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Proximate analysis of lipid composition in Moroccan truffles and desert truffles

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PAPER

Abstract

Lipid composition in truffle is essential for nutraceutical and medicinal purposes. Currently, there is no data regarding the lipid content in Moroccan truffles. Therefore, we determined the fatty acid and sterol composition of six Moroccan truffles and desert truffles. The gas chromatography analysis revealed the predominance of fatty palmitic, oleic and linoleic acids. The prominent sterol components were brassicasterol and ergosterol. Besides, the sterol analysis discriminated between the *Tuber* and *Terfezia* truffles. These differences seem to be exploitable at a taxonomic level. This is a preliminary report disclosing the fatty acid and sterol composition of Moroccan truffles, indicating the potential use of lipids analysis, especially sterol analysis, as biomarker for truffles distinction.

Keywords: desert truffles, discrimination, fatty acid, gas chromatography, Moroccan truffle, sterol

Introduction

The truffles are edible ascocarp of hypogenous ascomycetes fungi that grow underground (Khabar *et al.*, 2001; Lee *et al.*, 2020). The term 'desert truffles' is used to describe truffles growing particularly in arid and semi-arid areas (Khabar *et al.*, 2001; Morte *et al.*, 2021), such as Morocco, Algeria, Tunisia, Egypt, South Africa, Saudi Arabia, Iraq, Syria and Kuwait (Khabar, 2014; Lee *et al.*, 2020). The genera found abundantly in those areas are *Terfezia* and *Tirmania*. Besides, other genera exist as well, namely *Delastria* and *Picoa* (Khabar, 2014). In Mediterranean countries, especially in North Africa, the truffles are harvested in abundance and known as 'Terfass,' also called 'Kame,' 'Kholassi,' 'Zoubaïdi,' 'Truffles of the deserts' and 'Truffles of the sands' because of their development in sandy soil (Khabar, 2014).

The determination of lipid composition in truffles is essential both for lipid analysis as well as for nutraceutical and medicinal purposes. The truffles contain only 4–9% by dry weight of total lipids (Tang *et al.*, 2011). Fatty acids and phytosterols are the main lipid compounds in truffle fruiting bodies, which are well known for their potential human benefits. Several studies through global chromatographic analysis demonstrated that desert truffles are rich in fatty acids, both saturated and unsaturated that have many positive effects on health (Akyüz, 2013; Al-Shabibi *et al.*, 1982; Bokhary *et al.*, 1989; Doğan and Aydın, 2013; Veeraraghavan *et al.*, 2021). For example, in *Terfezia boudieri* from Tunisia, Hamza *et al.* (2016) reported that essential fatty acids, like linoleic and oleic acids, account for 76% of the total fat content. Linoleic acid or omega-6 is an essential fatty acid and one of the most aromatic compounds in most truffle species (Lee *et al.*, 2020), which has protective and antioxidative effects beneficial for human health (Sokoła-Wysoczańska *et al.*, 2018). While, oleic acid, a bioactive compound, has the aptitude in reducing cholesterol levels (Lee *et al.*, 2020). Another example of *Terfezia claveryi* from Saudi Arabia, which is closely related to *T. boudieri* (Dahham *et al.*, 2018), was found to have arachidic, myristic, palmitic, behenic, pentadecanoic, stearic, heneicosanoic, nonadecanoic and margaric acids

as saturated fatty acids along with unsaturated fatty acids (palmitoleic, oleic, erucic and linoleic) (Bokhary *et al.*, 1989). The lipid composition of desert truffles depends highly on the species as well as growing environments. For instance, *T. boudieri* from Saudi Arabia was rich in pentadecanoic, margaric, stearic and arachidic acids (Bokhary *et al.*, 1989). Whereas, the same species from Turkey contained mainly oleic, linoleic, linolenic, palmitic, palmitoleic, stearic and behenic acids (Akyüz, 2013). The most identified phytosterols in truffle reports were brassicasterol and ergosterol. Harki *et al.* (1996) reported that the prominent components identified in *Tuber melanosporum* were ergosterol and brassicasterol, accounting for about 90% of total sterols. As well, the major sterol components in the *Tuber* ascocarps were brassicasterol and ergosterol, accounting for about 17–64% and 25–67% of total sterols, respectively (Tang *et al.*, 2012). In *Terfezia* truffles, brassicasterol levels were 98% of the total sterols, while ergosterol was present in lower amounts (Tejedor-Calvo *et al.*, 2021). Other phytosterols such as beta-sitosterols, stigmasterol and campestanol were also present in low contents (Dahham *et al.*, 2018).

The six species included in this study were natives of Morocco. *Terfezia arenaria,* commonly called 'Pink Terfess of Mamora,' was harvested from acidic soil, in semi-arid climate under *Helianthemum guttatum*. It is an appreciated edible fungus, detected by the 'mark' method (Khabar, 2014) unlike *Delastria rosea*, known as 'Bitter Terfess of Taida' due to its bitter flavour. It was collected under *Pinus pinaster* var. atlantica and *Pinus halepensis* in Mamora forest between November and December (Khabar, 2014). Similarly, *Tuber oligospermum* was also collected under *P. pinaster* var. atlantica in Mamora forest, starting from December until April. The ascocarp of *T. boudieri* originated from Bouaarfa region, collected from limestone soil, under arid and sub-Saharan climate, and *Terfezia leptoderma* was obtained from Mamora forest from the acidic soil under *H. guttatum* at the beginning of February until May. *Tuber asa,* commonly called 'Terfass male of Terfass' because of its hard consistency, collected as well under *H. guttatum* on acidic soil of Mamora forest, towards the end of February at the same time as *T. leptoderma* (Khabar, 2014). The aims of this work were 1) to determine the lipid profile of the six Moroccan truffle species, 2) to determine the relation between the genus, species and lipid composition of truffles, and 3) to determine whether the lipid profile can be used as a tool for species or genus distinction.

Materials and methods

Fungus material

Six Moroccan truffle species were used in this experiment. The ascocarps of different species (Table 1) were collected directly from their natural environments and transported to the laboratory. Under the fume hood, the samples were surface sterilised with ethanol, peeled and then fragmented by hand. Several pieces were taken from the glebe and stored in pillboxes at –64°C. Alternatively, other samples were sun-dried for 2 months before being stored at –64°C.

Table 1. The name and location of six Moroccan truffle species used in this study.

Species name	Location
Terfezia leptoderma (1)	Mamora Forest under *Helianthemum*
T. leptoderma (2)	Mamora Forest under *Pinus pinaster*
Terfezia arenaria	Mamora Forest
Terfezia boudieri	Bouaarfa
Tuber asa	Mamora Forest
Tuber oligospermum	Mamora Forest under *P. pinaster*
Delastria rosea	Mamora Forest under *P. pinaster*

The different steps of extraction, separation and analysis of lipids were released at the Laboratory of Mycology, Phytopathology and Environment of the Littoral France University, following the method of Fontaine *et al.* (2001).

Extraction of total lipids

The extraction was performed with approximately 20–40 mg of freeze-dried material (pieces of truffle glebe). The solvent used for extraction was a mixture of dichloromethane and methanol (2:1 v/v) with 0.05% BHT (Butylated hydroxytoluene; Sigma) as antioxidant. The freeze-dried fungal material was first ground in 40 ml of the solvent using ultra-turrax homogenizer. The first extractions were performed in the dark to preserve the ergosterol, a photosensitive sterol. The extraction of total lipids was carried out under reflux (1 h at 70°C) with some pieces of pumice stone. After filtration, the lipid extract was recovered under nitrogen blowdown and rotary evaporator at 60°C. This step was repeated thrice.

Separation of fatty acids and total sterols by saponification

The crude lipid extract was used to separate fatty acids and total sterols by saponification. The crude extract was saponified under reflux (1 h at 90°C) in 2 ml of 6% (w/v) methanolic potash and some pieces of pumice. After cooling, two successive cold extractions with hexane were performed. The first extraction permitted the

recovery of unsaponifiable fraction (sterols), while the second one enabled the retrieval of saponifiable fraction (fatty acid). To perform the first cold extraction, one volume of distilled water was added to the cooled saponified extract, followed by six volumes of hexane. This mixture was then vigorously stirred for 1 min with a vortex. After decantation, the organic phase (upper layer), which contains the unsaponifiable elements, was taken out and dehydrated with anhydrous sodium sulfate. This step was repeated three times, and the recovered extract was concentrated in a rotary evaporator at 50°C. For the fatty acid recuperation, the aqueous phase was acidified to pH 1 with 1 M HCl to liberate them from their saline combination. Afterwards, the acidified phase was extracted by performing three extractions with hexane. The concentration of these extracts was done under nitrogen.

Fatty acid analysis

The fatty acids were solubilise in 1 ml boron trifluoride–methanol (14% w/v). The methylation reaction was carried out for 2 min at 90°C in a water bath and then stopped by immersing the tubes in ice. After addition of 1 ml of distilled water and 2 ml of hexane, the tubes were vortexed for 30 s. The organic phase (upper phase) was taken out and dehydrated by adding anhydrous sodium sulfate. This step was repeated thrice. The methylated fatty acids were purified on silica gel of TLC (20 × 20 cm, type Silicagel F 254, Merck) with a solvent system composed of diethyl ether/hexane (10/90; v/v). The fatty acid spots were detected by primuline and eluted in about 1 ml of dichloromethane. Thereafter, the fatty acids were immediately taken up in 25–100 µl of hexane and injected into the gas chromatograph. Fatty acids were identified by comparing their relative retention times with internal standard such as methyl C 17:0 (methyl margarate) as well as other known standards (Alltech).

Sterol analysis

To obtain a better separation in gas chromatography, sterols were acetylated either for 12 h at room temperature or 2 h at 60°C by the mixture of toluene/acetic anhydride/pyridine (1/2/1; v/v/v). Sterol acetates were purified on silica gel thin layer with dichloromethane as migration solvent. Cholesterol acetate (1 mg/ml) was used as a control to localise acetylated products after spraying with 0.01% (w/v) primuline solution. The acetylated sterols were taken up in 25–100 µl of hexane and injected into the chromatograph. The sterol acetates were identified by comparing their relative retention times against an internal standard, cholesterol in alcohol form (non-acetylated) along with brassicasterol and other known acetylated standards.

Statistical analysis

The data were analysed using R studio software. The means and standard deviations were calculated. The pairwise comparisons among means were performed using two-way ANOVA and Tukey HSD test. To indicate significant differences, we used the multcompLetters4() function from the multcompView package.

Results and discussions

The objective of this work was to define the nature and proportion of fatty acids and sterols in six species of Moroccan truffles and to determine if this lipid profile could be used as a classification tool to discriminate between species or genus. This study was conducted for the first time on Moroccan truffles, disclosing distinctly the fatty acid and sterol components of truffles and desert truffles grown in Morocco.

Fatty acid composition

The fatty acid composition of Moroccan truffles has not been reported previously, and studies on fatty acid content of other truffles are scarce. The first and most reported studies on fatty acid composition were focused on *Tirmania pinoyi*, *Tirmania nivea*, *T. boudieri*, *T. claveryi* and *Picoa lefebvrei* from Saudi Arabia (Bokhary *et al.*, 1989; Bokhary and Parvez, 1995) and *T. claveryi* from Iraq (Al-Shabibi *et al.*, 1982). Recently, other studies appeared on fatty acid composition of *T. boudieri* from Iraq (Dahham *et al.*, 2018), *T. boudieri* from Turkey (Hamza *et al.*, 2016), *T. claveryi* and *Picoa juniperi* from Spain (Murcia *et al.*, 2003) as well as *T. nivea* from Libya (Shah *et al.*, 2020).

The chromatographic analysis results for the identification of fatty acids compositions are presented in (Table 2 and Figure 1). Seven fatty acids were detected in the six truffles species used in this experiment; four saturated fatty acids [palmitic (C16:0), stearic (C18:0), arachidic (C20:0) and behenic (C22:0)], and three unsaturated fatty acids [palmitoleic (C16:1), oleic (C18:1) and linoleic (C18:2)]. Bokhary *et al.* (1989) reported that palmitic, stearic, oleic and linoleic acids were predominant in *T. nivea* and *T. boudieri* which originated from Saudi Arabia. As well, the Turkish *T. boudieri* was also rich in behenic, palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids (Akyüz, 2013), which agree with our results and particularly with *T. boudieri*. Furthermore, a recent study on fatty acid composition in *Tuber maculatum*, *Tuber aestivum/uncinatum*, *Tuber borchii*, *T. melanosporum* and *T. nivea* revealed the dominance of palmitic, stearic, oleic and linoleic acids followed

Table 2. Fatty acid composition of the six Moroccan truffle species through gas–liquid chromatography analysis (percentage of dry weight of the lipid fraction).

Species	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2	C 20:0	C 22:0
Terfezia leptoderma (1)	16.080 ± 0.936^{a}	0.913 ± 0.372^{a}	1.600 ± 0.185^{a}	27.530 ± 0.598^{a}	48.513 ± 1.179^{a}	4.570 ± 0.589^{a}	0.790 ± 0.147^{a}
T. leptoderma (2)	17.456 ± 2.167^{a}	1.733 ± 0.221^{a}	1.026 ± 0.047^{a}	26.106 ± 0.740^{a}	48.633 ± 1.075^{a}	5.753 ± 0.247^{a}	0.803 ± 0.100^{a}
Terfezia arenaria	21.470 ± 1.822^{b}	1.066 ± 0.133^{a}	3.253 ± 0.206^{a}	27.020 ± 0.727^{a}	38.803 ± 0.525^{b}	8.563 ± 0.577^{b}	0.900 ± 0.095^{a}
Terfezia boudieri	20.823 ± 1.019^{b}	0.830 ± 0.303^{a}	2.433 ± 0.508^{a}	28.333 ± 1.028^{a}	42.770 ± 1.127^{c}	4.580 ± 0.545^{a}	0.710 ± 0.156^{a}
Tuber asa	16.470 ± 1.483^{a}	0.940 ± 0.096^{a}	1.810 ± 0.259^{a}	15.983 ± 0.879^{b}	61.186 ± 0.315^{d}	4.773 ± 0.396^{a}	0.740 ± 0.070^{a}
Tuber oligospermum	16.670 ± 0.138^{a}	1.090 ± 0.389^{a}	2.023 ± 0.200^{a}	20.593 ± 0.450^{c}	54.926 ± 1.601^{e}	4.720 ± 0.500^{a}	0.780 ± 0.105^{a}
Delastria rosea	12.420 ± 1.574^{c}	1.230 ± 0.166^{a}	1.263 ± 0.112^{a}	60.160 ± 0.916^{d}	19.836 ± 1.237^{f}	4.503 ± 0.678^{a}	0.830 ± 0.052^{a}

Data shown as mean ± standard deviation (n = 3). Different superscript letters in the same column indicate a statistically significant difference ($P < 0.05$).

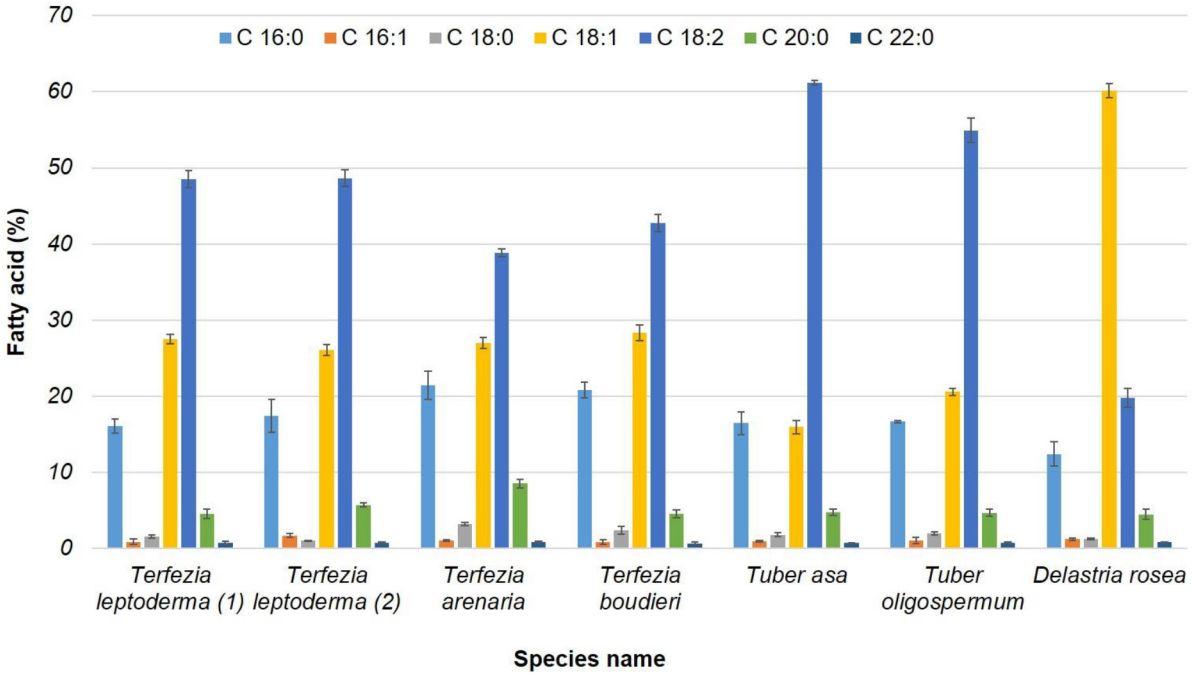

Figure 1. Fatty acid composition of the six Moroccan truffle species used in this study.

by traces of polyunsaturated fatty acids (Shah *et al.*, 2020).

The main fatty acids detected were palmitic, oleic and linoleic acids. The other fatty acids were also present but at lower levels. Similar findings were also reported in various species of *Terfezia* and *Tuber* (Hamza *et al.*, 2016; Tejedor-Calvo *et al.*, 2021). Our results demonstrated that the rate of palmitic acid (C16:0) is appreciably equal in all the species studied. We could also notice that the linoleic acid level was generally higher compared to the oleic acid (C18:1) level in all the species studied except for *D. rosea* where the opposite was true; the level of oleic acid was higher than linoleic acid. The same results were also reported by Hamza *et al.* (2016). The *T. boudieri* was characterised by its higher content of linoleic acid (54.10%) compared to oleic and palmitic acids that represented 22 and 20.40%, respectively (Hamza *et al.*, 2016). Linoleic acid level was considerably high in *Tuber* compared to *Terfezia* species. The rate of oleic acid was, on the other hand, slightly lower in *Tuber* genus compared to *Terfezia* genus. This remark goes with Tejedor-Calvo *et al.* (2021), who reported that linoleic acid content in *Tuber brumal* and *T. melanosporum* reached 78.3 and

61.12%, respectively, compared to *T. leptoderma* and *T. arenaria* which noticed only 51.3 and 30.9%, respectively. The fatty acid results of Moroccan truffles and desert truffles proved their richness in unsaturated and healthy fatty acids such as linoleic acid, suggesting their equivalent culinary value compared to European truffles.

Regarding fatty acid discrimination, it seems that these criteria could not differentiate clearly between the species of the two genera of *Tuber* and *Terfezia*. Indeed, the ratio of linoleic acid or oleic acid was approximately equal between the different species of the two genera. Nevertheless, the fatty acid composition could distinguish between *Delastria* and other two genera.

Sterol composition

The sterol composition of Moroccan truffles has never been reported before. The first report on sterol was that by Weete *et al.* (1985), which mentioned about both *Terfezia* and *Tuber* genera. Further, other studies principally on *Tuber* species including *T. melanosporum* (Harki *et al.*, 1996; Sancholle *et al.*, 1988), *Tuber magnatum*, *T. melanosporum*, *T. aestivum*, *Tuber albidum* and *Tuber indicum* were released (Sommer *et al.*, 2020).

Four sterols (brassicasterol, ergosterol and lanosterol) were identified in the ascocarps of the examined truffle species (Table 3 and Figure 2). Furthermore, the main

Table 3. Sterol composition of the six Moroccan truffle species through gel permeation chromatography analysis.

Sterols	Brassicasterols	Ergosterols	Lanosterol (1.31)	n.i. (1.42)
Terfezia leptoderma (1)	96.870 ± 1.260^{a}	3.016 ± 0.621^{a}	0 ± 0^{a}	n.d.
T. leptoderma (2)	92.410 ± 2.606^{a}	8.153 ± 0.143^{a}	0 ± 0^{a}	n.d.
Terfezia arenaria	96.833 ± 1.045^{a}	0 ± 0^{b}	2.003 ± 0.532^{a}	n.d.
Terfezia boudieri	97.190 ± 2.416^{a}	0 ± 0^{b}	3.110 ± 0.298^{a}	n.d.
Tuber asa	40.526 ± 2.377^{b}	23.196 ± 0.718^{c}	17.470 ± 0.856^{b}	12.220 ± 2.186^{b}
Tuber oligospermum	46.006 ± 2.001^{c}	21.470 ± 1.990^{c}	16.223 ± 4.441^{b}	17.286 ± 1.997^{b}
Delastria rosea	21.343 ± 1.770^{d}	42.9633 ± 0.621^{d}	22.826 ± 3.471^{c}	12.886 ± 1.806^{b}

Data shown as mean ± standard deviation (n = 3). Lanosterol and n.i. compounds are reported with their retention time (in minutes) between brackets. Different superscript letters in the same column indicate a statistically significant difference ($P < 0.05$). n.i., not identified; n.d., not detected.

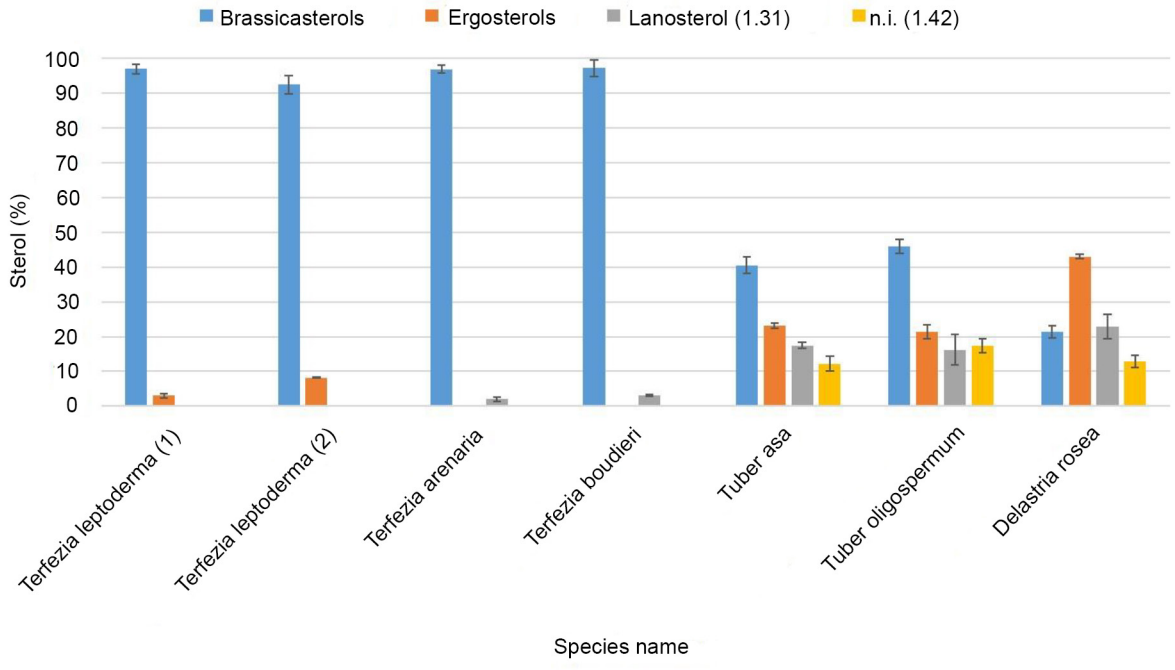

Figure 2. Sterol composition of the six Moroccan truffle species.

sterols were ergosterol and brassicasterol with the highest percentage in all the examined species. Similar results were disclosed in black truffle, where the sterol composition of *T. melanosporum* was analysed and ergosterol along with brassicasterol were identified as the major components (90%) (Harki *et al.*, 1996). As well, the examination of *Tuber sinense*, *T. aestivum*, *T. indicum*, *Tuber himalayense* and *T. borchii* revealed the predominance of brassicasterol and ergosterol, with 17–64% and 25–67% of total sterols, respectively (Tang *et al.*, 2012).

Besides, the ratio of ergosterol to brassicasterol changes according to the genera studied. In *Terfezia* species, brassicasterol was the main sterol identified, accounting for 92–97% of the total sterols, affirming the results of Weete *et al.* (1985), who reported that brassicasterol levels were 98% of total sterols in *Terfezia* truffles, while ergosterol was registered at very low amounts (0–8%). On the other hand, in *Tuber* species (*Tuber asa* and *Tuber oligospermum*) and *D. rosea*, the ergosterol represented a considerable amount compared to *Terfezia* species, accounting for 23, 21 and 43% of the sterols, respectively. These species also contained 40, 46 and 21% of brassicasterol, respectively. A recent study by Tejedor-Calvo *et al.* (2022) demonstrated that ergosterol and brassicasterol were the two main sterols in *T. claveryi* and *T. aestivum* ascocarps, with differences in ergosterol to brassicasterol ratio depending on the ascocarp genus. Lanosterol was also detected in *Tuber* species, as well as in *D. rosea*, in considerable quantities, accounting for 16 and 23% of the sterols, respectively. While in *Terfezia*, they were either totally absent or present in very small quantity (approximately 2–3%). The high amount of brassicasterol in *Terfezia* will increase the quality interest of the Moroccan genera, knowing that brassicasterol has several health benefits, such as antioxidative activity and anti-infective properties.

Finally, *Terfezia* genus was distinguished by the high brassicasterol content, while the *Tuber* genera and *Delastria* were characterised by the equivalent amount of ergosterol and brassicasterol. Hence, sterol analysis proved their importance to highlight differences between species and to separate the *Tuber* from *Terfezia* truffles collected in Morocco. These differences seem to be exploitable at the taxonomic level.

Conclusion

The lipid composition and concentration were highly influenced by truffle speciation and their growing area. This was the first report of lipid composition of Moroccan truffles, divulging the fatty acid and sterol compositions of six species of truffles and desert truffles and demonstrating the richness of Moroccan truffles in essential unsaturated fatty acid, such as linoleic acid. There was a slight difference between *Tuber* and *Terfezia* species in fatty acid component, which is not sufficient to differentiate between them. However, sterol analysis distinguished between these two genera. Hence, a comparison of their sterol composition with reported data seems to be plausible for *Tuber* and *Terfezia* distinction. Finally, a deeper study on other nutrient compounds and bioactive molecules of Moroccan truffles as well as their antioxidant evaluation is predetermined to improve their edible and culinary interest through their health benefits.

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