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Characterisation of *Tuber melanosporum* (Perigord Black Truffle) of French and Australian Origin Using Solid-Phase

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Abstract

In this study, a headspace, solid-phase microextraction, combined with gas chromatography mass spectrometry, was optimized to allow automated room temperature sampling of the aroma profile of *Tuber melanosporum* cultivated in France and Australia. A DVB/CAR/PDMS fibre with 10 minutes' exposure at room temperature was chosen as the most effective in extracting the highest amount of each of the esters, alcohols, and sulphides measured. The eight West Australian truffles studied showed large variations in the relative composition of the aroma compounds observed, however, the compounds present were similar to those found in the French grown truffle. Further standardization of the extraction and analysis methods are required determine whether the variation that occurs between aroma are environmentally (pre and post-harvest) or genetically (isolate or host/isolate specific) determined.

Keywords: Aroma; GC; SPME; Truffles; *Tuber*

Introduction

Truffles are the underground sporocarps (fruiting bodies) of ectomycorrhizal fungi that form through the association with roots of particular species of woody shrubs and trees (particularly oaks and hazels). Important economic species within this group belong to the genus *Tuber* and include European species such the Italian white truffle (*T. magnatum*) and the Perigord black truffle (*T. melanosporum*), and the Chinese species *T. indicum*. The European species are prized gourmet mushrooms that demand high prices and thus contribute to the livelihoods of those that collect them from natural habitats or grow them in plantations (truffieres). While it is not yet possible to grow the white truffle in plantations, the Perigord black truffle has been produced in truffieres for a number of years [1,2]. Despite this expansion in artificial cultivation, truffle production in Europe has been declining for many years from as high as 2000 t per year early in the 20th century to generally less than 150 t per year in the 21st century [1-3]. The reasons for this decline are unclear but it is probably due to habitat destruction or change, climate change, over exploitation [1,3,4] and possibly the introduction of disease and/or competition from other ectomycorrhizal fungi including *T. indicum* [5,6]. Recently, truffieres for the production of the Perigord black truffle have been established in the USA, New Zealand and Australia. The success of these truffieres has been variable, generally with only limited truffle production. However, their geographical distribution is providing valuable information about the environmental conditions required for truffle production and this is aiding in the selection of areas suitable for further establishment [3]. One truffiere in the south west of Western Australia, yielded over 30 kg per hectare 7 years after establishment with yields increasing annually [7].

The potential of these truffieres does not appear to have been fully realized and with further research and management are expected to produce higher yields. The production of these truffles outside of their natural range/habitat leads us to ask whether there are differences in the properties of these truffles; particularly their aroma profiles. Truffle aromas have been analysed using Gas Chromatography (GC) in association with a number of sampling techniques. Talou et al., [8,9] used dynamic headspace with Tenax trapping to sample the volatiles from French grown black truffles prior to GC analysis. Pacioni et al., [10] and Bellesia et al., [11,12]

used a similar approach to characterise the aroma of Italian grown truffles, while March et al., [13] used a pressure balanced headspace sampling technique for the analysis of truffles of French origin which included the black truffle. More recently, solid-phase microextraction (SPME) has been used to sample and concentrate the volatiles emitted by Spanish [14,15], Italian [16] and French [17] black truffles. This has been very successful in extracting a large number of volatiles when compared to the use of other techniques. However, the variety of extraction conditions employed for SPME to date make comparisons between studies difficult. For example, three different fibres have been employed to extract the analytes, sampling times and temperatures have ranged from room temperature for 10 minutes to 80°C for 30 minutes and both fresh and stored (frozen, or 4°C) material have been used.

In attempting to determine the aroma of *T. melanosporum* from published data, it becomes clear that there are three other factors which limit comparison. Firstly, the use of different sampling techniques (e.g. SPME-GC versus purge and trap-GC) is important. This is a particular issue when using SPME as, although it is more sensitive and extracts a greater number of analytes, it does not necessarily provide a representative sample. Secondly, the studies involve *T. melanosporum* grown in different geographical locations (Spain, Italy and France). The Italian grown truffle is well characterised in the literature using both purge and trap [11,12] and SPME GC techniques [16]. The Spanish grown truffles have also been analysed by SPME-GC [14,15], while Vernin et al., [18] used SPME-GC-MS to determine the aroma profile of French grown truffles. Thirdly, the condition of the truffle itself varies from study to study. In addition to the variation that can be expected from a natural product, the data presented in several papers are for truffles stored by a number of methods prior to analysis: frozen for several months [14,15], analysed within 24 hours of picking [16] and analysed after storage at 4°C for up to a month [19,20]. We were keen to compare the French grown black truffle with the Western Australian (WA) grown black truffle using SPME-GC. However, prior to any such characterisation of the WA truffle by SPME-GC, the French truffle also needed to be characterised using the same technique. This paper, therefore, describes: 1) the development of an optimised SPME-GC method for the analysis of truffle material using an automated sampling system at room temperature, 2) the aroma profile of the French black truffle and how it changes with storage at 4°C and 3) the first reported analysis of the aroma profile of WA grown black truffle.

Materials and Methods

Truffle Material

Certified fresh French and Spanish grown *T. melanosporum* was provided by the company Domaine D'Argens (Australian Quarantine and Inspection Service permit 2000113057). They were transported (3 days) at 4 oC and analysed within 24 h of receiving. Fresh Australian grown *T. melanosporum* was provided by the Wine and Truffle Company situated in Manjimup which is located in the South West of Western Australia (34o 16' S 116o 05' E). Truffles were harvested and inspected for truffle rot. The Australian truffles were randomly selected from the harvest and immediately transported (overnight on ice) to the laboratory. Standard Mixture of Key Truffle Aroma Compounds

A standard mixture of key aroma compounds known to be present in truffles was prepared. It consisted of: acetaldehyde, dimethylsulfide, 2-methylpropanal, 2-butanone, 2-methylpropanol, 3-methylbutanal,2-methylbutanal,3-methylbutanol,2-methylbutanol, hexanal, octen-3-ol, and benzaldehyde. Each component (50 µL) was dissolved in 10.0 mL of ethanol. This mixture was analysed each time the GC-MS was used to monitor any change in detector or fibre performance which would impact on a comparison of peak areas across different days.

Sampling Using Headspace Solid-phase Microextraction

Three SPME fibres supplied by Supelco were used for the study: a Polydimethylsiloxane (PDMS) with a 100 µm thick polymeric coating; a Polyacrylate (PA) fibre and a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre. They were conditioned following the manufacturer's instructions prior to use. The truffle material was ground to a fine powder using liquid nitrogen with a mortar and pestle and approximately 0.1 g was transferred to a 2 mL GC vial and sealed. Unless otherwise stated, the truffles and standard mixture were sampled in the following way: approximately 0.1 g of finely ground truffle or 10 µL of the standard mixture was transferred to a 2 mL GC vial. The fibre was exposed to the headspace for 10 minutes and then thermally desorbed in the GC injection port for 5 minutes. For elevated temperature work, the samples were heated and sampled at the elevated temperature.

Analysis by Gas Chromatography-mass Spectrometry

A Varian 3600 GC coupled to a Varian Saturn 2000 MS/MS mass spectrometer and a Varian auto-sampler was used for this study. An AT-waxms column (60 m x 0.25 mm i.d.) with a film thickness of 0.25 µm (supplied by Alltech Associates, Australia) and a VF-xms column (30 m x 0.25 mm i.d.) with a film thickness of 1.0 µm (supplied by Varian Associates, Australia) were used. The GC conditions were: injector 250°C; oven program 35°C for 2 minutes, then to 100°C at 3°C/min, then to 270°C at 20°C/min (held for 10 minutes); transfer line 270°C. The carrier gas was helium with a flow rate of 1 $mL.min^{-1}$.

Results and Discussion

Optimisation of a SPME-GC Method for Analyzing Truffle Aroma

Several SPME methods have been proposed for the extraction of truffle aroma. However, there is a high degree of inconsistency with respect to fibre type, extraction time and temperature, vial volume and sample size. Moreover, it would appear that few, if any, of the methods have been developed for automated sampling. In order to allow for automated sampling in this laboratory, 22°C (+/-2°C) sampling was necessary and a 2 mL vial volume (compatible with the auto-sampler carousel). The mass of truffle material was

limited to 0.1 g because of the small vial volume. Using 0.1 g portions of French black truffle in a 2 mL vial and sampling for 10 minutes at room temperature, three fibres were tested for their ability to extract the key volatiles, which were determined from the literature [14,16]. PDMS and a DVB/CAR/PDMS were tested as they have been the most commonly used fibres. The PA fibre was included because it has been successful in the extraction of polar analytes from other food products [21,22]. The DVB/CAR/PDMS fibre was superior in all respects; it extracted the maximum total peak area and also the highest amount for each class of compound i.e esters, aldehydes, alcohols and sulphides (Figure 1). This fibre was therefore used for the remainder of the study.

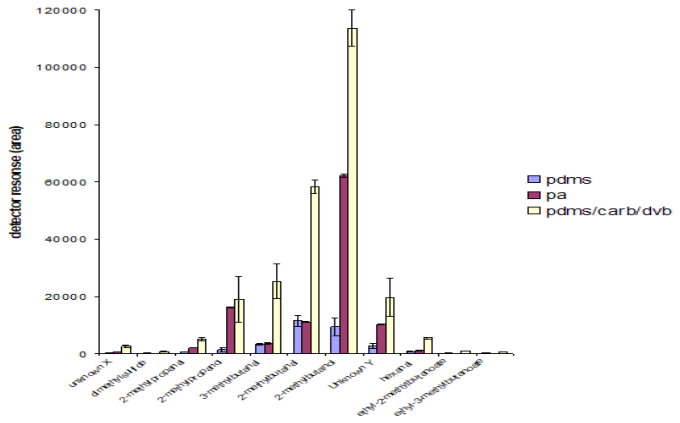


Figure 1: Comparison of the extraction efficiencies of the poly(dimethylsiloxane) (PDMS), poly(acrylate) (PA) and poly(divinylbenzene)/carboxen/poly (dimethylsiloxane) (DVB/CAR/PDMS) fibres using fresh black truffle.

The time that the sample was exposed to the fibre at room temperature was then studied. French truffle (analysed in triplicate) was sampled using absorption times of 5, 10, 15, 20 and 30 minutes. A plot of total peak area versus absorption time indicated that that absorption rate plateaued at 10 minutes. (Figure 2). This is similar to the results reported by Gioacchini et al., (2006) [23] for Italian black truffles analysed at 20°C. A 10-minute absorption time was chosen for the study.

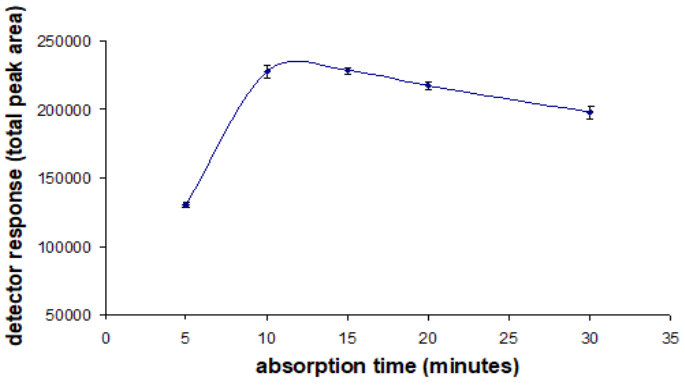


Figure 2: Effect of absorption time at room temperature on the extraction efficiency of the divinylbenzene/carboxen/poly(dimethylsiloxane) fibre using fresh black truffle.

As elevated extraction temperatures (as high as 80°C) were used in several studies [14,16,18] extraction at 25°C and 50°C was investigated to determine if a room temperature method severely compromised the extraction of volatiles. Western Australian black truffle was analysed in triplicate at the two temperatures and the results indicated that the extraction efficiency varied with the compound (Figure 3). Compounds that eluted early tended to be optimally extracted at room temperature, while elevated temperatures extracted the later eluting less volatiles analytes. However, extraction at room temperature was sufficient to extract all the analytes.

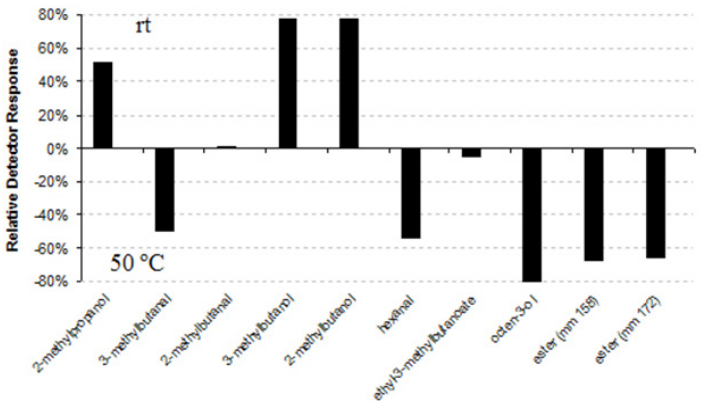


Figure 3: Effect of temperature on the extraction efficiency of the divinylbenzene/carboxen/poly(dimethylsiloxane) fibre using fresh black truffle.

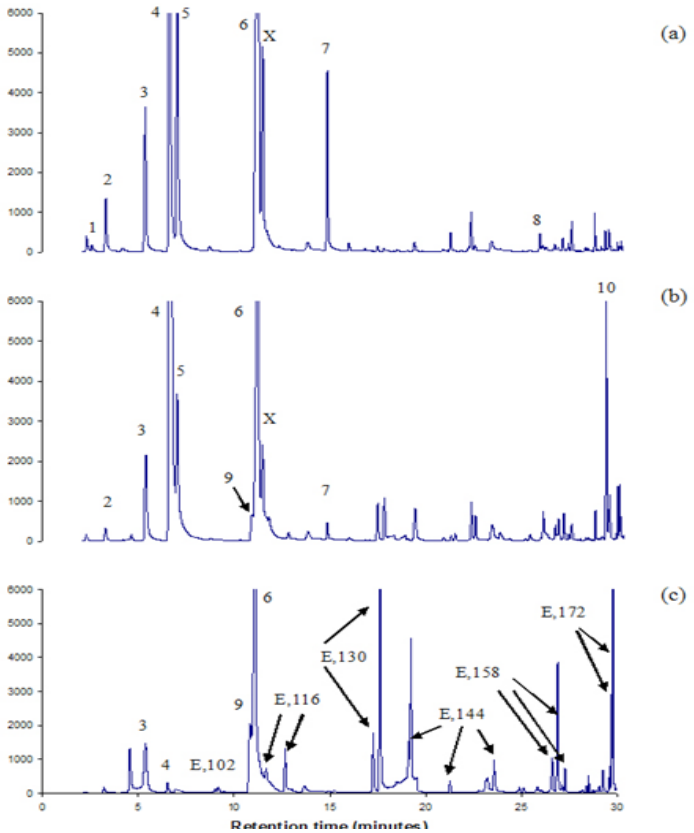
Two separation columns were evaluated for their ability to resolve the aroma compounds and, in particular, the early eluting alcohols and aldehydes. A 60 m polar column (AT-wax) and a non-polar column with a thick film (VF-Xms) were trialed. The latter provided resolution of all the key analytes and also resolved the key pairs, 3-methylbutanal and 2-methylbutanal and 2-methylbutanol and 3-methylbutanol. However, ethanol was not retained and when using MS detection it eluted prior to the filament being turned on. The polar column failed to resolve the aldehyde pair. The non-polar column was subsequently employed for this study because of its better resolving powers.

Characterisation of the French Grown Black Truffle

Three French grown black truffles certified fresh and stored at 4°C during transportation (duration of approximately 72 h) were purchased for this work. These truffles were analysed within 24 hours of arrival and using the optimised SPME method. The French grown truffles all exhibited the expected key compounds: 3-methylbutanal (21%), 2-methylbutanal (13 %), 2-methylbutanol (38%), 2-methyl-1-propanol (5%), and hexanal (5%). Dimethylsulfide and 2-methylpropanal were also present but at lower concentrations. Several minor alcohols were also identified, including octen-3-ol (Figure 4a).

To determine if storage impacts upon the percentage composition of the key volatiles the truffles were sampled after 7 and again after a further 14 days storage at 4 oC. After 7 days storage the profile (Figure 4b) did not change significantly: 3-methylbutanal, 2-methylbutanal and 2-methylbutanol still contributed to over 70% of the total peak area composition. However, dimethylsulfide was no longer detected, the contribution of hexanal and 3-methylpropanal was 1 % or less and the presence of the previously undetected 3-methylbutanol was evident. After further storage (14 days) some major changes in the aroma profile were evident (Figure 4c).

The contribution from 2-methylbutanal was significantly reduced while the contribution from 3-methylbutanal was maintained. Bellesia et al., [12], using purge and trap GC, analysed the Italian grown black truffle stored at 0 ˚C for a week and also found that the 2-methylbutanal concentration fell over time while the 3-methylbutanal concentration was maintained; the concentration of 3-methylbutanol in the French truffles also continued to be a major contributor to the aroma profile. Interestingly, March et al., [13] suggested that 3-methylbutanol could be used to characterise the French grown *T. miesentericum* truffle as it was not present in any of the other French truffles studied, which included *T. melanosporum*. However, our work indicates that it is present in French grown *T. melanosporum* especially when the truffle is stored at 4˚C for extended periods and, therefore, may potentially act as a marker for aging of the truffle. The esters ethyl-2-methylbutanoate and ethyl-3-methylbutanoate which eluted at 17.2 and 17.6 minutes respectively became more prominent with extended storage at 4˚C. The larger molecular weight esters such as propyl-3-methylbutanoate, 2-methylpropyl-3-methylbutanoate and 2-methylbutyl 2-methylbutanoate and their isomers, which have been reported for French grown truffles were not evident here [10,16,18]. However, analysis of the French truffles after storage at room temperature for just a week clearly showed the presence of these higher molecular weight esters (data not shown) suggesting that the presence of esters in truffle material may also serve as an indicator of freshness.



Figures 4(a-c): Gas chromatographic profile of the headspace components sampled by solid-phase microextraction from the fresh black truffle (a) initial ; (b) after a further 7 days storage at 4˚C and (c) after a further 14 days storage at 4˚C. Peak identification: 1 = dimethylsulfide, 2 = 2-methylpropanal, 3 = 2-methylpropanol, 4 = 3-methylbutanal, 5 = 2-methylbutanal, 6 = 2-methylbutanol, 7 = hexanal, 8 = octen-3-ol, 9 = 3-methylbutanol, 10 = ethyl-2-methylbutanoate, 11 = ethyl-3-methylbutanoate, X = unidentified compound.

Characterisation of the Western Australian Grown Black Truffle

Australian grown black truffles were harvested in June (2 truffles) and August (3 truffles) of one season and again in June/July (3 truffles) of the following season. These were stored at 4°C and analysed within 3 days of harvesting. (Figure 5) presents the aroma profile for three of these truffles when analysed by SPME-GC-MS. These three were chosen in order to highlight the variation observed. The key aroma compounds characteristic to truffles were all present, however, their relative concentrations varied substantially between individual truffles. The aldehyde 2-methylbutanal was the key contributor in five of the seven truffles analysed, its percentage peak contributing up to 67 % of the total peak area. Another aldehyde commonly found in black truffle, 3-methylbutanol, was only present in trace amounts. Of interest was the significant contribution by dimethylsulfide. Its contribution ranged from trace to over 30%, and for 3 truffles its contribution was in excess of 10%.

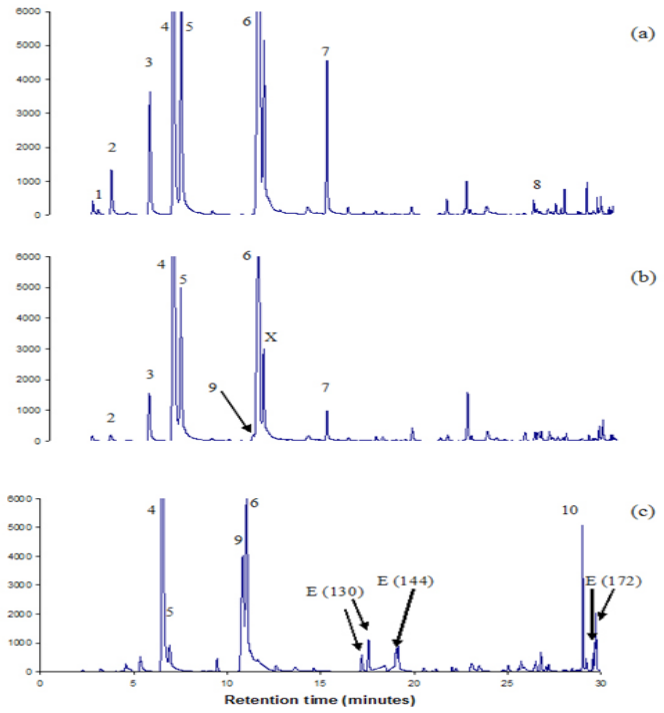


Figure 5: Gas chromatographic profile of the headspace components sampled by solid-phase microextraction for three fresh Western Australian grown black truffles Peak identification: 1 = dimethylsulfide, 2 = 2-methylpropanal, 3 = 2-methylpropanol, 4 = 3-methylbutanal, 5 = 2-methylbutanal, 6 = 2-methylbutanol, 7 = hexanal, 8 = octen-3-ol, 9 = 3-methylbutanol, 10 = ethyl-2-methylbutanoate, X = unidentified compound.

Three of the truffles contained significant amounts of what has been tentatively identified as a formic acid ester and had a retention time of 7.3 minutes. The esters identified in the storage study described earlier were not detected for the Western Australian truffles which is not surprising as these truffles were fresh and sampled within days of harvesting. The percentage composition of black truffle using SPME has shown similar variation between truffles, for example Mauriello et al., [17] analysed two black truffles of Italian origin and reported up to 100% variation in the percentage composition of two key compounds 2-methylbutanal and dimethylsulfide.

Conclusion

There is natural variation in the aroma profile of truffles. The aroma of a truffle varies with aging at 4 oC. In particular, the production of esters is apparent. The aroma of the Australian grown black truffle is similar to the French grown black truffle with the same key compounds present. The eight Australian grown truffles showed significant but not unexpected variation between samples. The acceptance of the WA grown truffles in restaurants worldwide, including France, is testament to its quality and characteristic aroma, however, the variation that is displayed needs to be examined more closely to determine whether it is of genetic or environmental origin.

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