; it could therefore be expected to find less *T. melanosporum* in C_RRn sample replicates than in the C_RR cream replicates. The black truffle, which was not indicated on the label, was therefore found. Supposing a correct recognition of the *T. borchii* species, the lack of its molecular detection could be explained either with its low quantity or by a detection system that was not sufficiently sensitive. A qPCR protocol should be developed also for this species in order to overcome the limit of the conventional PCR.

With regard to the C_MV and the C_MT creams and the BT butter, the quantification of *T. melanosporum*was not possible indicating the absence of fraudulent practices. The quantity of *T. magnatum* (0.032) found in the C_MV cream was very similar in the three replicates (standard deviation 0.02), thanks to the homogeneity of this cream (without visible pieces of truffles), and more than that found in the other samples. This last finding is in agreement with the fact that this is the only cream in which *T. magnatum*could be detected with the conventional PCR. *T. magnatum* was neither detected nor quantified in the last cream (C_MT), in agreement with the very few spores, which were seen under the light microscope. The other spores were ascribed to the *B. edulis* complex, which includes four different species that are difficult to distinguish only from the morphological point of view (Mello, Ghignone, et al., 2006). The molecular data allowed us to confirm the presence of *B. edulis*, which was listed on the label. The quantity of *T. magnatum*found in the BT butter was elevated but the three replicates showed high variability due to the patchy structure of the butter.

However, a bias in the qPCR protocol may still exist because the standard curve was built using fruiting bodies from the two species, *T. magnatum* and *T. melanosporum*, instead of a more reliable truffle processed food, since no suitable food product was available as a standard (same ingredients). For this reason, we are aware that the quantification is not completely reliable. However, this qPCR method allowed us to authenticate and quantify the prized truffles in different food matrices below the average limit of detection of conventional PCR, which was 0.032 ng/ μ l. The quantity we estimated was not absolute, because the DNA extraction yield could have been negatively influenced by the other non-fungal ingredients contained in the products. However, adopting this method, we have obtained the minimum estimate of the quantity of truffles present in the food.

4.1. Conclusions

A qPCR assay has been developed to authenticate and quantify *T. magnatum* and *T. melanosporum* in food matrices. The optimized assays, showing good values of efficiency, are specific, sensitive and applicable to products that have undergone intensive transformation processes, and have been successfully tested on commercial samples (cream in tubes and in pots, and butter). The designed method will help in detecting missing ingredients and/or the incorrect labeling of processed products. This tool can therefore be used to detect fraudulent practices, to protect the consumer and to assess food quality.

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