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First Steps in Developing a Fast, Cheap, and Reliable Method to Distinguish Wild Mushroom and Truffle Species

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Abstract: Wild mushrooms and truffles (MT) are important resources, which can contribute to the socioeconomic sustainability of forestry ecosystems. However, not all wild MT are edible. Fast, cheap, and reliable methods that distinguish wild MT species (including the deadly ones) can contribute to valuing these important forest resources. Here, we tested if wild MT species, and their edibility, could be distinguished based on their aroma profiles (i.e., smellprints). For that, we combined the use of the electronic nose with classification models (linear discriminant analysis (LDA) and partial least squares discriminant analysis (PLS-DA)) to distinguish between 14 wild MT species (including edible and non-edible species) collected in Portugal. The 14 wild MT species could be accurately distinguished using LDA (93% accuracy), while the edible and non-edible species could be accurately distinguished using both LDA and PLS-DA (97% and 99% accuracy, respectively). Keeping in mind that our methodological design's feasibility was verified using a small sample, the data show the potential of the combined use of the electronic nose with discriminant analysis to distinguish wild MT species and their edibility based on their aromatic profile. Although a larger dataset will be necessary to develop a quick and reliable identification method, it shows potential to be as accurate as the identification performed by mycologists and molecular biology, yet requiring less technical training, and the analyses are cheaper and faster.

Keywords: electronic nose; forest resources; identification method; volatile profile; wild mushrooms and truffles

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1. Introduction

Approximately 148,000 species of fungi have been identified so far. However, it is believed that more than 90% of the fungal species remain unknown, and the total number of fungal species worldwide could reach 2.2 to 3.8 million species [1,2]. Mushrooms and truffles (MT) are the better known fungal species [2]. Mushrooms and truffles both consist of the fruiting body of macrofungi, but mushrooms fruit aboveground while truffles fruit belowground [3]. Indeed, for millennia, humans have included wild MT in their diets, medicinal practices, and ceremonial traditions [4]. In the contemporary era, these natural wonders have evolved into non-timber forest commodities, embodying a vast genetic reservoir that holds profound ecological, sociocultural, economic, medicinal, and biotechnological importance worldwide [5–8]. In the last decades, we have seen a growing interest in MT for their rich composition and bioactive compounds, which make them a great resource of exciting ingredients for food and nutraceuticals [9–13].

However, not all MT species are safe for human consumption. From the 14,000 MT species identified so far [10], Li and colleagues [14] reviewed 2786 MT species from 99 countries. From that list, most MT species were considered edible, i.e., 79% of the species were

identified as edible, and 72% were considered safe for human consumption. An additional 7% of those species required specific pretreatment measures before they could be considered safe or had been associated with allergic reactions in some instances. Furthermore, 17% of those species were categorized as of uncertain edibility due to a lack of conclusive evidence of human safe consumption, while 3% remained unconfirmed due to ongoing debates and differing opinions regarding their edibility and potential toxicity.

Although wild MT constitute a highly esteemed delicacy in specific parts of the world [15,16], they face great skepticism in other regions and, therefore, they are not valued as an important forest resource. The valorization of wild MT has the potential to promote the socioeconomic sustainability of rural communities and forests [17,18]. Supporting the sustainable harvesting and cultivation of these resources can generate income for local people, conserve biodiversity, mitigate climate change [15,19], and contribute to forest ecosystem processes and services [20]. Wild MT are versatile resources with potential applications in medicine, food, cosmetics, and recreation, from developing novel drugs and therapies, to the development of culinary delights and skincare products [9,21–24]. Their diverse properties offer opportunities for innovation across various industries [20], while highlighting the importance of responsible and ethical use.

Counteracting this skepticism about wild MT in some regions, society has recently and progressively developed a strong interest for wild MT hunting and consumption [25]. The downside of this growing interest in wild MT is the cases of poisoning, which often occur as a common outcome of enthusiastic wild MT gathering and consumption by mushroom enthusiasts that are not highly skilled (i.e., people with insufficient training on wild MT species identification) [26]. As an example, a retrospective study showed that around 94% of the reported mushroom poisoning cases resulted from the consumption of incorrectly identified wild mushrooms [27]. To avoid cases of poisoning, the safe trade of wild MT must rely on the implementation of guidelines and the enactment of legislation that ensures food safety [26]. To bridge knowledge gaps and contribute to wild MT species identification, new identification methodologies and technologies (please see below for some examples) have been developed, further enhancing our understanding and use of these valuable natural resources [28–30].

The traditional methods for identifying wild MT species include mainly morphological identification, instrumental analysis (e.g., gas chromatography), and molecular biology approaches [30]. However, developing technologies (e.g., image recognition [31,32], integrating machine learning [33–37]) have been largely applied for wild MT species identification in the last decade. With the globalization of the Internet of Things (IoT), methods to identify wild MT species from field-collection images using a smartphone application have been developed [28,29]. These smartphone identification applications hold promise to aid clinical toxicologists and the general public in accurately identifying wild MT species, but their low accuracy makes them insufficient to distinguish edible wild MT from potentially toxic ones [29]. Similar phenomena are occurring with artificial-intelligence-generated books for foraging and MT identification, which contain inaccurate information such as "taste and smell" as an identifying feature [38]. This could lead amateurs to the incorrect assumption that tasting is an identification method, which can ultimately result in wild MT poisoning. Therefore, it is important to always follow reliable sources, and use highly accurate tools that build greater trust on wild MT species identification.

One of the traits that plays a significant role in identifying wild MT is their odor. In accordance, most field guides for foraging and identifying wild MT include details about the odor alongside the macroscopic description of the flesh. For example, the odor of *Agaricus xanthodermus* is reported as phenolic, that of fresh *Clitocybe odora* has a strong anise component, and that of young *Amanita phalloides* is described as very faint [39]. Although the odor may offer valuable insights into identifying specific wild MT species, these odor descriptions are typically quite vague and generalized. Therefore, such odor identification cannot be regarded as definitive on its own when it comes to wild MT species [40,41]. Nevertheless, this specific trait (i.e., the odor) is beginning to be explored

in the identification of wild MT species using technologies such as the electronic nose (or simply e-nose) [30].

The e-nose is a sensitive device that can obtain information about odors—the smellprint. Slight changes in volatile compounds' odor, composition, or concentration can result in a different sensor response [42]. The e-nose has been used to assess the volatile profiles of mushrooms for several applications in the food industry [43,44], including quality control during postharvest processing [45–47], monitoring of the maturation process [48], and assessment of the shelf life and packaging [49,50]. Recently, several studies have investigated the e-nose's capacity to accurately distinguish different wild MT species by analyzing their volatile profiles [16,40,41,43,44,51,52]. While the findings obtained using the e-nose are promising, a wild MT species identification based on the e-nose requires further research to expand the datasets, standardize the methodology, and explore the integration of machine learning algorithms to maximize its potential in wild MT species identification. Although a few studies have recently focused on this specific question, they covered a small number of wild MT species and genera. Further research is needed to explore a wider range of wild MT species and genera, including those species with similar morphological features, which can easily lead to incomplete or incorrect wild MT species identification.

Therefore, the present study aims to further explore wild MT's odors as an identification trait by using a fast, cheap, and reliable methodology to analyze their aroma profiles. The feasibility of the methodological design we propose was verified using a small sample, and included: (i) capturing the aroma composition of 14 wild MT species using the e-nose Cyranose-320, and (ii) applying multivariate analysis techniques (principal component analysis (PCA), linear discriminant analysis (LDA), and partial least squares-discriminant analysis (PLS-DA)) to the wild MT aroma profiles to develop classification models able to distinguish the wild MT species, and between edible and non-edible species.

2. Materials and Methods

2.1. Wild Mushrooms and Truffles (MT)

Fourteen wild-growing MT species were collected during field trips in the south and center of Portugal between the autumn of 2022 and the spring of 2023. At least 2 individuals of each species were collected from each site, adding up to a total of 28 samples. After relevant notes of their morphological and ecological features were taken, individuals were placed in separate paper bags and brought to the laboratory in a cooler bag. Specimens were freed from substrate debris at the site and further cleaned in the laboratory. The wild MT samples were kept at 4 °C until analysis in the first 48 h postharvest.

Each wild MT species was identified by an experienced mycologist, based on morphological features, and using standard reference field identification books [53–56]. When the morphological features were not enough to make a precise identification of the species, the specimens were sent for molecular analysis to confirm their identification. The identification of six wild MT species was confirmed by molecular analysis. Genomic DNA was extracted from 100 mg of each fresh mushroom/truffle. DNA extraction was performed in microtubes with 200 µL of degradation corrosion (Proteinase K at 0.5 mg/mL in 100 mM of Tris-HCl pH 9.0) preheated to 60 °C. The mixture was kept in a dry bath at 60 °C overnight (about 16 h), and initially (in the first 30 min of incubation), they were strongly vortexed for periods of 30 s. DNA extraction was followed by amplification using the forward primer (ITS4 F) 5′ TCCTCCGCTTATTGATATGC and the reverse primer (ITS5 R) 5′ GGAAGTAAAAGTCGTAACAAGG. Polymerase chain reactions (PCR) were carried out in a final volume of 20 µL. The reaction mixture consisted of 10 µL of MyTaq Red Mix 2× (Bioline, Paris, France), 1 µL each of primers F and R (at 10 µM/each), 1 µL of DNA sample, and 7 µL of ultrapure water. The mixture was placed in a Tpersonal cycler (Whatman Biometra, Göttingen, Germany), programmed as follows: initial denaturation at 95 °C for 5 min, then 35 cycles, each consisting of three steps: denaturation at 95 °C for 10 s, annealing at 43 °C for 10 s, and elongation at 72 °C for 60 s, and then a final elongation at 72 °C for 7 min. Aliquots of the PCR reactions were resolved on 0.7% agarose gels stained

with ethidium bromide. PCR products were purified by the Zymoclean DNA kit (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions. Purified PCR products were sequenced in both directions at StabVida (Caparica, Portugal) using the primers previously cited. The obtained sequences were compared with the sequences available from the National Centre for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov (accessed on 20 June 2023) using the BLAST algorithm to identify our wild MT species.

After the identification, each wild MT species' edibility was attributed based on existing literature. Finally, a literature survey identified other wild MT species that could be morphologically confused with the species we collected.

2.2. Exploring Wild MT's Odors Using the E-Nose

A Cyranose-320 e-nose (Sensigent, Pasadena, CA, USA) was used to develop a non-destructive, fast, cheap, and reliable method to distinguish between the 14 wild MT species and their edibility. The portable electronic nose (e-nose) Cyranose-320 can rapidly detect and identify samples based on their aroma profile. It is equipped with a nanocomposite sensor array of 32 nanosensors, an internal air sampling pump, and advanced pattern recognition algorithms. The sensor array measures the responses of the nanosensors to the chemical vapors in the air. The pattern recognition algorithms then use these responses to create a "smellprint" of the sample, which is a unique signature that can be used to identify it [42]. Therefore, we specifically used this e-nose to collect volatile profile information of the specimens belonging to the 14 wild MT species and create a smellprint for each species.

Two fresh samples of each wild MT species were analyzed separately. Four grams of each sample were introduced in a 10 mL vial and were incubated for 1 h at room temperature (i.e., 24 °C). The Cyranose-320 was mounted on a tripod, which could be adjusted for inserting the e-nose needle into the vials for headspace reading. Five readings per sample were performed, adding to a total of ten readings per species. The e-nose was coupled to the computer, and PCNose+ software (Version 10.13, Sensigent, Pasadena, CA, USA) was used to set the list of parameter settings of the Cyranose-320 (see Table S1) and data acquisition of the smellprint.

2.3. Smellprints' Statistical Analyses

2.3.1. Distinguishing the 14 Wild MT Species

To compare each sensor's response between the 14 wild MT species, we used the Kolmogorov–Smirnov test, a nonparametric test. To evaluate the possibility to distinguish the 14 wild MT species based on their smellprints, a principal component analysis (PCA) was carried out. This helped to identify patterns in the data, and to compare the smellprints of the different species.

Furthermore, we performed a linear discriminant analysis (LDA) to classify the smellprints of the different wild MT for each species. The LDA found linear combinations of the features of a sample to classify them into one of several classes (i.e., the 14 wild MT species). Then, the LDA detected the directions in which the classes were most separated, i.e., the discriminant functions. Finally, each sample was classified (i.e., identified as a wild MT species) by the discriminant function that yielded the highest value. The LDA model performance was evaluated for its accuracy using the following equation:

$$\text{Accuracy}(\%) = \frac{\text{True positive}}{\text{Total}} \times 100 \quad (1)$$

All analyses were performed using the software Microsoft Excel 2019 and XLSTAT-Premium (Version 2021.4.1, Addinsoft, Inc., Brooklyn, NY, USA).

2.3.2. Distinguishing the 14 Wild MT Species According to Their Edibility

To evaluate the possibility of distinguishing edible and non-edible species by their smellprints, two classification models were performed, the LDA and the partial least squares discriminant analysis (PLS-DA). These classification methods were used to test

if the groups (edible and non-edible) to which the observations belong are distinct and to reveal the properties of these groups using the e-nose sensor responses as explanatory variables. The LDA was tested as previously described but here, the classes were edible and non-edible. In the PLS-DA, the edibility classification was the dependent variable, and the e-nose sensor data were the independent variables. PLS-DA is a classification method that can simultaneously perform dimensionality reduction and discriminant analysis. PLS-DA is more flexible than LDA because it can handle cases where the classes are not linearly separable. The model performance for the LDA and PLS-DA was evaluated for accuracy, as described above using Equation (1). Multivariate analyses were performed using the software Microsoft Excel 2019 and XLSTAT-Premium (Version 2021.4.1, Addinsoft, Inc., Brooklyn, NY, USA).

3. Results

3.1. Distinguishing the 14 Wild MT Species

Based on the analysis of the morphological features by our experienced mycologist, we undoubtedly identified the following eight wild mushroom species: *Cantharellus cibarius*, *Craterellus lutescens*, *Craterellus tubaeformis*, *Cyclocybe cylindracea*, *Hydnum repandum*, *Lactarius deliciosus*, *Pisolithus tinctorius*, and *Suillus collinitus* (Table 1). The six species whose identification was confirmed by molecular analysis were the wild mushrooms *Agaricus xanthodermus*, *Amanita phalloides*, *Amanita subparvipantherina*, *Hygrocybe helobia*, and *Lepista nuda*, and the wild truffle *Terfezia arenaria*. Most of the wild MT species we collected (9 out of 14) were edible, and included the wild truffle *Terfezia arenaria* and the wild mushrooms *Cantharellus cibarius*, *Craterellus lutescens*, *Craterellus tubaeformis*, *Cyclocybe cylindracea*, *Hydnum repandum*, *Lactarius deliciosus*, *Lepista nuda*, and *Suillus collinitus*. The non-edible wild mushroom species included *Agaricus xanthodermus*, *Amanita phalloides*, *Amanita subparvipantherina*, *Hygrocybe helobia*, and *Pisolithus tinctorius*. All wild MT species had been reported to be morphologically confused with species from the same genus (e.g., *Amanita subparvipantherina* can be morphologically confused with *Amanita citrina* and other *Amanita* spp.) or from other genera (e.g., *Amanita phalloides* can be morphologically confused with *Agaricus* spp., *Russula* spp., and *Thricoloma* spp.).

The Cyranose-320 e-nose was able to detect the wild MT smellprints. From the 14 wild MT species analyzed, the truffle *Terfezia arenaria* was the species that induced higher responses for most of the 32 sensors (Figure 1). The sensors' responses induced by *Terfezia arenaria* were different from those induced by the other 13 wild MT species, except for 2 sensors (S30 and S31; Table S1). Furthermore, four edible wild MT species (mushrooms *Craterellus lutescens*, *Lactarius deliciosus*, and *Lepista nuda*, and truffle *Terfezia arenaria*) induced sensor responses for the 32 sensors different from those induced by the non-edible and deadly mushroom *Amanita phalloides*. However, the sensors' responses to the other non-edible wild mushroom species could not be distinguished from those of the edible wild mushrooms.

Table 1. List of the wild mushrooms and truffle (MT) species that were collected for this study, with the corresponding class, edibility, location, and species with which they can be morphologically confused.

Class and Species	Identified by	Edibility	Location	Morphologically Confused with	Reference
Ascomycetes					
Terfezia arenaria (Moris) Trappe	Molecular biology (100%)	Edible	Alentejo, Crato village, in montado	*Terfezia leptoderma*; *Choiromyces gangliformis*, *Choiromyces meandriformis*	[53,54,57]
Basidiomycetes					
Agaricus xanthodermus Genev.	Molecular biology (100%)	Not edible	Lisboa, Quinta das Conchas, in urban garden	*Agaricus* spp.	[53,54,57]
Amanita phalloides (Vaill. ex Fr.) Link	Molecular biology (99.70%)	Not edible	Sintra, Parques de Sintra, in pine forest	*Agaricus* spp., *Russula* spp., *Thricoloma* spp.	[53,54,57]

Table 1. *Cont.*

Class and Species	Identified by	Edibility	Location	Morphologically Confused with	Reference
Amanita subparvipantherina Zhu L. Yang, Q. Cai & Y.Y. Cui	Molecular biology (100%)	Not edible	Leiria, Carreira village, in mixed wood	*Amanita citrina* *Amanita* spp.	[55,58]
Cantharellus cibarius Fr.	Mycologist	Edible	Azambuja, in montado	*Hygrophoropsis aurantiaca* *Omphalatus olearius*; *Omphalatus illudens*	[53,54,57]
Craterellus lutescens (Fr.) Fr.	Mycologist	Edible	Leiria, Carreira village, in mixed wood	*Craterellus tubaeformis*	[53,54,57]
Craterellus tubaeformis (Fr.) Quél.	Mycologist	Edible	Leiria, Carreira village, in mixed wood	*Craterellus lutescens*	[39,40,44]
Cyclocybe cylindracea (DC.) Vizzini & Angelini 2014	Mycologist	Edible	Lisboa, Quinta das Conchas, in urban garden	*Amanita* spp.	[53,54,57]
Hydnum repandum L.	Mycologist	Edible	Leiria, Carreira village, in mixed wood	*Hydnum rufescens*, *Cantharellus cibarius*	[39,40,44]
Hygrocybe helobia (Arnolds) Bon	Molecular biology (99.33%)	Not edible	Leiria, Carreira village, in mixed wood	*Hygrocybe* spp.	[54]
Lactarius deliciosus (L.) Gray	Mycologist	Edible	Leiria, Bajouca village, in mixed wood	*Lactarius* spp.	[53,54,57]
Lepista nuda (Bull.) Cooke	Molecular biology (100%)	Edible	Lisboa, Quinta das Conchas, in urban garden	*Lepista sordida*, *Cortinarius* spp.	[53,54,57]
Pisolithus tinctorius (Mont.) E. Fisch	Mycologist	Not edible	Leiria, Carreira village, in eucalyptus forest	*Pisolithus* spp.; *Scleroderma* spp.	[53,54]
Suillus collinitus (Fr.) Kuntze	Mycologist	Edible	Leiria, Bajouca village, in mixed wood	*Suillus* spp.; *Boletus* spp.	[53,54]

The first two components of the PCA (based on the response of the 32 sensors for each of the 14 wild MT species) explained 96% of the total variance (PC1 = 90.3% and PC2 = 5.3%) (Figure 2). Despite explaining most of the variance, the PCA model showed overlapping of the smellprints of most wild MT species, with only two species showing a clearly different smellprint: *Agaricus xanthodermus* and *Terfezia arenaria*. Finally, the clusters that were formed based on the wild MT species' smellprints included edible and non-edible species. Therefore, the PCA was not able to clearly distinguish the 14 wild MT species or their edibility.

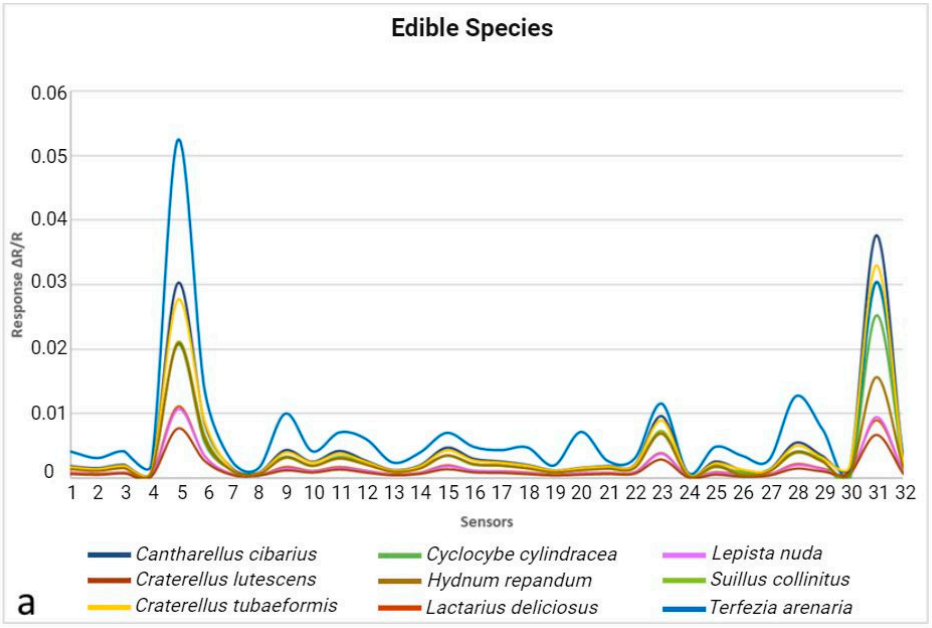

Figure 1. *Cont.*

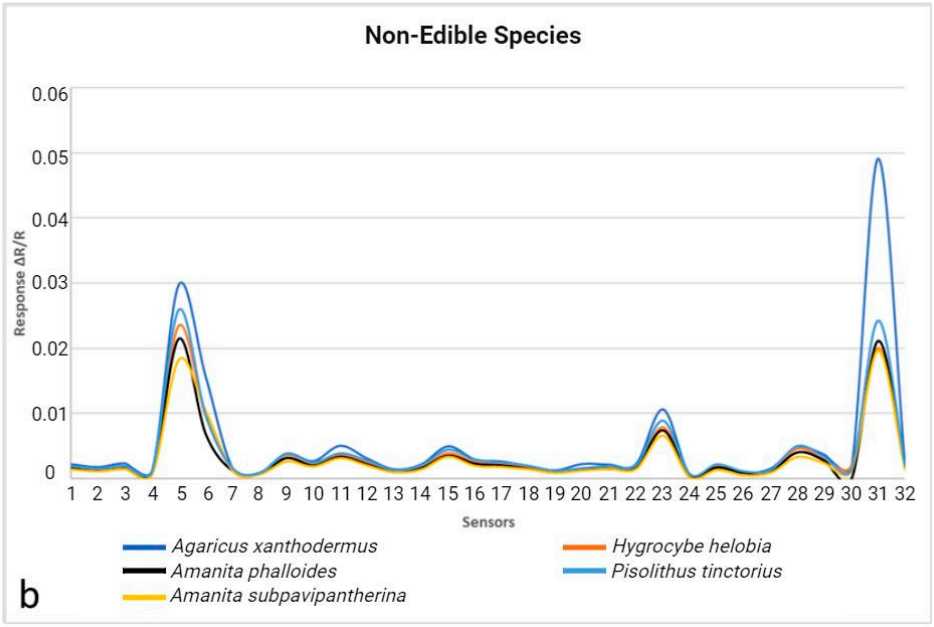

Figure 1. Cyranose-320 e-nose smellprints, integrating the responses of the 32 sensors for each of the 14 wild MT species. The wild MT species were grouped according to their edibility: edible (**a**) and non-edible (**b**) species. Each peak represents the response of a different sensor for each species. Each line represents the average response per species (n = 10 replicates). Created with BioRender.com.

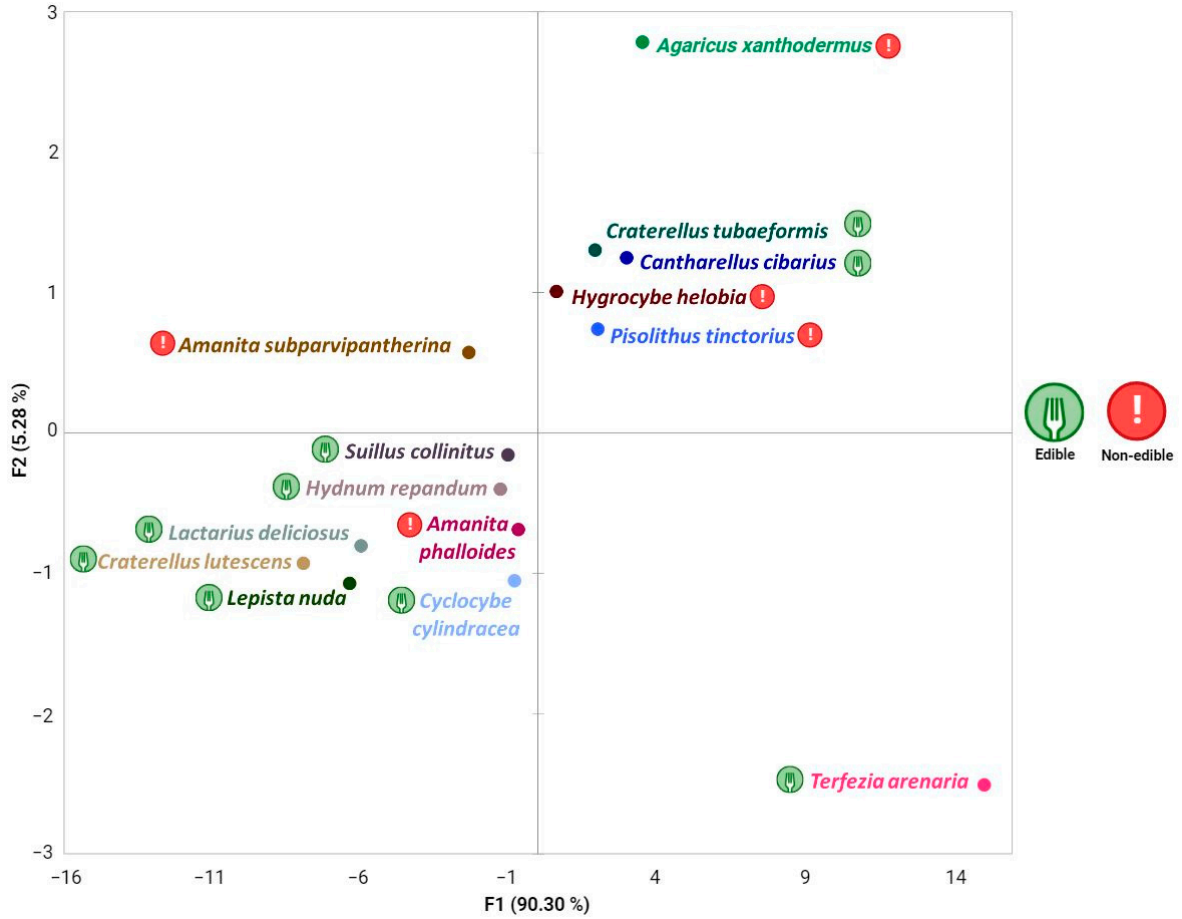

Figure 2. PCA of the smellprints, integrating the responses of the 32 sensors for each of the 14 wild MT species. The wild MT species are further classified as edible or non-edible. Symbols are the mean (n = 10) per species. Created with BioRender.com.

The LDA classification model presented an overall accuracy of 93% for distinguishing the 14 wild MT species. This overall accuracy level integrates the cases when species were always identified correctly, and those that were identified incorrectly. The LDA classification model correctly identified 9 out of the 14 species analyzed, with 100% accuracy (Figure 3). The species correctly identified by the LDA classification model (i.e., the species where all samples were correctly identified, with no incorrect identifications) included: (i) the edible *Cyclocybe cylindracea*, *Hydnum repandum*, *Suillus collinittus*, and *Terfezia arenaria*, and (ii) the non-edible *Agaricus xanthodermus*, *Amanita phalloides*, *Amanita subparvipantherina*, *Hygrocybe helobia*, and *Pisolithus tinctorius*. It is important to highlight that all non-edible species that we tested were correctly classified (i.e., 100% accuracy).

Figure 3. Overall species identification accuracy of the LDA classification model based on the e-nose smellprints of the 14 wild MT species. Created with BioRender.com.

The LDA classification model, on the other hand, showed misclassifications (i.e., incorrect identifications) for five wild edible mushroom species, with an accuracy of 70% to 90% (Figure 3). In a few samples, the LDA classification model misclassified *Craterellus tubaeformis* as *Cantharellus cibarius* (and vice versa), *Craterellus lutescens* as *Lepista nuda*, *Lactarius deliciosus* as *Craterellus lutescens*, and *Lacatrius deliciosus* as *Lepista nuda* (and vice versa). This can be related to the proximity of the smellprints of these species, which was also observed by the overlapping in the PCA (Figure 2). However, although *Amanita phalloides*, *Cyclocybe cylindracea*, *Hydnum repandum*, and *Suillus collinitus* were also close in the PCA, the LDA correctly identified these species.

3.2. Distinguishing the 14 Wild MT Species' Edibility

The confusion matrixes for the LDA and PLS-DA models for classifying (i.e., identifying) edible and non-edible species are shown in Figure 4. For both classification models (LDA and PLS-DA), we observed a very high percentage of correct identifications. Using the LDA classification model, we only observed five incorrect identifications of samples, thus reaching 97% accuracy. Using the PLS-DA classification model, we only observed one incorrect identification of samples, thus reaching 99% accuracy (Figure 4). Both classification models (LDA and PLS-DA) were highly accurate in distinguishing the smellprints of edible from those of the non-edible species.

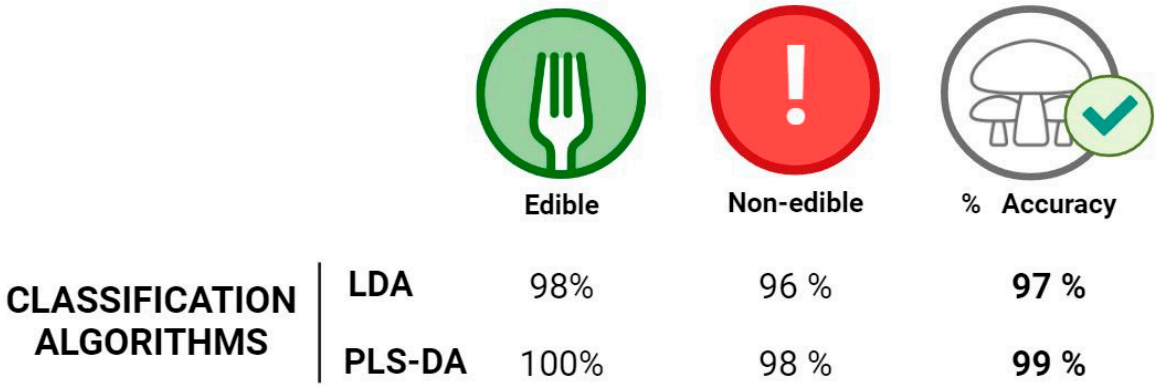

Figure 4. Edibility identification accuracy of the LDA and PLS-DA classification models based on the e-nose smellprints of the 14 wild MT species. Created with BioRender.com.

4. Discussion

By combining the use of the e-nose with discriminant analysis, we were able to distinguish 14 wild MT species, and their edibility (i.e., distinguish the edible from the non-edible species), using an accurate, fast, and cheap method. Our study used the highest number of wild MT species in similar studies so far, including wild MT species with similar morphological features, which can easily lead to incomplete or incorrect identifications.

4.1. Fast, Cheap, and Reliable Method to Distinguish Wild MT Species and Their Edibility

The smellprint of the truffle *Terfezia arenaria* stood out from those of the other wild mushrooms (Figures 1 and 2). Being a belowground fruiting fungus, *Terfezia arenaria*'s unique aroma plays an important ecological role by mediating this truffle's communication with below- and above-ground communities [59], including attracting animals that help disperse the truffle spores [60]. Despite the variability in the aromatic profile, this truffle species can have volatile organic compounds that act as a species-specific fingerprint [16,60], which can help explain its distinctive smellprint when compared to that of the wild mushrooms.

Similar phenomena can help explain why the smellprint of the non-edible and lethal wild mushroom *Amanita phalloides* was so different from that of the nine edible wild MT species of this study (Figure 3). While fifty *Amanita* spp. have been described as lethal worldwide [61], two *Amanita* spp. are considered delicacies and of economic interest in the Mediterranean region (*Amanita caesarea* and *Amanita ponderosa*) [62]. The use of the e-nose

for successfully distinguishing between *Amanita* mushrooms was demonstrated by Portalo-Calero and colleagues [40,41,51]. These studies obtained an accuracy of 97.7% to 99.9% using multivariate analysis for smellprint classification, and included two lethal species (*Amanita phalloides* and *Amanita verna*) and the two edible delicacies *Amanita caesarea* and *Amanita ponderosa* [41,51]. The use of electronic devices, such as the e-nose, that accurately distinguish between potentially dangerous wild MT and the safe ones can add an extra layer of safety to wild MT hunting. Improving wild MT's safe consumption can contribute to the socioeconomic sustainability of forest ecosystems since several of the wild edible MT studied here are highly appreciated and represent an important bio-resource of food and income for rural populations [63]. For example, *Cantharellus cibarius*, *Hydnum repandum*, and *Lactarius deliciosus* are among the wild edible mushroom species authorized for trade and commercialization in at least twelve European Union countries [15], and their international trade has increased in recent years [64].

In accordance with previous studies [43,44,65], the LDA classification model was able to accurately identify the 14 wild MT species by their smellprint (Figure 3), and both the LDA and PLS-DA classification models could accurately distinguish between the edible and non-edible species (Figure 4). These classification models have been largely used for statistical treatment of the volatile compounds of food matrices, and have been showing high accuracy [66–68]. For example, the combined use of the e-nose and PLS-DA contributed to the successful identification of filamentous fungal [67] and plant species [66], monitoring product quality during and after production processing [68–71], product quality [72], and establishing geographic origin [73]. Moreover, comprehensive datasets encompassing a wide range of wild MT species should be established to enhance the accuracy and robustness of the e-nose's identification capabilities. This would require the collection and analysis of smellprints of a diverse range of wild MT, including rare and lesser-studied species.

Since the incubation temperature has been shown to influence the compounds emitted by the MT samples [16], the fact that we used a fixed ambient temperature (24 °C) for incubating the wild MT samples must have contributed to our method's accuracy. However, when the temperature or humidity changes (i.e., when identifying the wild MT species in the forest), the e-nose response will drift, which may deteriorate the detection performance. Therefore, in future research, machine learning methods can be considered to design drift compensation methods to promote the intelligent process of e-noses [74,75]. By training algorithms with large datasets of smellprints and corresponding wild MT species, it may be possible to develop automated, real-time identification systems that can identify wild MT species accurately, quickly, and at low costs.

Althought our approach allowed us to accurately distinguish wild MT species and their edibility based on their smellprints, the number of samples we used was limited. The reduced number of wild MT species and specimens we analyzed reflected a reduction in the fruiting of the wild MT due to climate change. Specifically, these wild MT species require high soil water availability for producing their fruit bodies in arid and semi-arid areas (e.g., most of mainland Portugal) [76]. The severe droughts that affected mainland Portugal during the sampling period and previous years [77] negatively impacted the wild MT abundance. Furthermore, wildfires also resulted in a loss of productive forest areas and, consequently, a lower wild MT abundance. Despite our efforts to include more wild MT species and specimens, further research is needed to: (i) increase the number of species (including rare and lesser-studies ones) and specimens, (ii) account for the different phenological phases of the wild MT (e.g., maturation), (iii) assess the importance of geographic origin, and (iv) test product quality.

4.2. Perspectives for Wild MT Identification

One direct application of our approach (combined use of the e-nose and discriminat analysis) is to support accurate wild MT species identification, which can significantly enhance the identification accuracy of mycologists and wild MT enthusiasts. Traditional methods of wild MT identification typically involve time-consuming processes, such as

microscopic analysis and chemical reagent tests (Figure 5). Although professional and experienced collectors can avoid the harvest of hazardous and non-edible wild MT, the increasing number of amateurs collecting wild MT increases the risk of poisining [41]. Wild MT identification can include morphological features, instrumental analysis, and molecular biology methods [30] (Figure 5). Morphological identification is a long-established approach, based in fungal taxonomy, requires long training, and greatly benefits from experience. Wild MT species identification by a mycologist is critical to guarantee safe consumption. For example, in Switzerland, a free service is offered to the population to confirm the edibility of self-harvested wild mushrooms to promote the safe and sustainable harvesting of these forest resources [78]. Although the identification of wild MT species by experts (mycologists) is a highly accurate process (i.e., almost 100% accuracy) (Figure 5), we did not find any study demonstrating or quantifying it. On the other hand, there is a limited number of experts because this work's complexity makes it challenging to find people with a high level of knowledge in this area [79,80]

Sometimes, even expert identification needs support from other methodologies, such as molecular biology. In agreement, during this study, we faced difficulties identifying some species based on their morphological characteristics alone, namely *Agaricus xanthodermus* (could also be *Agaricus silvestris*), *Amanita subparvipantherina* (could also be *Amanita citrina*), and *Hydnum helobia* (we could only identify the genus, but not the species). Thus, we performed molecular identification of these species and another three as controls. Therefore, even experienced mycologists may need to use other methodologies to identify (or confirm) wild MT species.

Molecular biology is more expensive than mycologists' identification (see Figure 5) [81,82] and is mainly applied to fresh or dried fruiting bodies, primary processed products, and deeply processed products [30,83–85]. Molecular analysis is a precise method for identifying fungi (accuracy of almost 100%), but its performance can be affected by several factors. For example, (i) it is important to avoid cross-contamination during sample collection and processing [86], (ii) kit-based approaches are recommended to obtain good-quality DNA, but they can be expensive [86], and (iii) the choice of primer is essential for obtaining high-quality PCR products. The ITS region is the most commonly used primer for fungi, but it is not always suitable for all species. If the species of fungus is unknown, it can be difficult to choose the most appropriate ITS primer [80,87].

The three identification techniques (mycologist, molecular biology, and e-nose) have high accuracy but the e-nose stands out due to the ease of the process (simple and with minor material handling) and the short analysis time. On the other hand, both the mycologist and molecular biology require time-consuming expert training, some equipment, time, and costs for the analysis (especially in the case of molecular biology—Figure 5). Therefore, using an e-nose has the potential to become a fast, cheap, and reliable method for wild MT species identification because (i) it is a technique that is easy to learn and training can be completed in a few weeks, (ii) the previously trained e-nose allows fast identification within a few hours, (iii) once the e-nose is purchased, it only requires maintenance costs and, therefore, analysis will be cheap, and (iv) it accurately distinguishes wild MT species and their edibility (Figures 3 and 4). Therefore, this can be a valuable alternative or complementary approach, especially in cases where the morphological features of wild MT do not provide definitive results.

The potential impact of using an e-nose for wild MT identification extends beyond its immediate applications. A better understanding of the volatile profile—smellprint—of different wild MT species can contribute to the knowledge of their biology and ecology. Using tools such as the e-nose can lead to advancements in mycology, food security, and environmental monitoring while improving the socioeconomic sustainability of forest ecosystems. To validate the use of e-noses to identify wild MT and their edibility, it is essential to develop new methodologies and test new models. E-noses have the potential to add value to this forest bio-resource, highlighting the aromatic profile of wild MT that can be of interest to the food industry, and by certifying products and gaining consumers' trust.

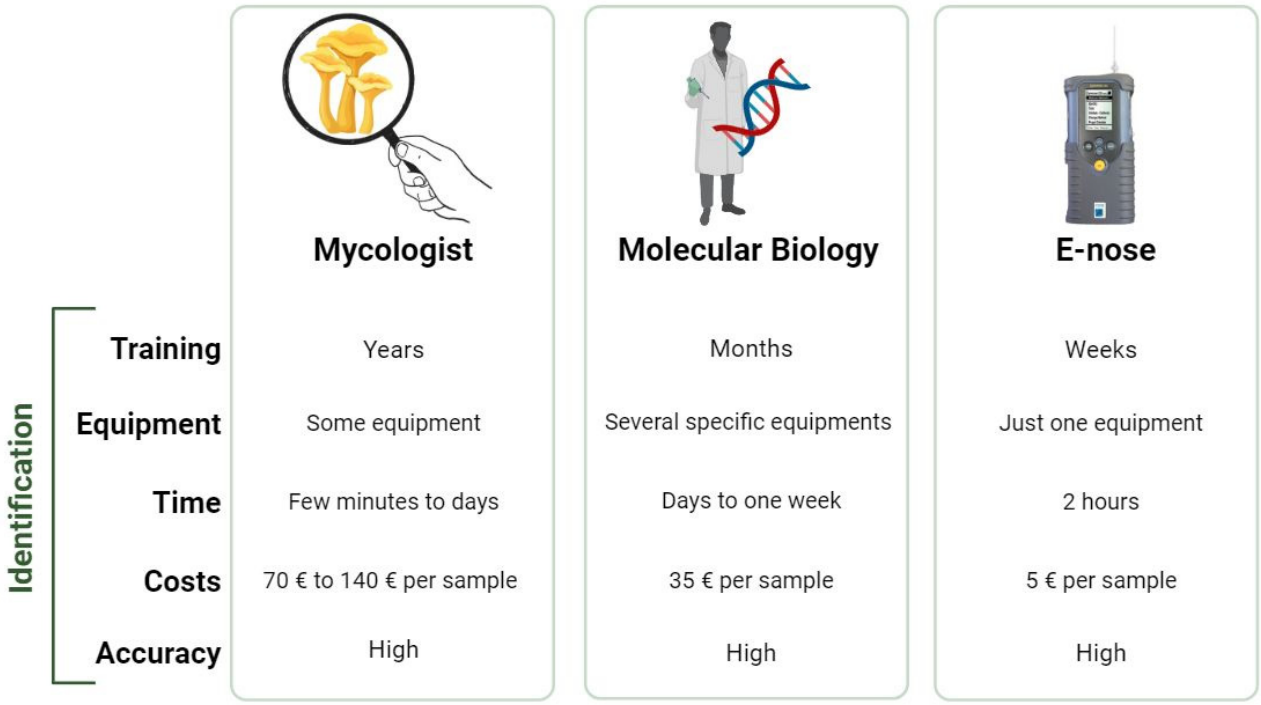

Figure 5. Comparison of methods to identify wild MT species based on their training duration, equipment, time, costs, and accuracy involved in the analysis [78–85,87] and this study. Created with BioRender.com.

5. Conclusions

The combined use of the e-nose and discriminant analysis accurately distinguished between 14 wild MT species and their edibility (i.e., between edible and non-edible species). These results suggest that the e-nose could be a valuable tool for wild MT species identification; for example, in cases when the morphological features of wild MT do not provide definitive results.

The e-nose proves to be a fast, cost-effective, and accurate method for wild MT identification, with potential applications in preventing mushroom poisoning. However, since this method is hampered by a small sample size, further research is needed to develop and validate e-nose-based identification methods for a broader range of wild MT species. The potential applications of the e-nose extend beyond identification, including assessing ripeness for harvest and detecting spoilage, suggesting its utility in improving efficiency in wild mushroom harvesting and processing. This study underscores the need for additional research to validate the e-nose's accuracy and highlights its potential in quality control for the food industry, ultimately enhancing the value of forest bio-resources and consumers' confidence in these commodities.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/resources12120139/s1, Table S1: Parameter setting of the Cyranose-320; Table S2: Comparison via the Kolmogorov–Smirnov test of the 32 sensors' responses between the 14 wild species of edible and non-edible mushrooms and truffles.

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