Abstract: Truffles are the rarest species and appreciated species of edible fungi and are well-known for their distinctive aroma and high nutrient content. However, their chemical composition largely depends on the particularities of their grown environment. Recently, various studies investigate the phytoconstituents content of different species of truffles. However, this research is still very limited for Romanian truffles. This study reports the first complete metabolites profiles identification based on gas chromatography-mass spectrometry (GC-MS) and electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-QTOF-MS) of two different types of Romania truffles: *Tuber magnatum pico* and *Tuber brumale*. In mass spectra (MS) in positive mode, over 100 metabolites were identified from 14 secondary metabolites categories: amino acids, terpenes, alkaloids, flavonoids, organic acids, fatty acids, phenolic acids, sulfur compounds, sterols, hydrocarbons, etc. Additionally, the biological activity of these secondary metabolite classes was discussed.

Keywords: secondary metabolites; truffles; GC-MS; mass-spectra; bioactive compounds

1. Introduction

At present, truffles (*Tuberaceae* family, *Tuber* genus) are considered an emblem of culinary refinement. Because of their nutritive and very particular organoleptic properties, they are considered as one of the most precious foodstuffs. Truffles were assigned mythical qualities in antiquity and then later in the Middle Age because they grow in the ground and are rarely found [1–7].

The high content of nutrients (proteins, fatty acids, minerals, amino acids) and, most of all, their recognizable flavor and aroma are, most probably, key factors that propelled these fungi into a highly precious and exclusive ingredient [1–8].

From ancient times, truffles have been considered aphrodisiacs. This property is attributed to the outstanding chemical constituents able to mimic the male reproductive hormones (androsterone). There are reports about the truffle flavor is associated with perspiration, clay, garlic, mildew, and a faint onion smell [1,5]. There have been several studies on the volatile organic compounds (VOC) and the components involved in flavor. However, the chemical composition of truffles largely depends on the soil characteristics, environmental conditions, and especially the host trees [1–5,7–12].

The truffle’s growth in natural conditions depends on continuously changing climate conditions causing a restriction of their natural area, which directly influences their prices. Preserving truffles and their complex flavor still represents a challenge for the
modern food industry, and is most probably the main factor in their market evaluation worldwide [1,3,8–13].

The increasing market demands (food, cosmetic industry) have brought forth new studies on the extension of truffle cultivation. The quality of truffles is attributed, in particular, to the different soil conditions (pH, organic substances, minerals, etc.), climate, and vegetation characteristics of each region [1,3,4,8–13]. It is appreciated that Central and South European forests have the highest phyllogenetic variety, and are practically the origin growth area of these ectomycorrhizal fungi species. Romania is renowned in Europe for its truffle quality [13,14]. In Romania, the most widespread truffle variants are *Tuber brumale* and *Tuber aestivum*. Nevertheless, in Romania, there are other types of truffles, such as *Tuber aestivum*, *Tuber Macrosporum*, *Tuber Mesentericum*, *Tuber magnatum pico*, and *Choiromyces meandriformis*. The more flavorful truffles (*Tuber magnatum pico* and *Tuber melanosporum*) are the most valuable. *Tuber magnatum pico* (white truffle), with a smooth garlic flavor, is considered one of the rarest varieties and cannot be cultivated. In South and Central Europe, *Tuber brumale* (winter truffle) can be found [13,14].

*Tuber* spp. are organisms adapted to habitats with a low concentration of oxygen by default. These symbiotic fungi most probably contain large quantities of antioxidant agents. The polyphenolics derivates from mushrooms induce a high antioxidant activity [3,15–17].

Recently, special attention was given to the potential biomedical application of hypogean fungus bioactive compounds, in particular, phytosterols, fatty acids, phenols, amino acids, volatile components, etc. [1,3,6–8,11,15–29]. However, still only a few scientific studies have been undertaken on secondary metabolites with therapeutic potential and on truffles’ biology [3,6,7,19–29].

There are relatively few studies on Romanian truffles, despite their high economic value being recognized. Furthermore, biologically active compounds from Romanian truffles have not been assessed through modern analytical methods. Research has only investigated the influence of soil particularities on truffle development [13]. Additionally, in a previous study, our team reported a comparative study on antioxidant activity through the electrochemical method (cyclic voltammetry), morphology (scanning electron microscopy), and semi-quantitative elemental analysis (EDAX) to estimate the diversity from two different types of truffles: *Tuber magnatum pico* and *Tuber melanosporum* [30].

The inclusion of the metabolomics approach in the study of secondary metabolites with therapeutic potential is paramount [31–35]. In this study, was used a qualitative untargeted metabolomics methodology based on the combination of gas-chromatography coupled with mass spectroscopy (GC-MS) and electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-QTOF-MS) to analyze the metabolic profiles from two Romanian truffles species with high economic value, namely *Tuber magnatum pico* and *Tuber brumale*, or winter truffle.

2. Results and Discussion

The truffles chemical composition is highly complex and it is not yet fully described, especially since it is directly dependent on several factors, of which the most important are: host tree and soil parameters. Two solvents were selected with low polarity to achieve the extraction of truffles metabolites.

Thus, in dichloromethane, a polar aprotic solvent is expected to extract lipophilic compounds, such as fatty acids, terpenes, steroids, etc. Moreover, high polarity fractions (amino acids, alkaloids, carbohydrates, etc.) were extracted in methanol. The bioactive compounds screening from the truffles sample were tentatively identified by gas-chromatography coupled with mass spectroscopy (GC-MS) and electrospray ionization-quadrupole time-of-flight mass spectrometry (ESI-QTOF-MS) analysis.

Even though gas-chromatography coupled with mass spectroscopy (GC-MS) is one of the most common analytic techniques and is essential in the investigation of natural products due to their features, robustness and high sensitivity allowing affordable and highly accurate separation and identification of metabolites [36].
Usually, gas-chromatography (GC) is used mainly for the separation of relatively low molecular weight metabolites such as amino acids, carbohydrates, organic acids, fatty acids, sterols, etc. [36].

A comparison of the total ion chromatographs of both truffle extracts presents the similarities and the differences regarding the metabolite types separated from the analyzed samples. The results are summarized in Table 1, which presents the GC-MS tentative compounds identification corresponding to *Tuber magnatum pico* and *Tuber brumale* samples.

Table 1. Main compounds identified by GC-MS analysis in both truffle samples.

| Sample                  | Compounds Identified from GC-MS Library | RT  | RI (Determined) |
|-------------------------|----------------------------------------|-----|-----------------|
| *Tuber magnatum pico*   | 3-octanol                              | 20.452 | 1087            |
|                         | dimethyl sulfoxide                     | 28.769 | 516             |
|                         | stearic acid                           | 32.974 | 216             |
|                         | squalene                               | 34.536 | 2745            |
|                         | beta-sitosterol                        | 36.167 | 3292            |
|                         | campesterol                            | 36.680 | 3297            |
|                         | stearic acid                           | 38.211 | 2163            |
|                         | dimethyl sulfoxide                     | 51.286 | 924             |
|                         | benzothiazole                          | 55.461 | 1184            |
| *Tuber brumale*         | 3-octanol                              | 20.452 | 1087            |
|                         | 1,2-butanediol                         | 21.968 | 811             |
|                         | lupeol                                 | 21.971 | 3265            |
|                         | 2,4-octanediene                        | 35.445 | 1082            |
|                         | tris(methylthio)methane                | 51.275 | 1364            |
|                         | ergosterol                             | 52.008 | 3085            |

2.1. Mass Spectrometry Analysis of *Tuber magnatum pico* and *Tuber brumale*

Truffle samples were diluted in methanol and characterized by ESI-TOF mass spectrometry (ESI-QTOF-MS). The spectra revealed a complex mixture of molecules from which a few molecules were detected. Thus, mass spectra analysis showed the presence of 103 compounds in *Tuber magnatum pico* and 105 compounds from the *Tuber brumale*. Major of these phytochemicals are fatty acids, fatty esters, and sterols. The truffles samples were carried out in positive mode.

About 54% of the identified compounds were detected in the *m/z* range from 50 to 180. Identified compounds are listed in Table 2 and classified on the base of their *m/z* ratio (both theoretical and measured), chemical name, molecular formula, and the related literature. In sample 2 (*T. brumale*) another six additional compounds were detected: dipropyl trisulfide (*m/z*: 183.40), bis (2-methyl-3 furyl) disulfide (*m/z*: 227.34), sinapine (*m/z*: 311.37), ergosta-5,7,22-trien-ß-ol (*m/z*: 397.61), ergosta-5,7,22-trien-ß-ol (*m/z*: 397.66), and brassicasterol (*m/z*: 399.69).

The spectra disclose a very complex mixture of molecules from which only some molecules were detected. A total of 109 identified metabolites were attributed to different chemical classes such as amino acids, saccharides, flavonoids, aldehyde, ketone, esters, sulfur compounds, terpenoids, phenolic acids, steroids, hydrocarbons, and other data confirming results already published in the literature [7,10,15,17,19–29,35–59]. The results of the GC-MS were confirmed by ESI-QTOF-MS analysis.
Table 2. Phytochemicals identified in both truffles sample detected by the MS method.

|Sample Fraction| Compound No. | m/z Detected| Theoretic m/z| Formula| Tentative of Identification| Ref. |
|---------------|--------------|--------------|--------------|--------|-----------------------------|-----|
|1              | 65.17        | 65.15        | C₇H₆S⁺        | dimethyl sulfide | [7,10,23,37]             |     |
|2              | 89.18        | 89.15        | C₉H₁₂O⁺       | isomyl alcohol   | [7,10]                     |     |
|3              | 89.14        | 89.12        | C₉H₁₇O₂⁺      | 3-hydroxy-2-butane | [38]        |     |
|4              | 90.11        | 90.097       | C₈H₁₄O⁺       | alanine          | [7,25,39]                  |     |
|5              | 95.15        | 95.14        | C₉H₁₅O₂⁺      | dimethyl sulfone | [10,37]                  |     |
|6              | 95.23        | 95.20        | C₈H₁₇S⁺       | dimethyl disulfide | [10,23]           |     |
|7              | 99.17        | 99.15        | C₈H₁₄O       | 1-hexen-3-one    | [38]                     |     |
|8              | 105.19       | 105.18       | C₈H₁₄O⁺       | methional        | [7,10,38]                 |     |
|9              | 107.17       | 107.13       | C₈H₁₄O⁺       | benzaldehyde     | [25,59]                   |     |
|10             | 107.20       | 107.19       | C₈H₁₄O⁺       | 3-(methylthio)propanol | [38,40]             |     |
|11             | 109.07       | 109.06       | C₈H₁₄O⁺       | methoxybenzene (anisole) | [10]         |     |
|12             | 109.25       | 109.24       | C₈H₁₅S⁺       | bis(methylthio)methane | [6,39]     |     |
|13             | 110.15       | 110.14       | C₈H₁₄NO⁺      | 4-amino-phenol   | [7,10,22]                 |     |
|14             | 117.17       | 117.16       | C₈H₁₅O₂⁺      | butanoic acid ethyl ester | [39,40]       |     |
|15             | 117.19       | 117.17       | C₈H₁₄O₂      | ethyl butyrate   | [40]                     |     |
|16             | 118.11       | 118.14       | C₈H₁₅NO₂⁺     | valine           | [7,25,39]                 |     |
|17             | 120.08       | 120.03       | C₈H₁₅NO₂⁺     | threonine        | [7,10,25,39]             |     |
|18             | 121.18       | 121.16       | C₈H₁₄O⁺       | benzeneacetaldehyde | [38,59]       |     |
|19             | 123.07       | 123.67       | C₈H₁₄O₂⁺      | 2-phenylethanol  | [10]                      |     |
|20             | 123.10       | 123.08       | C₈H₁₄O⁺       | p-cresyl methyl ether | [40]       |     |
|21             | 123.19       | 123.17       | C₈H₁₄O       | 3-ethylphenol    | [41]                      |     |
|22             | 125.16       | 125.15       | C₈H₁₅O₂⁺      | 2-acetyl-5-methyl furan | [10,23,25]       |     |
|23             | 125.27       | 125.24       | C₈H₁₅O⁺       | nonanal          | [10,59]                   |     |
|24             | 127.16       | 127.13       | C₈H₁₅O⁺       | 6-methyl-5-hepten-2-one | [38]       |     |
|25             | 127.23       | 127.21       | C₈H₁₄O⁺       | 3,4-dimethyl-3-hexen-2-one | [38]       |     |
|26             | 127.29       | 127.27       | C₈H₁₅S⁺       | dimethyl trisulfide | [3,38]         |     |
|27             | 129.21       | 129.18       | C₁₀H₁₄⁺       | napththalene     | [10]                      |     |
|28             | 129.25       | 129.22       | C₁₀H₁₄O⁺      | 1-octen-3-ol     | [3]                       |     |
|29             | 131.20       | 131.19       | C₈H₁₄O₂⁺      | butanoic acid propyl ester | [38]       |     |
|30             | 132.17       | 132.75       | C₁₀H₁₃N₂O₂⁺   | ornithine        | [7,10,25,38,39]           |     |
|31             | 132.19       | 132.18       | C₁₀H₁₃NO₂⁺    | leucine          | [7,10,25,38,39]           |     |
|32             | 133.08       | 133.06       | C₁₀H₁₄O⁺      | asparagine       | [7,10,25,38]             |     |
|33             | 135.25       | 135.23       | C₁₀H₁₃⁺       | p-cymene         | [25,37,38]               |     |
|34             | 136.20       | 136.19       | C₁₀H₁₄NO⁺    | benzothiazole    | [37,60]                   |     |
|35             | 137.22       | 137.20       | C₁₀H₁₄O      | 3-methyl-5-ethylphenol | [40,41,59]       |     |
|36             | 137.26       | 137.24       | C₁₀H₁₃⁺       | D-limonene       | [38]                      |     |
|37             | 137.27       | 137.25       | C₁₀H₁₄⁺       | cis-cisimene     | [38]                      |     |
|38             | 141.31       | 141.29       | C₁₀H₁₃S⁺      | methyl(methylthio)dimethyl sulfide | [3,38]       |     |
|39             | 143.23       | 143.21       | C₁₀H₁₄O₂⁺     | 2,4-octanedione  | [37]                      |     |
|40             | 145.22       | 145.21       | C₁₀H₁₄O₂⁺     | isobutyl hexanoate | [40]         |     |
|41             | 147.21       | 147.19       | C₁₀H₁₃O₂⁺     | lysine           | [7,10,25]                 |     |
|42             | 149.19       | 149.17       | C₁₀H₁₄O⁺      | cinnamic acid    | [38]                      |     |
|43             | 150.21       | 150.20       | C₁₀H₁₃NO₂⁺    | methionine       | [7,10,25]                 |     |
|44             | 151.23       | 151.22       | C₁₀H₁₄O⁺      | thymol           | [21]                      |     |
|45             | 155.27       | 155.25       | C₁₀H₁₃O⁺      | α-terpineol      | [38]                      |     |
|46             | 155.28       | 155.26       | C₁₀H₁₄O⁺      | eucalyptol       | [38]                      |     |
|47             | 155.35       | 155.32       | C₁₀H₁₃S⁺      | tris(methylthio)methane | [3,41]       |     |
|48             | 156.18       | 156.16       | C₁₀H₁₄NO₂⁺    | histidine        | [7,10,25,39]             |     |
| Sample Fraction | Compound No. | m/z Detected | Theoretic m/z | Formula | Tentative of Identification | Ref. |
|-----------------|-------------|--------------|---------------|---------|-----------------------------|-----|
| 48              | 157.25      | 157.23       | C_{14}H_{16}O_2+ | 2-pentyl-3-butenoic acid | [59] |
| 49              | 159.26      | 159.25       | C_{14}H_{16}O_2+ | 2-isopropyl-hexanoic acid | [41] |
| 50              | 165.19      | 165.17       | C_{14}H_{18}O_2+ | p-coumaric acid | [22] |
| 51              | 165.23      | 165.21       | C_{14}H_{16}O_2+ | eugenol | [38] |
| 52              | 169.18      | 169.16       | C_{14}H_{18}O_2+ | homogentisic acid | [22] |
| 53              | 169.31      | 169.29       | C_{14}H_{20}O_2+ | 2-methylisoborneol | [21] |
| 54              | 171.15      | 171.13       | C_{14}H_{18}O_2+ | gallic acid | [22,25] |
| 55              | 171.26      | 171.26       | C_{14}H_{20}O_2+ | 3-methyl-2-nonenioic acid | [38,60] |
| 56              | 171.34      | 171.34       | C_{14}H_{18}O_2+ | 2,4-dimethyl-decane | [38] |
| 57              | 173.11      | 173.13       | C_{14}H_{20}O_2+ | capric acid | [22,25,38] |
| 58              | 173.27      | 173.27       | C_{14}H_{20}O_2+ | isobutyl hexanoate | [40] |
| 59              | 177.14      | 177.13       | C_{14}H_{18}O_2+ | ascorbic acid | [22] |
| 60              | 179.28      | 179.24       | C_{14}H_{20}O_2+ | benzene-1,2-dimethoxy-4-(2-propenyl) | [39] |
| 61              | 181.19      | 181.17       | C_{14}H_{18}O_2+ | caffeic acid | [22,25] |
| 62              | 183.19      | 183.17       | C_{14}H_{18}O_2+ | D-alitrol | [51] |
| 63              | 187.24      | 187.22       | C_{14}H_{20}O_2+ | 2-naphthylacetic acid | [38] |
| 64              | 195.21      | 195.19       | C_{14}H_{20}O_2+ | ferulic acid | [7,10,25] |
| 65              | 203.35      | 203.35       | C_{14}H_{20}O_2+ | α-cubebene | [10,38] |
| 66              | 203.37      | 203.35       | C_{14}H_{20}O_2+ | caryophyllene | [10,39] |
| 67              | 203.38      | 203.36       | C_{14}H_{20}O_2+ | β-elemene | [10,38] |
| 68              | 217.33      | 217.32       | C_{14}H_{20}O_2+ | trisopropyl-S-trisane | [3,38] |
| 69              | 227.35      | 227.35       | C_{14}H_{20}O_2+ | 8-dodecyl acetate | [10,38] |
| 70              | 230.31      | 230.31       | C_{14}H_{20}O_2+ | 1-ergothioneine | [7] |
| 71              | 235.40      | 235.39       | C_{14}H_{20}O_2+ | sparteine | [7] |
| 72              | 239.35      | 239.34       | C_{14}H_{20}O_2+ | agroclavine | [7] |
| 73              | 241.33      | 241.31       | C_{14}H_{20}O_2+ | cystine | [7,10,39] |
| 74              | 255.42      | 255.42       | C_{14}H_{20}O_2+ | palmitoleic acid | [22,25] |
| 75              | 257.27      | 257.27       | C_{14}H_{20}O_2+ | palmitic acid | [22] |
| 76              | 272.43      | 272.43       | C_{14}H_{20}O_2+ | androstenone | [52] |
| 77              | 278.24      | 278.24       | C_{14}H_{20}O_2+ | neuraminic acid | [7] |
| 78              | 281.40      | 281.40       | C_{14}H_{20}O_2+ | linoleic acid | [22,25] |
| 79              | 281.45      | 281.45       | C_{14}H_{20}O_2+ | octadecadienoic acid | [22,25,38] |
| 80              | 283.50      | 283.50       | C_{14}H_{20}O_2+ | oleic acid | [22,25] |
| 81              | 289.45      | 289.45       | C_{14}H_{20}O_2+ | stearic acid | [22,25] |
| 82              | 291.11      | 291.09       | C_{14}H_{20}O_2+ | catechin | [21] |
| 83              | 298.28      | 298.28       | C_{14}H_{20}O_2+ | 7-methylguanosine | [7] |
| 84              | 300.29      | 300.29       | C_{14}H_{20}O_2+ | sphing-4-enzyme | [54] |
| 85              | 303.05      | 303.05       | C_{14}H_{20}O_2+ | eicosapentaenoic acid | [22] |
| 86              | 305.51      | 305.51       | C_{14}H_{20}O_2+ | arachidonic acid | [7,22] |
| 87              | 309.51      | 309.51       | C_{14}H_{20}O_2+ | ethyl linolate | [21,22] |
| 88              | 322.36      | 322.36       | C_{14}H_{20}O_2+ | S-methyl glutathione | [1] |
| 89              | 329.51      | 329.51       | C_{14}H_{20}O_2+ | docosahexaenoic acid | [22] |
| 90              | 341.35      | 341.34       | C_{14}H_{20}O_2+ | behenic acid | [22] |
| 91              | 343.31      | 343.31       | C_{14}H_{20}O_2+ | trehalose | [22] |
| 92              | 369.62      | 369.62       | C_{14}H_{20}O_2+ | lignoceric acid | [22,25] |
| 93              | 387.37      | 387.37       | C_{14}H_{20}O_2+ | cholesterol | [48,50,53,57–60] |
| 94              | 401.69      | 401.70       | C_{14}H_{20}O_2+ | campestanol | [48,50,53,57–60] |
| 95              | 411.72      | 411.72       | C_{14}H_{20}O_2+ | squalene | [7,23,45] |
| 96              | 413.70      | 413.70       | C_{14}H_{20}O_2+ | fucosterol | [48,50,53,57–60] |
| 97              | 415.73      | 415.73       | C_{14}H_{20}O_2+ | beta-sitosterol | [7,45] |
Table 2. Cont.

| Sample Fraction | Compound No. | m/z Detected | Theoretic m/z | Formula | Tentative of Identification | Ref. |
|-----------------|--------------|--------------|---------------|---------|----------------------------|------|
| 98              |              | 419.71       | 419.70        | C_{27}H_{46}O_{2} | cholest-5-en-3β,6,24-triol | [48,50,53,57-60] |
| 99              |              | 425.72       | 425.70        | C_{26}H_{44}O_{2} | luponene                  | [7,22,45] |
| 100             |              | 427.74       | 427.73        | C_{30}H_{44}O_{2} | lupeol                     | [7,22,45] |
| 101             |              | 537.92       | 537.91        | C_{48}H_{56}O_{2} | lycopene                   | [22] |
| 102             |              | 596.51       | 586.50        | C_{28}H_{30}O_{13}+ | kolaflavone               | [7] |
| 103             |              | 812.72       | 812.70        | C_{44}H_{66}NO_{3} | glucosylceramide           | [7,53,54] |

| 1               |              | 95.23        | 95.20         | C_{2}H_{5}S_{2}+ | dimethyl disulfide          | [3,7,10,23,37] |
| 2               |              | 99.17        | 99.15         | C_{8}H_{10}O     | 1-hexen-3-one               | [38] |
| 3               |              | 105.19       | 105.18        | C_{8}H_{14}O_{2} | methional                  | [7,10,39] |
| 4               |              | 107.17       | 107.13        | C_{10}H_{16}O_{2} | benzaldehyde                | [23,60] |
| 5               |              | 107.20       | 107.19        | C_{10}H_{16}O_{3} | 3-(methylthio)propanol       | [38,40] |
| 6               |              | 109.07       | 109.06        | C_{10}H_{18}O_{2} | methoxybenzene (anisole)     | [10] |
| 7               |              | 109.25       | 109.24        | C_{11}H_{16}S_{2}+ | bis(methylthio)methane       | [6,38] |
| 8               |              | 110.15       | 110.14        | C_{5}H_{9}NO     | 4-amino-phenol              | [7,10,22] |
| 9               |              | 117.17       | 117.16        | C_{12}H_{20}O_{2} | butanoic acid ethyl ester   | [38,41] |
| 10              |              | 117.19       | 117.17        | C_{10}H_{12}O_{2} | ethyl butyrate              | [40] |
| 11              |              | 118.11       | 118.14        | C_{12}H_{14}O_{2} | valine                      | [10,25,39] |
| 12              |              | 120.14       | 120.13        | C_{10}H_{14}NO_{2}+ | threonine               | [7,10,25,39] |
| 13              |              | 121.18       | 121.16        | C_{11}H_{16}O_{2} | benzeneacetaldehyde         | [38,59] |
| 14              |              | 123.07       | 123.67        | C_{12}H_{20}O_{2} | 2-phenylethanol             | [10] |
| 15              |              | 123.19       | 123.17        | C_{12}H_{20}O_{2} | 3-ethylphenol               | [41] |
| 16              |              | 123.10       | 123.08        | C_{12}H_{18}O_{2} | p-creosyl methyl ether      | [40] |
| 17              |              | 125.16       | 125.15        | C_{12}H_{20}O_{2} | 2-acetyl-5-methylfuran       | [10,23,25] |
| 18              |              | 125.27       | 125.24        | C_{12}H_{20}O_{2} | nonanal                     | [10,59] |
| 19              |              | 127.16       | 127.13        | C_{13}H_{22}O_{2} | 6-methyl-5-hepten-2-one      | [38] |
| 20              |              | 127.23       | 127.21        | C_{13}H_{22}O_{2}+ | 3,4-dimethyl-3-hexen-2-one | [38] |
| 21              |              | 127.29       | 127.27        | C_{13}H_{22}S_{2}+ | dimethyl trisulfide         | [10,23] |
| 22              |              | 129.21       | 129.18        | C_{13}H_{22}O_{2} | napthalene                  | [10] |
| 23              |              | 129.25       | 129.22        | C_{13}H_{22}O_{2} | 1-octen-3-ol                | [3] |
| 24              |              | 131.20       | 131.19        | C_{13}H_{22}O_{2}+ | butanoic acid propyl ester  | [38] |
| 25              |              | 132.17       | 132.75        | C_{12}H_{22}NO_{2}+ | ornithine               | [7,10,25,38,39] |
| 26              |              | 132.19       | 132.18        | C_{13}H_{24}NO_{2}+ | leucine                   | [7,10,25,38,39] |
| 27              |              | 133.08       | 133.06        | C_{14}H_{20}O_{4}+ | asparagine                 | [7,10,25,39] |
| 28              |              | 135.25       | 135.23        | C_{15}H_{24}O_{4}+ | p-cymene                   | [25,37,38] |
| 29              |              | 137.22       | 137.20        | C_{15}H_{24}O_{2}+ | 5-methyl-5-ethylphenol       | [40,41,59] |
| 30              |              | 137.26       | 137.24        | C_{14}H_{24}O_{4}+ | D-limonene                 | [38] |
| 31              |              | 137.27       | 137.25        | C_{15}H_{24}O_{4}+ | cis-cimene                 | [38] |
| 32              |              | 141.31       | 141.29        | C_{15}H_{25}S_{2}+ | (methyl(methylthio)dimethyl sulfoxide | [3,38] |
| 33              |              | 143.23       | 143.21        | C_{16}H_{24}O_{4}+ | 2,4-octanedione             | [37] |
| 34              |              | 145.22       | 145.21        | C_{16}H_{24}O_{2} | isobutyl hexanoate          | [40] |
| 35              |              | 147.21       | 147.19        | C_{16}H_{24}NO_{2}+ | lysine                     | [7,10,25] |
| 36              |              | 149.19       | 149.17        | C_{16}H_{24}O_{4}+ | cinnamic acid               | [39] |
| 37              |              | 150.21       | 150.21        | C_{16}H_{24}NO_{3}+ | methionine                 | [7,10,25] |
| 38              |              | 151.23       | 151.22        | C_{16}H_{24}O_{5}+ | thymol                     | [21] |
| 39              |              | 155.27       | 155.25        | C_{16}H_{24}O_{4}+ | α-terpineol                | [38] |
| 40              |              | 155.28       | 155.26        | C_{16}H_{24}O_{4}+ | eucalyptol                 | [38] |
| 41              |              | 155.35       | 155.32        | C_{16}H_{24}S_{2}+ | trimethyl(thio)methane       | [3,41] |
| 42              |              | 156.18       | 156.16        | C_{16}H_{24}NO_{4}+ | histidine                  | [7,10,25,39] |
| 43              |              | 157.25       | 157.23        | C_{16}H_{24}O_{5}+ | 2-pentyl-3-butenolic acid   | [39] |
| Sample Fraction | Compound No. | m/z Detected | Theoretic m/z | Formula | Tentative of Identification | Ref. |
|-----------------|--------------|--------------|--------------|---------|-----------------------------|------|
| 44              | 159.26       | 159.25       | C₂₃H₄₀O₂⁺   | 2-isopropyl-hexanoic acid   | [41] |
| 45              | 162.15       | 162.13       | C₁₉H₂₃NO₃⁺  | carnitine                       | [7]  |
| 46              | 165.19       | 165.17       | C₁₀H₁₇O₂⁺   | p-coumaric acid                | [22] |
| 47              | 165.23       | 165.21       | C₅₀H₃₀⁺    | eugenol                         | [38] |
| 48              | 169.18       | 169.16       | C₁₉H₂₂O₂⁺  | homogentisic acid              | [22] |
| 49              | 169.31       | 169.29       | C₁₁H₂₀O⁺   | 2-methylisoborneol             | [21] |
| 50              | 171.15       | 171.13       | C₁₀H₁₈O₂⁺  | gallic acid                     | [22,25] |
| 51              | 171.26       | 171.26       | C₁₀H₁₈O₂⁺  | 3-methyl-2-nonenenoic acid      | [38,59] |
| 52              | 171.36       | 171.34       | C₁₁H₂₀⁺   | 2,4-dimethyl-decane             | [38] |
| 53              | 173.11       | 173.15       | C₁₀H₂₁O₂⁺  | capric acid                     | [22,25,38] |
| 54              | 173.29       | 173.27       | C₁₀H₂₀O₂⁺  | isobutyI hexanoate              | [40] |
| 55              | 177.14       | 177.13       | C₁₀H₂₀O₂⁺  | ascorbic acid                   | [22] |
| 56              | 179.28       | 179.24       | C₁₁H₂₀O⁺   | benzene-1,2-dimethoxy-4-   (2-propenyl) | [38] |
| 57              | 181.19       | 181.17       | C₁₁H₂₂O⁺   | caffeic acid                    | [22,25] |
| 58              | 183.19       | 183.17       | C₁₀H₁₈O₂⁺  | D-alitoll                       | [51] |
| 59              | 183.40       | 183.38       | C₁₀H₂₁S₂⁺  | dipropyl trisulfide             | [10,23] |
| 60              | 187.24       | 187.22       | C₁₀H₂₀O₂⁺  | 2-naphthylacetic acid           | [38] |
| 61              | 195.21       | 195.19       | C₁₀H₂₂O₄⁺  | ferulic acid                    | [7,10,25] |
| 62              | 205.36       | 205.35       | C₁₁H₂₃⁺    | α-cubebeene                     | [10,38] |
| 63              | 205.37       | 205.35       | C₁₁H₂₃⁺    | caryophyllene                   | [7,38] |
| 64              | 205.38       | 205.36       | C₁₁H₂₃⁺    | β-elemene                       | [10,38] |
| 65              | 217.35       | 217.33       | C₁₂H₂₆O₂⁺  | trisopropyl-5-trioxane          | [5,38] |
| 66              | 227.34       | 227.30       | C₁₃H₁₆O₂S₂⁺| bis(2-methyl-5-(furylidisulfide) | [40] |
| 67              | 227.36       | 227.35       | C₁₃H₂₀O₂⁺  | 6-dodecyl acetate               | [10,38] |
| 68              | 230.32       | 230.31       | C₁₃H₁₄N₂O₅S⁺| L-ergothioneine               | [7]  |
| 69              | 235.40       | 235.39       | C₁₃H₁₆N₁⁺  | sparteine                       | [7]  |
| 70              | 239.35       | 239.34       | C₁₃H₁₈N₂   | agroclavine                     | [7]  |
| 71              | 241.03       | 241.31       | C₁₁H₂₁N₂O₅S₂⁺| cystine                        | [7,10,39] |
| 72              | 255.43       | 255.42       | C₁₃H₂₈O₂⁺  | palmitoleic acid                | [22,25] |
| 73              | 257.27       | 257.25       | C₁₄H₂₈O₂⁺  | palmitic acid                   | [22] |
| 74              | 273.45       | 272.43       | C₁₃H₂₇O    | androstenedione                 | [5]  |
| 75              | 278.25       | 278.24       | C₁₄H₂₂NO⁺  | neuraminic acid                 | [7]  |
| 76              | 281.41       | 281.40       | C₁₃H₂₆O₂⁺  | limoleic acid                   | [22,25] |
| 77              | 281.46       | 281.45       | C₁₃H₂₂O₂⁺  | octadecadienoic acid           | [22,25,38] |
| 78              | 283.51       | 283.50       | C₁₃H₂₆O₂⁺  | oleic acid                      | [22,25] |
| 79              | 289.47       | 289.45       | C₁₄H₂₈O₂⁺  | stearic acid                    | [22,25] |
| 80              | 291.11       | 291.09       | C₁₄H₂₈O₂⁺  | catechin                        | [21] |
| 81              | 298.30       | 298.28       | C₁₄H₂₉N₁O₃⁺| 7-methylguanosine               | [7]  |
| 82              | 300.27       | 300.29       | C₁₄H₂₉NO₂⁺  | sphing-4-ene                    | [56] |
| 83              | 303.06       | 303.05       | C₁₅H₂₉O₂⁺  | eicosapentaenoic acid           | [22] |
| 84              | 305.53       | 305.51       | C₁₅H₂₉O₂⁺  | arachidonic acid                | [7,22] |
| 85              | 309.53       | 309.51       | C₁₅H₂₉O₂⁺  | ethyl linolate                  | [21,22] |
| 86              | 311.37       | 311.36       | C₁₅H₂₉NO₃⁺| sinapine                        | [7]  |
| 87              | 322.38       | 322.36       | C₁₅H₂₉N₂O₃S| S-methyl glutathione            | [1]  |
| 88              | 329.52       | 329.51       | C₁₆H₂₉O₂⁺  | docosahexaenoic acid            | [22] |
| 89              | 341.35       | 341.34       | C₁₇H₂₉O₂⁺  | behenic acid                    | [22] |
| 90              | 343.32       | 343.31       | C₁₇H₂₉O₁⁺  | trehalose                       | [22] |
| 91              | 369.62       | 369.61       | C₁₅H₂₉O₂⁺  | lignoceric acid                 | [22,25] |
| 92              | 387.38       | 387.37       | C₁₇H₂₉O⁺   | cholesterol                     | [48,50,53,57-60] |
Table 2. Cont.

| Sample Fraction | Compound No. | m/z Detected | Theoretic m/z | Formula | Tentative of Identification | Ref. |
|-----------------|--------------|--------------|---------------|---------|-----------------------------|------|
| 93              | 397.61       | 397.60       | C_{28}H_{44}O | ergosta-5,7,22-trien-3-ol   | [48,50,53–60] |
| 94              | 397.66       | 397.65       | C_{28}H_{44}O | ergosterol          | [51,52,57–60]  |
| 95              | 399.69       | 399.67       | C_{28}H_{44}O | brassicasterol      | [7,45,48,50,53–60]|
| 96              | 401.71       | 401.69       | C_{28}H_{46}O | campestanol         | [7,45,48,50,53–60]|
| 97              | 411.74       | 411.72       | C_{28}H_{48}O+| squalene            | [7,23,45]   |
| 98              | 413.71       | 413.70       | C_{29}H_{46}O | fucosterol          | [46,50,53–60] |
| 99              | 415.73       | 415.71       | C_{29}H_{50}O | beta-sitosterol     | [7,45,48,50,53–60]|
| 100             | 419.71       | 419.70       | C_{29}H_{50}O | cholest-5-en-3β,6,24,25-tetrol | [46,50,53–60] |
| 101             | 425.72       | 425.70       | C_{30}H_{50}O+| lupeol              | [7,45]   |
| 102             | 427.74       | 427.73       | C_{30}H_{50}O | lupeol              | [7,45]   |
| 103             | 537.92       | 537.91       | C_{29}H_{50}O+| lycopene            | [22]   |
| 104             | 596.51       | 586.50       | C_{29}H_{50}O+| kolaflavanone       | [7]  |
| 105             | 812.72       | 812.70       | C_{46}H_{89}NO | glucosylceramide    | [1,7,74] |

The proportion of each metabolite categories distributed in two species truffles investigated was presented in the figures below. There is a distinction regarding the metabolite numbers accumulated in *T. brumale* (105), which was slightly larger than in *T. magnatum pico* (103). It was found that for *T. brumale*, the number of steroids and sulfur compounds was significantly higher than in *T. magnatum pico*. More amino acids were present in *T. magnatum pico* than *T. brumale*. In both truffle samples investigated, different amino acids were identified, and most of them are essential amino acids (valine, threonine, leucine, lysine, methionine) with few non-essential amino acids (ornithine, asparagine, cysteine) [7,25].

Previous studies revealed that each of these categories of metabolites identified in truffle samples exhibit biological activity [7,22–24,52]. For instance, sinapine, an alkaloid from *T. brumale*, possesses antioxidant and anti-inflammatory properties [7]. Aldehydes, alcohols, esters, and sulfur compounds are considered as responsible for the special truffle flavor [7,22,53,59]. Despite numerous studies, there is no complete description of the truffles’ very complex VOC assemble. Moreover, it is even more difficult to distinguish between each flavor component [7,10,38,40,45,53]. Some of them have been identified and presented in Table 3 [1,7,10,39,40,45]. In black truffles, such as *T. brumale*, the presence of sulfur compounds in large numbers is considered to be decisive for their specific aroma [1,7,10,39,40,45]. The environmental conditions lead to differences in the VOC profile between the same type of truffles harvested in different seasons.

Table 3. TOF-MS identified VOC odor compound in truffle samples.

| No. | VOC Name          | Odor          |
|-----|-------------------|---------------|
| 1   | dimethylsulfone   | sulfuric      |
| 2   | dimethylsulfide   | cabbage, sulfurous onion |
| 3   | dimethyl disulfide| cabbage, onion |
| 4   | methional         | mold, French fry, yeasty |
| 5   | isoamyl alcohol   | alcoholic, fruity |
| 6   | 3-hydroxy-2-butanoic acid | dairy, buttery |
| 7   | 1-hexen-3-one     | vegetable metallic |
| 8   | benzaldehyde      | sweet almond  |
| 9   | 3-(methylthio)propanol | onion, garlic |
| 10  | methoxybenzene (anisole) | anise seed |
Table 3. Cont.

| No. | VOC Name                          | Odor                        |
|-----|----------------------------------|-----------------------------|
| 11  | bis(methylthio)methane           | garlic, sulfurous, mushroom |
| 12  | 4-amino-phenol                   | sweet, balsamic             |
| 13  | butanoic acid ethyl ester        | sweet, fruity (apple)       |
| 14  | ethyl butyrate                   | fruity, sweet               |
| 15  | benzeneacetaldelyde              | earthy, chocolate, floral   |
| 16  | 2-phenylethanol                  | floral                      |
| 17  | p-cresyl methyl ether            | nutty, camphor              |
| 18  | 3-ethylphenol                    | phenolic                    |
| 19  | 2-acetyl-5-methylfuran           | nutty, dusty                |
| 20  | nonanal                          | citrus                      |
| 21  | 6-methyl-5-hepten-2-one          | citrus, green, nutty        |
| 22  | 3,4-dimethyl-3-hexen-2-one       | blue-cheese, nutty          |
| 23  | dimethyl trisulfide              | onion, leek                 |
| 24  | naphthalene                      | naphthalene                 |
| 25  | 1-octen-3-ol                     | earthy, green, mushroom     |
| 26  | butanoic acid propyl ester       | fruity, pineapple           |
| 27  | benzothiazole                    | sulfurous, nutty            |
| 28  | 3-methyl-5-ethylphenol           | fruity                      |
| 29  | methyl(methylthio)dimethyl sulfoxide | sulfurous, broccoli       |
| 30  | 2,4-octanodione                  | earthy, dill                |
| 31  | isobutyl hexanoate               | sweet, fruity               |
| 32  | tris(methylthio)methane          | earthy, mushroom            |
| 33  | carnitine                        | fishy                       |
| 34  | 2-methylisoborneol               | earthy, musty               |
| 35  | 3-methyl-2-nonenoic acid         | fruity                      |
| 36  | isobutyl hexanoate               | fruity, green               |
| 37  | benzene-1,2-dimethoxy-4-(2-propenyl) | spicy, woody              |
| 38  | dipropyl trisulfide              | sulfurous, garlic, pungent  |
| 39  | triisopropyl-S-trioxane          | dairy                       |
| 40  | bis(2-methyl-3 furyl)disulfide   | sulfurous, meaty            |
| 41  | 8-dodecenyl acetate              | fruity, pineapple           |
| 42  | androstenone                     | urine, sweet, floral        |
| 43  | S-methyl glutathione             | allium, sulfurous           |

Winter truffles have to develop more VOC molecules than white truffles, since the growing conditions are quite different between them [1,7,10,38,40,45]. Our results support this hypothesis. Among the winter truffles investigated, *T. brumale* contains more VOC molecules than white truffle, *T. magnatum pico*. Dipropyl trisulfide and bis (2-methyl-3 furyl) disulfide are the two sulfur compounds that have been identified only in our black truffle sample (*T. brumale*). More recently, truffles’, ergosteroid have been integrated into the VOC category with a characteristic sulfurous aroma [54]. Ergosta-5,7,22-trien-8-ol, ergosterol, and brassicasterol were tentatively identified by ESI-QTOF-MS in *T. brumale*. 
It should be mentioned that in both truffles, androstenone was identified, a steroidal pheromone with a distinct scent with various and completely different descriptions (floral, vanilla, sandalwood, sweaty, urine, or even without any odor [1,57]). It is estimated that due to the presence of this pheromone it is possible to train pigs or dogs to detect truffles [1,57]. The predominant sulfur compounds in white truffle aroma are dimethyl sulfide and bis(methylthio)methane and dimethyl sulfide in black truffle aroma [40]. Disulfides derivates has bacteriostatic and antifungal properties [43]. The phenolic compound 4-aminophenol has shown to have an anti-inflammatory role [7].

Fatty acids were found in both truffles samples and represent a significant proportion of the total metabolites identified. Research has demonstrated that fatty acids have antibacterial and antimicrobial activity, as well as hypocholesterolemic properties [1,23,42,45]. Although absolute contents are, percentage-wise, basically the same (12%), the composition of terpenoids is varied and consists of squalene, β-elemene, α-terpineol, p-cymene, D-limonene, eucalyptol, thymol, lupenone, α-cubebene, 2-methyl-isoborneol, and lupeol. These compounds act mainly as antibacterial and antioxidant agents [7,45]. Moreover, previous investigations revealed that squalene presents antibacterial, anticancer, antioxidant, tumoural protective, immunostimulant, and chemoprotective activity [23,45–47].

The steroid compounds found in truffles are involved in the mechanism of tumor protection and angiogenesis [7,23,26,46–50]. Furthermore, truffles contain stigmasterol and beta-sitosterol, compounds with similar chemical structures to cholesterol. Studies indicate that phytosterols act as hypercholesterolemic, immunomodulatory, and antitumor agents [52]. Recent studies report that ergosterol has shown antioxidant, anti-inflammatory, immunomodulating, and lowering hyperlipidemic effects [22,23,58,59].

The glycosylceramide identified in both truffles investigated is a sphingolipid type containing glucose residue [20,54]. This compound is highly bioactive with multiple roles in the organism: cell growth apoptosis, antitumor activity, and lowering cholesterol [20,54].

The flavor of the VOC metabolites identified in the investigated truffles is displayed in Table 3 and Figure 1. The key aroma of the investigated Romanian truffles is influenced by environmental conditions (soil parameters, tree host, etc.). Their fragrances are unique: medium sulfuric with sweet fruity, nutty, and floral notes [40].

![VOC flavor profile](image-url)
2.2. Screening and Classification of Metabolites

A total of 109 metabolites were assigned to different chemical categories: amino acids, saccharides, nucleoside, flavonoids, organic acids, phenols and alcohol, esters, sulfur compounds, terpenoids and sesquiterpenes, aldehyde and ketones, phenolic acids, fatty acids, hydrocarbons, vitamins, alkaloids, and other (Table 4).

Table 4. Classification of metabolites identified in truffles samples on chemical categories.

| Sample Fraction  | Chemical Class                  | Metabolite Name               |
|------------------|---------------------------------|-------------------------------|
| Tuber magnatum   | Amino acids                     | Alanine                       |
| pico             |                                 | Valine                        |
|                  |                                 | Threonine                     |
|                  |                                 | Ornithine                     |
|                  |                                 | Leucine                       |
|                  |                                 | Asparagine                    |
|                  |                                 | Lysine                        |
|                  |                                 | Methionine                    |
|                  |                                 | Histidine                     |
|                  |                                 | Cystine                       |
|                  | Saccharides and nucleoside      | Trehalose                     |
|                  |                                 | 7-Methylguanosine             |
|                  |                                 | Glucosylceramide              |
|                  | Flavonoids                      | Sparteine                     |
|                  |                                 | Agroclavine                   |
|                  |                                 | Kolaflavanone                 |
|                  | Organic acids                   | Cinnamic acid                 |
|                  |                                 | 2-Pentyl-3-Butenoic acid      |
|                  |                                 | 2-Isopropyl-Hexanoic acid     |
|                  |                                 | P-Coumaric acid               |
|                  |                                 | 3-Methyl-2-Nonenoic acid      |
|                  |                                 | Capric acid                   |
|                  |                                 | 2-Naphthylacetic acid         |
|                  |                                 | Neuraminic Acid               |
|                  |                                 | Homogentisic Acid             |
|                  | Phenols and alcohols            | 4-Amino-phenol                |
|                  |                                 | Isoamyl Alcohol               |
|                  |                                 | D-Allitol                     |
|                  |                                 | 2-Phenylethanol               |
|                  |                                 | 3-Ethylphenol                 |
|                  |                                 | 1-Octen-3-ol                  |
|                  |                                 | 3-Methyl-5-Ethylphenol        |
|                  | Esters                          | Butanoic Acid Ethyl Ester     |
|                  |                                 | Butanoic Acid Propyl Ester    |
|                  |                                 | Ethyl Butyrate                |
|                  |                                 | 8-Dodecenyl Acetate           |
Table 4. Cont.

| Sample Fraction | Chemical Class | Metabolite Name |
|-----------------|----------------|-----------------|
| Tuber magnatum | Sulfur compounds | dimethylsulfide |
| pico           |                 | dimethylsulfone |
|                |                 | dimethyl disulfide |
|                |                 | methional |
|                |                 | bis(methylthio)methane |
|                |                 | methyl(methylthio)dimethyl sulfoxide |
|                |                 | 3-(methylthio)propanol |
|                |                 | tris(methylthio)methane |
|                |                 | trisopropyl-S-trioxane |
|                |                 | L-ergothioneine |
|                |                 | S-methyl glutathione |
|                |                 | dimethyl trisulfide |
|                |                 | benzothiazole |
|                | Terpenoids and sesquiterpenes | p-cymene |
|                |                 | α-terpineol |
|                |                 | D-limonene |
|                |                 | cis-ocimene |
|                |                 | thymol |
|                |                 | eucalyptol |
|                |                 | 2-methylisoborneol |
|                |                 | α-cubebene |
|                |                 | caryophyllene |
|                |                 | β-elemene |
|                |                 | squalene |
|                |                 | lupenone |
|                |                 | lupeol |
|                | Aldehyde and ketone | benzaldehyde |
|                |                 | 3-hydroxy-2-butanone |
|                |                 | benzeneacetaldehyde |
|                |                 | nonanal |
|                |                 | 1-Hexen-3-one |
|                |                 | 6-methyl-5-hepten-2-one |
|                |                 | 3,4-dimethyl-3-hexen-2-one |
|                |                 | 2,4-octanedione |
|                | Phenolic acids | ferulic acid |
|                |                 | gallic acid |
|                |                 | caffeic acid |
|                |                 | catechin |
Table 4. Cont.

| Sample Fraction | Chemical Class | Metabolite Name |
|-----------------|----------------|-----------------|
| Tuber magnatum  | Fatty acids     | palmitoleic acid|
| pico            |                 | palmitic acid   |
|                 |                 | linoleic acid   |
|                 | Sterol and steroids | cholesterol   |
|                 |                 | campestanol     |
|                 |                 | fucosterol      |
|                 |                 | beta-sitosterol |
|                 | Hydrocarbons    | 2,4-dimethyl-decane |
|                 |                 | 2-acetyl-5-methylfuran |
|                 | Other           | sphing-4-enine (ceramide) |
|                 |                 | isobutyl hexanoate (fatty acid esters) |
|                 |                 | ascorbic acid (vitamin) |
|                 |                 | lycopene (carotenoid) |
|                 |                 | benzene-1,2-dimethoxy-4-(2-propenyl) |
|                 |                 | p-resyl methyl ether |
|                 |                 | methoxybenzene (anisole) |
| Tuber brumale   | Amino acids     | valine          |
|                 |                 | threonine       |
|                 |                 | ornithine       |
|                 |                 | leucine         |
|                 |                 | asparagine      |
|                 |                 | lysine          |
|                 |                 | methionine      |
|                 | Saccharides and nucleoside | 7-methylguanosine |
|                 |                 | glucosylceramide |
Table 4. Cont.

| Sample Fraction | Chemical Class | Metabolite Name |
|-----------------|----------------|-----------------|
| Tuber brumale   | Flavonoids     | sparteine       |
|                 |                | agroclavine     |
|                 |                | kolaflavanone   |
|                 | Organic acids  | cinnamic acid   |
|                 |                | p-coumaric acid |
|                 |                | 3-methyl-2-nonenonic acid |
|                 |                | capric acid     |
|                 |                | 2-naphthylacetic acid |
|                 |                | neuraminic acid |
|                 |                | homogentisic acid |
|                 |                | 2-pentyl-3-butenoic acid |
|                 |                | 2-isopropyl-hexanoic acid |
|                 | Phenols and alcohols | 4-amino-phenol |
|                 |                | 3-ethylphenol   |
|                 |                | 1-octen-3-ol    |
|                 |                | 3-methyl-5-ethylphenol |
|                 | Esters         | 2-phenylethanol |
|                 |                | D-allitol       |
|                 |                | butanoic acid ethyl ester |
|                 |                | butanoic acid propyl ester |
|                 |                | ethyl butyrate  |
|                 |                | 8-dodecenyl acetate |
|                 | Sulfur compounds | dimethyl trisulfide |
|                 |                | benzothiazole   |
|                 |                | methional       |
|                 |                | bis(methylthio)methane |
|                 |                | methyl(methylthio)dimethyl sulfoxide |
|                 |                | 3-(methylthio)propanol |
|                 |                | tris(methylthio)methane |
|                 |                | triisopropyl-S-trioxane |
|                 |                | L-ergothioneine  |
|                 |                | S-methyl glutathione |
|                 |                | dipropyl trisulfide |
|                 |                | bis(2-methyl-3 furyl)disulfide |
| Sample Fraction | Chemical Class     | Metabolite Name                      |
|-----------------|--------------------|--------------------------------------|
|                 | Terpenoids and sesquiterpenes |                                      |
| Tuber brumale   | 2-methylisoborneol  |                                      |
|                 | α-cubebene         |                                      |
|                 | Caryophyllene      |                                      |
|                 | β-elemene          |                                      |
|                 | Squalene           |                                      |
|                 | Lupenone           |                                      |
|                 | Lupeol             |                                      |
|                 | Benzaldehyde       |                                      |
|                 | 3-hydroxy-2-butanone|                                      |
|                 | Benzeneacetaldehyde|                                      |
|                 | Nonanal            |                                      |
|                 | 1-Hexen-3-one      |                                      |
|                 | 6-methyl-5-hepten-2-one|                                  |
|                 | 3,4-dimethyl-3-hexen-2-one|                               |
|                 | 2,4-octanedione    |                                      |
|                 | Gallic acid        |                                      |
|                 | Ferulic acid       |                                      |
|                 | Caffeic acid       |                                      |
|                 | Catechin           |                                      |
|                 | 2,4-dimethyl-decane|                                      |
|                 | 2-acetyl-5-methylfuran|                                |
|                 | Naphthalene        |                                      |
|                 | P-cymene           |                                      |
|                 | Eugenol            |                                      |
|                 | Palmitoleic acid   |                                      |
|                 | Palmitic acid      |                                      |
|                 | Linoleic acid      |                                      |
|                 | Octadecadienoic acid|                                  |
|                 | Oleic acid         |                                      |
|                 | Stearic acid       |                                      |
|                 | Eicosapentaenoic acid|                                 |
|                 | Arachidonic acid   |                                      |
|                 | Ethyl linolate     |                                      |
|                 | Docosahexaenoic acid|                                 |
|                 | Behenic acid       |                                      |
|                 | Lignoceric acid    |                                      |
The data analysis reported in Table 4 allowed obtaining charts for *T. magnatum pico* and *T. brumale*, which are presented in Figures 2 and 3.

![Figure 2](image-url)
3. Materials and Methods

Fresh fruiting bodies of *Tuber magnatum pico* (50 g) and *Tuber brumale* (50 g) were collected in late November 2019 from the area of the Eastern Carpathians and offered by Cromatec Plus after prior taxonomically and authentication. The truffles samples were rapid frozen in liquid nitrogen (−196 °C), ground and sieved to obtain a particle size lower than 0.5 mm, and kept at −80 °C to avoid enzymatic conversion or metabolites degradation.

For each analysis, 2 g of dried sample was subject to sonication extraction in 25 mL solvent (methanol/dichloromethane = 1:1) for 20 min at 45 °C, with a frequency of 50 kHz. The solution was concentrated using a rotavapor and the residue was dissolved in MeOH. The extract was centrifuged and the supernatant was filtered through a 0.2-μm syringe filter and stored at −18 °C until analysis.

3.1. Reagents

All used reagents were GC grade. Methanol and dichloromethane were purchased from VWR (Wien, Austria).

3.2. GC-MS Analysis

Gas chromatography was carried on the ClarusSQ8 GC/MS (PerkinElmer) apparatus with a nonpolar column Agilent 1909 s-433 (5% phenyl methyl siloxane); carrier gas, He, flow rate, 1 mL/min.

3.3. GC-MS Separation Conditions

The oven temperature program was 80 °C for 9 min, then raised to 220 °C (5 °C/min), to 280 °C (10 °C/min.), and finally held at this temperature for 20 min. The temperature of the injector was 260 °C and the temperature at the interface was 200 °C.

3.4. Mass Spectrometry

MS experiments were conducted on an EIS-QTOF-MS analysis from Bruker Daltonics, Billerica, MA, USA. All mass spectra were acquired in the positive ion mode within a mass range of (100–2500) m/z, with a scan speed of 2.1 scans/second. The source block
temperature was kept at 80 °C. The reference provided in positive ion mode a spectrum with fair ionic coverage of the m/z range scanned in full-scan MS. The resulting spectrum is a sum of scans over the total ion current (TIC) acquired at 25–85 eV collision energy to provide the full set of diagnostic fragment ions.

Peak assignment to specific ion was based on the standard library, the NIST/NBS-3 (National Institute of Standards and Technology/National Bureau of Standards) spectral database. According to the peak, the resolution area was determined from the total ion current (TIC) or from the estimated selected ions integration. The results are presented in Table 1. The mass spectra of the compounds were compared with those from NIST/EPA/NIH Mass Spectral Library, and the identified compounds are presented in Table 2.

4. Conclusions

The proposed analytical methodology for the chemical screening of these Romanian truffles type allowed obtaining their metabolite profile. The number of metabolites (amino acids, steroids, and sulfur compounds) was different in both truffle species.

The different proportion of total metabolites identified between 

\textit{T. brumale} and 

\textit{T. magnatum pico} can be considered as evidence of the influence exerted by genetic and environmental conditions. Each of the chemical categories were detailed, including their biological activity. Moreover, we evaluated the profile of the key aroma compounds. However, studies on Romanian truffles are in the early stages considering that these fungi are still an unvalued source of compounds with high economic value. Further investigations are necessary to disclose the influence of the external factors (environmental condition, host tree, etc.) on the metabolic mechanism of truffles.

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