School of Molecular and Life Sciences

Extraction, Encapsulation, and Evaluation of Volatile Compounds from Black Périgord Truffle (*Tuber melanosporum*) for Innovative Value-added Products

Win Nee Phong
0000-0001-6440-0132

This thesis is presented for the Degree of Doctor of Philosophy of Curtin University

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DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Human Ethics: The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Number # HRE2019-0374.

Signed: Win Nee Phong

Date: 21.03.2022

Abstract

Black Périgord truffle (*Tuber melanosporum* Vittad. 1831) is highly appreciated for its unique aroma and rarity but its seasonal and perishable nature have restricted its opportunity for a broader market. Current preservation methods could not effectively prolong the shelf life while maintaining the organoleptic properties of truffles. The limited availability of fresh truffles puts pressure on the food industry to rely on synthetic truffle aroma for food application. Nonetheless, the synthetic truffle aroma is less desirable due to poor quality and safety concerns raised by some consumers. In view of the growing demand for natural truffle aroma, processing fresh truffles into a natural flavouring ingredient by extraction and encapsulation of volatile compounds could be a potential strategy. There are limited reports on the extraction of truffle aroma whereas the encapsulation method has yet to be reported in truffles.

Truffles are usually sold whole, offcut pieces, or in freeze-dried form but there is insufficient information about their microbial populations during storage. Surface cleaning is usually used to clean off any surface dirt from truffles, but this step does not completely remove microbes. Residual microbes can multiply during storage, causing spoilage and quality loss, therefore the microbiological and decontamination aspects of truffle were also studied. The microbial changes of the vacuum-packaged whole, sliced, and freeze-dried truffles stored at 4 ± 2°C were evaluated on day 0, 4, 8, 15, and 30. Based on the results, the freeze-dried truffles exhibited lower counts in the total aerobic microbes, *Pseudomonas* spp., and yeast than the whole and sliced truffles. Whilst mould, *Listeria* spp., and *Salmonella* spp. were undetectable, a very low number of *Bacillus* spp. were detected in all sample types. The results suggest the need to establish an effective decontamination treatment for fresh truffles before any postharvest processing, packaging, and storage to delay microbial spoilage.

The extraction and isolation of ten key volatiles that are reported to produce the distinct aroma of black truffle using four different solvents (ethanol, acetone, hexane, and liquefied butane) was first evaluated. Steeping truffles in ethanol produced the best outcome in terms of the key volatile profile and mass recovery, ethanol was therefore used in the subsequent study. Soxhlet extraction was compared with supercritical carbon dioxide (SC-CO_2) extraction. Of the ten key volatile compounds, both extracts contained six volatiles (methanethiol, dimethyl disulfide, isoamyl alcohol, dimethyl trisulfide, 1-octen-3-ol, and *p*-cresol), suggesting that the aroma of natural extracts were more

complex than the synthetic truffle aroma (typically contains only two to five molecules). The elimination of microbes from both extracts to below the detection limit (< 2.00 log CFU/g) indicates that obtaining a safe truffle flavouring ingredient via extraction is attainable.

Encapsulation can be another option to produce a natural truffle flavouring ingredient. To the best of our knowledge, this is the first time the potential of capturing the key volatiles of truffle by encapsulation using β-cyclodextrin (β-CD) was investigated. The key volatile profile and microbial population between products derived from different encapsulation methods namely direct mixing method (M1), direct mixing followed by ethanol addition method (M2), and paste method (M3) were compared over 90 days of storage. M3 was deemed the most optimal method and was compared with freeze-drying. Freeze-dried truffles retained an overall higher relative percentage of volatiles compared to the M3-derived product on day 0 but some volatile changes occurred (ethyl-3-methylbutanoate, isoamyl alcohol, and *p*-cresol showed a significant reduction) after 90 days of storage. No volatile changes were detected in the M3-derived product on day 90. This indicates that capturing the truffle volatiles by encapsulation using β-CD would be a promising application to preserve truffle volatiles for a longer period over freeze-drying so that a truffle flavouring ingredient that is shelf-stable can be produced. However, the presence of microbes in all products on day 0 and 90 suggests the need to incorporate a decontamination step prior to the processing of truffles.

The impacts of freeze-drying and encapsulation on the key volatiles, consumer discrimination and overall sensory impression of truffles was evaluated. Although some volatile and sensory differences between products were detected, there was no preference towards any product and all of them were acceptable from a consumer perspective. The overall finding implies the potential value of processing truffles into a natural flavouring ingredient via freeze-drying or encapsulation.

In developing an effective decontamination treatment, fresh truffles were treated with ozonated water (3 ppm and 6 ppm) and citric acid (5% and 10%) for 5, 10, 15, and 20 min, respectively. While ozonated water did not significantly reduce the microbial counts in truffles, citric acid exhibited a positive antimicrobial effect without compromising the colour and firmness of truffles. Treatment with 10% citric acid for 15 min showed the most effective microbial reduction without affecting the key volatile profile of truffles.

The finding suggests that a single-step washing with citric acid could be a promising decontamination treatment for fresh truffles before any postharvest processing.

This thesis showcases the potential application of extraction and/or encapsulation to process fresh truffles into a natural flavouring ingredient. A readily available natural truffle aroma is expected to add value to the smaller, offcut or excessive unsold lower-priced truffles and promote the development of new truffle-flavoured food products, thereby benefiting both the truffle and food industries.

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<tr><td>Ranil Coorey</td><td>10</td><td>-</td><td>-</td><td>-</td><td>-</td><td>35</td><td>25</td></tr>
<tr><td colspan="8">Co-Author 1 Acknowledgement:
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<tr><td>Alan Payne</td><td>5</td><td>-</td><td>-</td><td>-</td><td>-</td><td>20</td><td>20</td></tr>
<tr><td colspan="8">Co-Author 2 Acknowledgement:
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<tr><td>Mark Gibberd</td><td>5</td><td>-</td><td>-</td><td>-</td><td>-</td><td>20</td><td>20</td></tr>
<tr><td colspan="8">Co-Author 3 Acknowledgement:
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<tr><td>Gary Dykes</td><td>5</td><td>-</td><td>-</td><td>-</td><td>-</td><td>25</td><td>20</td></tr>
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Gary Dykes	5	0	0	0	0	25	15
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Chau Chun Beh	5	15	5	0	0	5	5
Co-Author 5 Acknowledgement: I, as the co-author, acknowledge that this index represents my contribution to the above research output. Signed:							
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Co-Author 4 Acknowledgement: I, as the co-author, acknowledge that this index represents my contribution to the above research output. Signed:							
Hani Al-Salami	5	5	0	0	0	5	5
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Win Nee Phong	60	90	100	85	90	0	15
I, as the first author, endorse that this level of contribution by the candidate indicated above is appropriate. Signed:							
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Win Nee Phong	75	100	100	100	100	0	20
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List of Symbols and Abbreviations

%	:	Percent
µm	:	Micrometer
a*	:	Red/green coordinate
ANOVA	:	One-way analysis of variance
b*	:	Yellow/blue coordinate
°C	:	Degree Celsius
C1	:	Control 1
C2	:	Control 2
C3	:	Control 3
CDs	:	Cyclodextrins
CFU/g	:	Colony-forming unit per gram
CO_2	:	Carbon dioxide
DMDS	:	Dimethyl disulfide
DMS	:	Dimethyl sulfide
DMTS	:	Dimethyl trisulfide
DRBC	:	Dichloran Rose-Bengal Chloramphenicol
DSE-SAFE	:	Direct solvent extraction-solvent-assisted flavour evaporation
EFSA	:	European Food Safety Authority
EI	:	Electron impact
e-Noses		Electronic noses
e-Tongues		Electronic tongues
FD	:	Freeze-dried truffles
FDA	:	Food and Drug Administration
FEMA	:	The Flavor and Extract Manufacturers Association of the United States
FSA	:	UK Food Standards Agency
FSANZ	:	Food Standards Australia New Zealand
g	:	Gram
GC×GC	:	Two-dimensional gas chromatography
GC–C–IRMS	:	Combustion-isotope ratio mass spectrometry
GC-MS	:	Gas chromatography–mass spectrometry
GC-O	:	Gas chromatography-olfactometry
h	:	Hour

HS-SPME	:	Headspace solid-phase micro-extraction
Inc.	:	Incorporated
kGy	:	KiloGray
L*	:	Lightness value
log	:	Logarithm
LSA	:	*Listeria* selective agar
m/z	:	Mass-to-charge ratio
M1	:	Mixing method
M2	:	Direct mixing followed by ethanol addition method
m^3	:	Cubic metre
M3	:	Paste method
MAP	:	Modified atmosphere packaging
mbar	:	Millibar
mg	:	Milligram
min	:	Minute
mL	:	Milliliter
mm	:	Millimeter
MSC	:	Mannitol Selenite Cystine
MSD	:	Mass Selective Detector
N	:	Newton
ND	:	Not detected
NIST	:	National Institute of Standards Technology
NSW	:	New South Wales
OS-SD	:	Organic solvent and steam distillation
p	:	Probability
p	:	para
Pa	:	Pascal
PEMBA	:	Polymyxin Egg Yolk Mannitol Bromothymol Blue Agar
pH	:	Potential of hydrogen
ppm	:	Parts per million
psi	:	Pounds per square inch
PTR-MS	:	Proton Transfer Reaction-Mass Spectrometer
Pty Ltd	:	Proprietary limited
rpm	:	Revolutions per minute
RTP	:	Research Training Program

RV	:	Rappaport-Vassiliadis
SC-CO_2	:	Supercritical carbon dioxide
SE	:	Standard error
sec	:	Second
SFE	:	Supercritical fluid extraction
spp.	:	Species
SPSS	:	Statistical Package for the Social Sciences
TIC	:	Total Ion Chromatogram
TOF	:	Time-of-fight
TPWA	:	Truffle Producers Western Australia
TSA	:	Tryptic soy agar
TSB	:	Tryptic soy broth
USA	:	United States of America
vs	:	Versus
X^2	:	Chi-square
XLD	:	Xylose-Lysine-Desoxycholate
α-CD	:	Alpha-cyclodextrin
β	:	Beta
β-CD	:	Beta-cyclodextrin
γ-CD	:	Gamma-cyclodextrin
μ	:	Micro

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CHAPTER 1: Introduction

Black Périgord truffle (*Tuber melanosporum* Vittad. 1831) has a high culinary and commercial value mainly due to its unique aroma and rarity (Reale et al., 2009a). Nevertheless, several problems restrict its opportunity for a broader market (Campo et al., 2017). Black truffle has a short harvesting season which only occurs in winter. The process of senescence begins immediately after harvest and is further exacerbated by dehydration, microbial growth, and a high respiration rate (Brennan et al., 2000; Rivera et al., 2010b). This underground fungus is typically colonised by diverse microbial communities (Splivallo et al., 2015; Vahdatzadeh et al., 2015). A simple surface cleaning is commonly used as an industrial practice to clean off any surface dirt from truffles (Reale et al., 2009a; Rivera et al., 2011b), but this procedure does not completely remove microbes from truffles (Rivera et al., 2010a). The residual microbes can proliferate in truffles during storage, causing spoilage and quality loss (Campo et al., 2017). A few preservation methods such as refrigeration, freeze-drying, freezing, packaging, irradiation, hot air drying, and canning have been studied in the past to preserve truffles but none of them could prolong the shelf life while maintaining the organoleptic properties of truffles (Campo et al., 2017; Rivera et al., 2010b; Wang & Marcone, 2011).

The truffle aroma availability is limited especially during the off-season. To meet a constant consumer demand for truffle aroma throughout the year (Torregiani et al., 2017), the food industry has relied on synthetic truffle aroma for application in food products (Wernig et al., 2018). However, the synthetic truffle aroma is less desirable as it does not have the complexity of flavour that comes from the actual truffle aroma (Culleré et al., 2010; Splivallo et al., 2011; Wernig et al., 2018). Furthermore, there has been a growing demand for natural truffle aroma as consumers are becoming increasingly concerned about the safety of consuming synthetic ingredients (Torregiani et al., 2017).

Most of the recent studies have been focusing on truffle cultivation. The production of truffles is expected to surge in the near future following the introduction of inoculation techniques for more truffle growth (Oliach et al., 2021). Due to their high perishable nature, fresh truffles need to be sold quickly once harvested (Reale et al., 2009a). Without an effective postharvest processing technique for fresh truffles that has a minimal impact on its aroma, there will be many leftover fresh produces unsold, leading to an issue of oversupply that may result in wastage in the near future as production increases (Garvey & Cooper, 2001).

In response to the current challenges facing the truffle industry and the growing consumer demand for natural truffle aroma, processing fresh truffles into a natural flavouring ingredient that closely resembles the actual truffle aroma could be a potential strategy. Extraction and encapsulation are the two promising techniques that can be used to achieve this notion. Extraction has been widely used to obtain flavouring compounds from various food materials but it has not been well studied in truffles. On the other hand, the application of encapsulation on truffles has not yet been reported. Encapsulation has been widely used in the food industry to protect sensitive substances such as volatile compounds from degradation or loss and achieve their stability. We hypothesised that a similar approach could also be applied to the fresh truffles for the production of a natural flavouring ingredient.

The main aim of this thesis is to explore the applicability of extraction and encapsulation techniques to process the smaller, offcut or excessive unsold lower-priced truffles into a natural flavouring ingredient that closely resembles its actual aroma for food application. Given that the quality of truffle aroma is closely associated with microbial spoilage and the limitation of the current surface cleaning practice to decontaminate fresh truffles, the microbial quality of different truffle products during storage and the development of an additional decontamination treatment prior to postharvest processing of truffles are another important aspect to study in this thesis.

The following specific objectives were set to achieve the aim of the study:

1) To investigate the microbial changes of the whole, sliced, and freeze-dried black truffles (*Tuber melanosporum*) under vacuum packaging and cold storage.
2) To produce truffle extract containing key volatiles that are responsible for the final aroma impression of black truffle which may be used as a flavouring ingredient for food application.
3) To investigate the possibility of capturing and stabilising compounds responsible for black truffle aroma by encapsulation using β-cyclodextrin (β-CD) and compare the developed method with freeze-drying.
4) To evaluate the effects of encapsulation and freeze-drying on the key volatile profile, sensory, and consumer acceptance of black truffle.
5) To evaluate the effects of ozonated water and citric acid decontamination treatments on the microbial population and organoleptic qualities of fresh black truffle.

1.1 Overview of thesis structure and chapters

This thesis consists of seven chapters written in the format of one review paper, five research papers, and the last chapter is the general conclusion of the entire thesis and suggestions for future directions. A more detailed synopsis of each chapter presented in this thesis is described as follows:

Chapter 2 presents a comprehensive review of the current challenges facing the truffle industry and the shortcoming of each existing preservation method. Research gaps were identified and potential approaches such as extraction and encapsulation were proposed in response to these challenges. This chapter also briefly discusses the microbiological aspect of truffles which are responsible for spoilage and the loss of the organoleptic quality in truffles. This chapter emphasises the importance of processing fresh truffles at the postharvest stage and supports the need of multi-disciplinary studies to establish a process for the production of a natural flavouring ingredient for food application.

Chapter 3 investigated the microbial changes of different types of truffle products namely the whole, sliced, and freeze-dried truffles under vacuum packaging and cold storage on day 0, 4, 8, 15, and 30.

In Chapter 4, the impacts of different extraction methods on the mass recovery, microbial quality, and key volatile profile of truffle extracts were investigated. Two experiments were conducted in this study. In Experiment 1, the extractability of different types of solvents (ethanol, acetone, hexane, and liquefied butane) were first evaluated and the preferred solvent (ethanol) for Soxhlet extraction was identified. Soxhlet extraction was then compared with SC-CO_2 extraction in Experiment 2.

In Chapter 5, three different encapsulation methods using β-CD (M1, M2, and M3) to capture truffle volatiles were evaluated over 90 days of storage and the most preferred encapsulation method (M3) was selected for comparison with freeze-drying (has been preferred among all the existing preservation methods). This is the first research to determine the possibility of encapsulating truffle aroma for food application.

In Chapter 6, the impacts of freeze-drying and encapsulation on the key volatiles, consumer discrimination, and overall sensory impression (aroma intensity, truffle aroma rating, overall liking, and overall acceptability) of truffles was evaluated based on the GC-MS analysis and consumer sensory evaluation.

In Chapter 7, truffles were treated with ozonated water (3 ppm and 6 ppm) and citric acid (5% and 10%) for 5, 10, 15, and 20 min, respectively. Subsequently, the effects of each treatment on microbial populations (total aerobic microbes, total anaerobic microbes, and *Pseudomonas* spp.), firmness, and colour of truffles were assessed. Based on the results of these analyses, the preferred treatment (10% citric acid treatment for 15 min) was identified and its impact on the key volatile profile of the truffles treated with these parameters was evaluated.

In Chapter 8, all results were summarised and discussed in a broader context. The entire thesis is concluded with limitations and some suggestions for potential future directions.

Supplementary information in relation to particular chapters (Chapter 3 and Chapter 6) are provided in the appendices.

CHAPTER 2: Literature review

A major part of the content in this chapter has been published as follows:

Phong, W.N., Gibberd, M., Payne, A. D., Dykes, G. A. & Coorey, R. Methods used for extraction of plant volatiles have potential to preserve truffle aroma: a review. *Comprehensive Reviews in Food Science and Food Safety,* 1-25. DOI:10.1111/1541-4337.12927

2.1 Truffles

Truffles (*Tuber* spp.) are the fruiting bodies of subterranean ascomycetes that usually grow in association with the roots of certain tree species such as oak and hazelnut (Liu et al., 2017a; Splivallo et al., 2011). These truffles species form a symbiotic relationship with host trees to complete their life cycle as they are unable to synthesise sugars and other carbohydrates. The tree provides truffles with a place to live and supplies a source of photofed carbohydrates for growth, and in return the fungus assists the tree with mineral and other nutrient uptake (Lee, 2008).

The physical characteristics of truffles are very different from the commonly consumed mushroom. Mature truffles are generally firm, dense, woody and have neither stalk nor gills (Hall et al., 2008; Wang & Marcone, 2011). These symbiotic fungi are usually globular in shape with a wrinkled external surface (Liu et al., 2017a; Raymond E. March et al., 2006). Specially trained dogs or pigs are normally used to locate this type of underground fungus due to their ability to detect distinctive scents through a layer of earth (Zambonelli et al., 2016).

Truffles are considered one of the most luxurious and sought after food items in the world (Romanazzi et al., 2015) owing to their desirable organoleptic properties, rarity, and high perishability (Reale et al., 2009a). Due to the strength of aroma and high price, truffles are commonly used as a flavour enhancer rather than as a food product in and of themselves (Pacioni et al., 2014a).

2.1.1 Types of truffle

There are hundreds of truffle species but not all of them are edible. Only the fruiting body of some (mostly belong to the genus *Tuber*, *Terfezia*, and *Tirmania*) are edible (Paolocci

et al., 2004; Wang & Marcone, 2011). *Tuber* species (also known as true truffles) include the French black (*Tuber melanosporum* Vittad. 1831), the Italian white (*Tuber magnatum* Picco 1788), the Bianchetto white (*Tuber borchii* Vittad. 1831), the black musk (*Tuber brumale* Vittad. 1831), the pecan truffle (*Tuber lyonii* Butters 1903) and the summer black truffle (*Tuber aestivum* Vittad. 1831) (Healy et al., 2016; Merényi et al., 2016; Wang & Marcone, 2011). The genera *Terfezia* and *Tirmania* are known as desert truffles since they are mostly found in arid and semi-arid areas and are popular mainly in the Mediterranean, Middle East, and Northern Africa regions. The white desert truffle (*Tirmania nivea* (Desf.) Trappe 1971) and the black desert truffle (*Terfezia boudieri* Chatin 1892) and (*Terfezia claveryi* Chatin 1892) are among the most scientifically studied species (Mustafa et al., 2020; Shavit, 2014). The different edible truffle species from around the world are summarised in Table 2.1.

Truffles have different aromatic notes which vary widely among species, leading to diverse levels of desirability (Wang & Marcone, 2011). For example, the white truffle (*T. magnatum*) has a garlic cheese aroma with subtle methane overtones (Wang & Marcone, 2011). On the other hand, the black truffle (*T. melanosporum*) has a musty, earthy flavour and pungent odour with a slight taste of radish and a tint of hazelnut (Bellesia et al., 1998; Islam et al., 2013; Wang & Marcone, 2011; Xiao et al., 2015). Desert truffles are relatives of European truffles, but their aroma and flavours are less intense compared to European truffles (Mustafa et al., 2020; Shavit, 2014).

Table 2.1: Common edible truffle species from around the world

Common name	Scientific name	References
	True truffles	
French black	*Tuber melanosporum* Vittad. 1831	(Healy et al., 2016; Merényi et al., 2016; Wang & Marcone, 2011)
Bianchetto white	*Tuber borchii* Vittad. 1831	
Italian white	*Tuber magnatum* Picco 1788	
Black musk	*Tuber brumale* Vittad. 1831	
Pecan truffle	*Tuber lyonii* Butters 1903	
Summer black truffle	*Tuber aestivum* Vittad. 1831	
	Desert truffles	
White desert truffle	*Tirmania nivea* (Desf.) Trappe 1971	(Mustafa et al., 2020; Shavit, 2014)
Black desert truffle	*Terfezia boudieri* Chatin 1892	
Black desert truffle	*Terfezia claveryi* Chatin 1892	

2.1.2 Microbial communities on truffle

A diverse and complex microbial community of unknown function including bacteria, yeasts and filamentous fungi has been reported to intensively colonise truffles at all stages of their life cycle (Splivallo et al., 2015; Vahdatzadeh et al., 2015), with some of them (such as *Pseudomonas* spp., yeasts, and moulds) may cause postharvest spoilage whereas some of them (such as *Bacillus* spp. and *Listeria* spp.) are pathogens (Rivera et al., 2010a; Saidi et al., 2015). Both the external (peridium) and internal parts (gleba) of truffles are colonised by microbes and the microbiome varies significantly between the peridium and the gleba (Antony-Babu et al., 2014). A higher total microbial load was observed in the peridium than in the gleba of healthy *T. aestivum* and *T. melanosporum* truffles (Rivera et al., 2010a). This suggests the surface structures of truffles provide an excellent habitat for the attachment of microbes.

The role of these microbes in the life cycle, nutrition and formation of aroma in truffles remains unknown (Antony-Babu et al., 2014; Splivallo et al., 2015). Bacteria such as those from the genus *Pseudomonas* and members of the *Enterobacteriaceae* are believed to be beneficial in the development and maturation of truffles (Rivera et al., 2010a; Romanazzi et al., 2015). It has also been speculated that truffle-associated microbes might play a key role in the formation of characteristic truffle volatiles (Buzzini et al., 2005; Carrasco & Preston, 2020; Vahdatzadeh et al., 2015). An experimental study involved the independent synthesis of volatile compounds by yeasts isolated from black and white truffles (*T. melanosporum* and *T. magnatum*, respectively) supported this hypothesis (Buzzini et al., 2005). Yeast strains isolated from the truffles demonstrated the ability to produce volatile organic compounds when they were cultured on culture medium supplemented with L-methionine (Buzzini et al., 2005). Another study demonstrated that the thiophene volatile characteristics of white truffle (*T. borchii*) was a result of the biotransformation of the non-volatile precursors of thiophenes into volatile compounds by the bacterial community and not by the truffle itself (Splivallo et al., 2015). The bacterial community was also suggested to be an important contributor to odour in other truffle species such as *T. melanosporum*, *T. magnatum*, and *T. aestivum* (Vahdatzadeh et al., 2015).

A genomic analysis of *T. melanosporum* discovered the presence of genes hypothetically encoding for flavour-related metabolites (Martin et al., 2010). These genes encode for enzymes that degrade truffle amino acids through the Ehrlich pathway, giving rise to

distinctive aroma and flavour compounds, such as 2-methyl-1-butanal, which has a cocoa almond-like aroma (Vahdatzadeh & Splivallo, 2018). This suggests that the production of some volatiles in *T. melanosporum* might be derived from the truffle fungus itself without requiring the participation of the microbial community (Martin et al., 2010). Other volatiles, namely octan-3-one and oct-1-en-3-ol, which give rise to the characteristic mushroom flavour, were also believed to be strictly fungal origin (Vahdatzadeh et al., 2015). On the other hand, the formation of dimethyl sulfide (DMS), a common volatile which occur in 85% of the species investigated to date, may be produced by both truffle (through the Ehrlich pathway) and microbes (α- and β-Proteobacteria) (Vahdatzadeh et al., 2015). The origin of some rare aromatic odourants such as 2-methylfuran-3-thiol that is specifically present in certain truffle species such as *T. melanosporum* and *T. aestivum* remains elusive. This odourant was speculated to be synthesised by truffles only, possibly during their sexual reproduction stage, as this compound can only be detected in the fruiting bodies of truffles but not in axenic mycelial cultures or microbes (Vahdatzadeh et al., 2015).

2.1.3 Truffle flavour

The development of advanced analytical techniques has led to the identification of a significant number of volatile compounds from various truffle species. Up to now, over 200 volatiles have been discovered from truffles of various species and the number of volatiles identified is expected to grow as the analytical tools used for aroma detection become increasingly sensitive (Culleré et al., 2010). Truffle flavour research is important as it may advance an understanding of the contribution and impact of each volatile compound on the overall flavour impression of the different truffle species (Wang & Marcone, 2011).

There are different metabolic pathways involved in the production of aroma volatiles (Martin et al., 2010; Vahdatzadeh et al., 2015), which give rise to complex profiles and vary from species to species. The volatile constituents generally contain alcohols, esters, aldehydes, ketones, acids, amines, aromatic ethers, and sulfur-containing compounds (Culleré et al., 2010). It is widely accepted that not all volatile constituents are responsible for what humans perceive as truffle aroma active compounds (Feng et al., 2019; Mustafa et al., 2020; Strojnik et al., 2020). The commonly used analytical instruments such as headspace solid-phase micro-extraction combined with gas chromatography-mass spectrometry (SPME/GC-MS) are known to discriminate volatiles merely by their boiling

points and the analytical instruments should be complemented by a sensomics approach to characterise the key volatile compounds that are responsible for the overall aroma impression of truffles (Schmidberger & Schieberle, 2017). The sensomics approaches include aroma extract dilution analysis (AEDA), aroma dilution analysis (ADA) in combination with GC-MS, quantitation by stable isotope dilution assays (SIDAs), calculation of odour activity values (OAVs) and aroma recombination experiments (Schieberle & Hofmann, 2011; Schmidberger & Schieberle, 2017). Some other technologies have also been employed to improve the GC-MS based methods for volatile measurement in truffles as reviewed by Lee et al. (2020). These include:

1) time-of-flight (TOF–MS) based Proton Transfer Reaction-Mass Spectrometer (PTR-MS) technology (Vita et al., 2015)
2) direct solvent extraction-solvent-assisted flavour evaporation (DSE-SAFE) technique coupled with a comprehensive two-dimensional gas chromatography (GC×GC) high resolution-TOF/MS and an electronic nose (Zhang et al., 2016)
3) GC coupled with combustion-isotope ratio mass spectrometry (GC–C–IRMS) (Wernig et al., 2018)

Instrumental techniques can be accurate by objectively measuring chemical compounds. Still, it has some limitations (Chambers & Koppel, 2013) such as requiring a prior sample extraction before analysis (Andrewes et al., 2021) and a GC heating step (Chambers & Koppel, 2013) which would add further complexity to the instrumental analysis (Andrewes et al., 2021). Given that human olfaction is the ultimate discriminator of aroma and taste quality (Andrewes et al., 2021; Fan et al., 2016), cross-referencing the instrumental data with the human sensory response (Lawless, 1991) is also necessary for advancement in the truffle flavour research. However, challenges exist in using traditional sensory methods to determine the quality of foods and beverages. The use of a human panel for sensory evaluation can be time-consuming and expensive, especially if a large number of panellists are required (Hough et al., 2006). Depending on the nature of the samples, sample carryover and sensory fatigue can also be problematic (Torrico et al., 2018). Over the last few decades, electronic senses such as electronic noses (e-noses) and taste sensors or electronic tongues (e-tongues) have been developed to address some of these concerns. The use of electronic senses would allow larger sample sets to be assessed, overcoming the fatigue and carryover challenges that exist in using traditional sensory methods (Schlossareck & Ross, 2019).

The key odour notes of truffles vary from one species to another, giving rise to their own unique aroma (Strojnik et al., 2020). Table 2.2 shows the key volatiles identified in the two most highly valued truffle species on the market, namely *T. magnatum* Pico and *T. melanosporum.* The distinctions in the aromas of *T. magnatum* and *T. melanosporum* can be attributed to the variability and predominance in their key volatile profile. Some volatiles are common to many truffle species, while certain volatiles appear to be species-specific (Mustafa et al., 2020; Torregiani et al., 2017). For instance, 2,4-dithiapentane is only present in *T. magnatum* Pico and can thus be used as one of the key markers to distinguish this truffle species from the others (Torregiani et al., 2017). The concentration of a single volatile compound also varies significantly among species (Torregiani et al., 2017; Wang & Marcone, 2011). For example, it has been reported that the black truffle (*T. melanosporum*) emits over 100 times the volume of volatile compounds than the less aromatic summer truffle (*T. aestivum*) (Culleré et al., 2010). This finding plausibly explains why black truffle are regarded by most truffle experts as one of the most aromatic and desirable truffle species (Culleré et al., 2010; Wang & Marcone, 2011). Aroma variability had also been reported in truffles of the same species (Molinier et al., 2015; Torregiani et al., 2017) which may be due to factors such as geographical origin, environmental factor, maturation stage or storage conditions (Strojnik et al., 2020; Torregiani et al., 2017).

Of all the volatile compounds that have been identified in truffles, sulfur-containing volatiles such as dimethyl sulfide (DMS), dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) represent the most important group of odorants in truffles (Strojnik et al., 2020; Vahdatzadeh et al., 2015). They confer the typical garlicky and sulfurous notes that characterise all truffle species (Vahdatzadeh et al., 2015). The most common sulfur-containing volatile in truffles is DMS, which has been extensively reported to occur in a wide variety of truffles including black truffles *T. melanosporum* (Culleré et al., 2010), *T. indicum* (Culleré et al., 2013b), *T. sinensis* (Feng et al., 2019); white truffles *T. magnatum* (Gioacchini et al., 2008), *T. sinoalbidum*, *T. sinoexcavatum* (Feng et al., 2019); summer truffle *T. aestivum* (Díaz et al., 2009).

Table 2.2: Key volatiles in white truffle (*Tuber magnatum* Pico) and black truffle (*Tuber melanosporum*)

Key volatiles	Aroma descriptor	White Alba Truffle (*Tuber magnatum* Pico)	Black truffle (*Tuber melanosporum*)
Dimethyl sulfide (DMS)	Truffle, sulfur	✓	✓
2,3-Butanedione/ diacetyl	Butter, cream	✓	✓
3-(Methylthio)propanal/ Methional	Boiled potatoes	✓	✓
Dimethyl trisulfide (DMTS)	Pungent	✓	✓
1-Octen-3-one	Mushroom	✓	✓
1-Octen-3-ol	Mushroom, fungal	✓	✓
Bis(methylthio)methane/ 2,4-Dithiapentane	Sulfury, garlic-like	✓	✗
(1H)-pyrroline/3,4-dihydro-2-(H)pyrrol	Amine-like, sperm-like	✓	✗
3-Methylbutanal	Malty	✓	✗
3-(Methylthio)propanol	Boiled potatoes	✓	✗
2-Methylbutanal	Malty	✓	✗
2-Methylbutanoic acid	Sweaty	✓	✗
Trans-4,5-epoxy-(E)-2-decenal	Metallic	✓	✗
1,2,4-Trithiolane	Sulfury, onion-like	✓	✗
3-Methylbutanoic acid	Sweaty	✓	✗
3-Hydroxy-4,5-dimethyl-2(5H)-furanone	Seasoning-like	✓	✗

Phenylacetic acid	Honey-like	✓	×
2,3-Diethyl-5-methylpyrazine	Earthy	✓	×
4-Methyl-2-methoxyphenol	Vanilla-like, clove-like, smoky	✓	×
2-Methylpropanal/Isobutyraldehyde	Malty	✓	×
Methanethiol	Cooked cabbage	×	✓
Ethyl-3-methylbutanoate/ Ethyl 3-methylbutyrate	Fruit, anise	×	✓
Dimethyl disulfide (DMDS)	Truffle, sulfur	×	✓
Isoamyl alcohol/ 3-methyl-1-butanol	Cheese	×	✓
p-Cresol	Phenolic/ leather	×	✓
3-Ethyl-5-methylphenol	Phenolic/leather	×	✓
2-Methylisoborneol	Mould, earth	×	✓
Dimethyl sulfoxide (DMSO)	Garlic-like note	×	✓
Ethyl butyrate	Fruity, green apple	×	✓
1-Hexen-3-one	Metallic	×	✓
2-Methyl-3-furanthiol	Onion, meaty	×	✓
5-Methyl-2-propylphenol	Phenolic, gasoline	×	✓
β-Phenylethanol	Roses	×	✓
Furaneol	Cotton candy	×	✓
3-Ethylphenol	Leather, animal, truffle	×	✓
3-Propylphenol	Phenolic, leather	×	✓

Adapted from Schmidberger and Schieberle (2017), Culleré et al. (2010) and Culleré et al. (2013a), with modification.

✓ : Key volatile

✗ : Not key volatile

2.2 Current challenges faced by the truffle industry

At present, the truffle industry faces a number of challenges. These issues are summarised in Figure 2.1 and each of them will be further explained below.

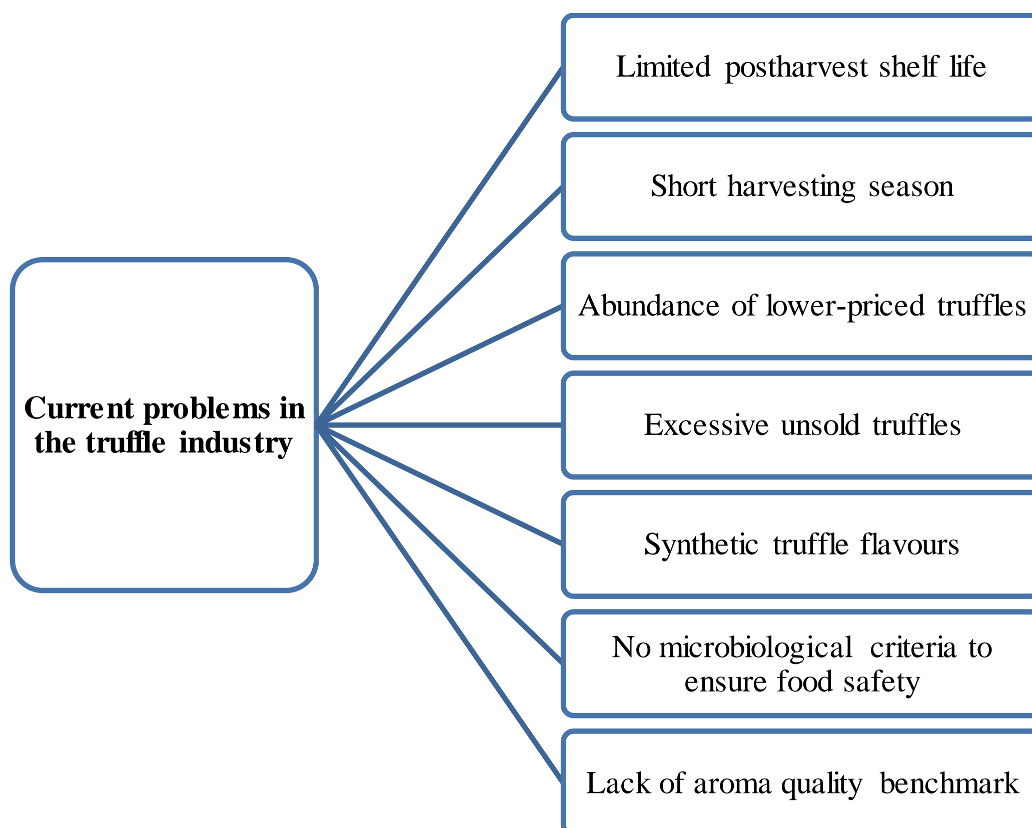


Figure 2.1: List of challenges faced by the truffle industry

2.2.1 Limited postharvest shelf life

Despite possessing a high culinary and economic value, the possibilities for a wider market for truffles remain restricted as they are extremely perishable and their postharvest shelf life is limited to about 7 to 10 days (Campo et al., 2017; Renowden, 2005; Rivera et al., 2011b). Truffles display their ideal organoleptic properties only when fresh, and their volatile profile changes rapidly with time following harvest. There is not only a decrease in the intensity of the aroma but also a complete shift occurs in the profile (Campo et al., 2017; Rivera et al., 2011b; Vahdatzadeh et al., 2019). The process of senescence commences immediately after harvest and is further exacerbated by dehydration, the development of superficial mycelium, the growth of insect larvae, and a high respiration rate (Brennan et al., 2000; Rivera et al., 2010b). Collectively these adversely affect the visual appearance and the aroma quality (Rivera et al., 2010b; Rivera et al., 2011b). Truffles growing underground play host to a significant number of microorganisms that

as indicated above, play a beneficial role in their life cycle. However, once the truffles are harvested, these microorganisms may act as spoilage agents and proliferate using the truffle as a substrate (Rivera et al., 2010a; Romanazzi et al., 2015). For this reason, truffles must be utilised quickly once they are harvested (Patel, 2012).

2.2.2 Seasonality

Truffles are seasonal products that require very specific growing conditions and are harvested within a short window of opportunity, after which they deteriorate and they are therefore unavailable during the rest of the year (Campo et al., 2017; Renowden, 2005; Rivera et al., 2011b). Different truffles species generally require different climates and soils for growth and maturation. For instance, black truffles (*T. melanosporum*) typically grow in a Mediterranean climate during summer, mature slowly through autumn and are ready for harvest in the winter (Büntgen et al., 2019; Zambonelli et al., 2016). Whereas white truffles (*T. magnatum*), which require an alkaline, well-drained and aerated soil environment for growth (Bragato et al., 2004), are harvested during late autumn (Pieroni, 2016). On the other hand, summer truffles (*T. aestivum*) which are mainly harvested in the summer can mature in totally dried-out soil (Bragato et al., 2004; Büntgen et al., 2017; Büntgen et al., 2019).

2.2.3 The abundance of lower-priced truffles

Truffles occur naturally or can be cultivated. These fungi can be found in both the Northern and Southern hemispheres (Hall et al., 2017). The marketing and commercial quality control of truffles are defined by the truffle grading standard. Although there is no worldwide accepted standard for truffles at present, the grading standard which is commonly adopted by the truffle industry requires truffles to be graded mainly based on physical appearances such as shape, size, and weight (The Australian Truffle Growers Association, 2014; UNECE, 2017). Larger truffles with regular shape are considered of the highest quality and fetch a premium at the market (Büntgen et al., 2017). There is no specific taste and aroma-based measurements for grading despite this being important to the consumer. As a result, despite their high aroma quality, smaller second and offcuts are graded as poor quality and fetch a reduced price at market (Hall et al., 2017).

Apart from the truffle grading standards, there are other factors that may lead to a price drop. One of the main factors is the increase in global production due to a rapid expansion in cultivation however the size of the market continues to remain unchanged without a

strong marketing strategy and a good understanding of the truffle supply chain, leading to market fatigue (Oliach et al., 2021). Efforts to identify truffle production that could help develop truffle consumption culture and marketing are still inadequate. The promotion of truffle consumption via tourism and gourmet marketing especially in countries without a tradition of truffle consumption has not been well established. Moreover, the use of synthetic aroma in restaurant cuisine and truffle products has been identified as another factor causing the price drop (Oliach et al., 2021).

Excess unsold or offcut truffles could potentially be made into a flavouring ingredient for food applications which would be available throughout the year. Research on value addition to these lower quality truffles is necessary as it could benefit both the consumer and the industry.

2.2.4 Excessive unsold truffles due to increasing truffle production

Truffles production is expected to increase in the near future as recent research has focused on its cultivation (ABARES, 2019; Diss, 2016; Oliach et al., 2021). For example, truffle production has grown rapidly over the past few years in Australia, with the farm gate value trebling since 2011 to $8.5 million as of 2016 and the production is expected to continue to soar in the future (ABARES, 2019; ABS, 2019; Diss, 2016). As truffles need to be sold quickly once harvested, there are concerns about the impact of an increase in production (Garvey & Cooper, 2001). The truffle industry runs the risk of an oversupply that cannot be sold, leading to possible food waste in the near future (Boa, 2004; Garvey & Cooper, 2001; Ishangulyyev et al., 2019), which could also impact its sustainability. Given that extracting volatile compounds from truffles is a potential solution to the oversupply problem, it is important for the truffle industry to explore the possibility of obtaining natural truffle aroma via extraction (The Australian Truffle Growers Association, 2014).

2.2.5 Synthetic truffle flavours

To circumvent the scarcity of truffles, synthetic truffle flavours have been developed. Synthetic truffle flavours can be made with natural (not originating from truffles but from other different natural sources) or chemical flavouring substances (Torregiani et al., 2017; Wernig et al., 2018). The flavouring substances used to produce truffle flavours must be clearly labelled, safe for human consumption and on the approved list of food regulatory bodies such as the Food Standards Australia New Zealand (FSANZ), Food and Drug

Administration (FDA), European Food Safety Authority (EFSA), UK Food Standards Agency (FSA) and The Flavor and Extract Manufacturers Association of the United States (FEMA) (EFSA, 2018; FDA, 2010; FEMA; FSA, 2018; FSANZ, 2018). Currently, this is a practical way to add synthetic truffle aroma to food products during the off-season (Splivallo et al., 2011; Wernig et al., 2018).

Nevertheless, possible food applications are curtailed due to the lack of a common standard regulating the use of synthetic truffle flavour compounds and effective traceability methods to determine the authenticity of compounds used in truffle products (Oliach et al., 2021). This has led to issues such as the misuse of synthetic flavour compounds and inconsistencies in product labelling, giving consumers a confusing and/or false image that products contain actual truffle flavour compounds. Such issues have the potential to tarnish the prestige and value of natural truffles (Oliach et al., 2021; Wernig et al., 2018). It costs much less to produce artificially flavoured truffle products than natural truffle products (Torregiani et al., 2017). As a result, most of the truffle-flavoured food products that are sold in retail markets, such as truffle oils and sauces, contain either a very low percentage of truffle (typically around 1% or less) or no real truffles at all. Instead, they use a combination of manufactured flavouring substances that do not correspond to the type of truffle declared on the label (Pacioni et al., 2014a; Renowden, 2005; Torregiani et al., 2017; Wernig et al., 2018).

2,4-Dithiapentane (also known as bis(methylthio)methane), which imparts sulfury, mustard, and garlicy aroma notes, is exclusive to the white truffle *T. magnatum* Pico (Splivallo & Culleré, 2016; Torregiani et al., 2017; Vahdatzadeh & Splivallo, 2018). For this reason 2,4-dithiapentane has been misused and widely added into many commercial truffle-flavoured products, including the black truffle oil that is not supposed to contain this compound (Wernig et al., 2018). As indicated above DMS and 2-methylbutanal impart sulfurous and pungent aroma notes, respectively (Culleré et al., 2010; Xiao et al., 2015). When these chemicals are mixed at a specific ratio, a synthetic aroma that resembles a typical black truffle aroma is achieved (Culleré et al., 2010; Wernig et al., 2018). Although this mixture has long been used by the food industry to imitate black truffle aroma, it does not present the complexity of natural truffle aroma (Culleré et al., 2010; Splivallo et al., 2011; Wernig et al., 2018). This is because the synthetic aroma contains only about two to five molecules in total as opposed to a natural truffle aroma that contains many more volatiles (Wernig et al., 2018).

Foodstuffs containing artificial food additives are often avoided by consumers as they are generally perceived as artificial (Bicas et al., 2010) and potentially harmful to human health with long-term consumption (Madene et al., 2006). Studies have linked artificial food additives with an increased risk of some illnesses such as kidney damage, tumours, skin rashes, migraine, asthma and sleep disturbance (Ramesh & Muthuraman, 2018). For this reason, there are concerns over the safety of consuming food products that contain artificial additives including artificially truffle-flavoured foodstuffs (Torregiani et al., 2017).

The attempt to produce a natural truffle aroma using ultrasound have been investigated. A patent filed by Talou et al. (2001) claimed that using ultrasound to extract natural aroma from truffles into oil or vinegar could produce truffle-flavoured products with a remarkable taste quality and offer advantages such as technological simplicity, short extraction time, and high extraction yield. Nonetheless, as far as we know, there is no such commercial product derived from this method found in the market as of now, possibly due to some underlying drawbacks such as the lack of a kill step for microbial control of the extract and the use of vinegar as one of the extraction mediums, which may impart a strong acetic acid note to the final product. Hence, the current market is still relying on synthetic truffle flavours for food application.

Due to consumer demand for natural food ingredients (Cataldo et al., 2016; Lukin et al., 2018), there is a pressing need to produce a natural truffle aroma as a flavouring agent for food application. The development of a cost-effective natural aroma extract from truffles therefore offers the commercial potential to the food industry.

2.2.6 Absence of microbiological safety and quality criteria

Fresh truffles typically carry a high number of microbes, some of which could be pathogenic and capable of causing food-borne disease (Rivera et al., 2010a). Truffles are usually consumed uncooked, but current food legislation does not establish any microbiological criteria for their use in culinary applications (Rivera et al., 2010a; Sorrentino et al., 2018). It has been reported that surface cleaning of truffles prior to usage reduces the microbial loads (Reale et al., 2009a; Rivera et al., 2011b). However, surface cleaning is not considered an effective decontamination step to completely remove microbes, especially pathogenic species (Rivera et al., 2010a).

The residual microbial population post-cleaning can still multiply rapidly even during refrigerated storage (Saltarelli et al., 2008). Considering that microbial spoilage is the main reason for the loss of organoleptic quality in truffles (Campo et al., 2017), there is a need to supplement this simple cleaning procedure with other more effective decontamination treatments (Saltarelli et al., 2008; Sorrentino et al., 2018). For this reason, a better understanding of the microbial populations on truffles is needed to establish an effective disinfection treatment for truffles while maintaining the truffle aroma (Rivera et al., 2010a). One possible solution to this problem may be the development of an effective aroma extraction process that inactivates microbes so that a safe food flavouring ingredient such as ginger extract (Martinez, 2007) can be developed.

2.2.7 Lack of aroma quality benchmark

Identification of volatiles that are present in truffles can be achieved via gas chromatography-mass spectrometry (GC-MS) (Díaz et al., 2003). Ranking the volatiles according to their potential importance with respect to truffle aroma, on the other hand, is more complicated and involves a combination of mass spectrometric, olfactometric and sensory techniques. As a consequence, there is still a gap in knowledge in this area (Culleré et al., 2010). For example, the aromatic characteristic of black truffle was studied using gas chromatography-olfactometry (GC-O), a method that uses human assessors to identify and evaluate odour-active compounds eluting following separation by a GC (Culleré et al., 2010). At least 17 different volatile molecules that seem to be uniquely responsible for the final distinguishable smell of black truffle have been identified (Culleré et al., 2010). Of these, the most significant contributors to the final aroma are thought to be 2,3-butanedione (butter, cream), DMDS (characteristic truffle, sulfur), ethyl butyrate (also known as ethyl butanoate, with a characteristic of fruity, green apple flavour), DMS (characteristic truffle, sulfur), 3-methyl-1-butanol (cheese) and 3-ethyl-5-methylphenol (phenolic, leather) (Culleré et al., 2010). Whereas quantitatively, black truffle emits mostly 3-ethyl-5-methylphenol (phenolic, leather) (> 50 % of the total volatiles released), 5-methyl-2-propylphenol (phenolic, gasoline), β-phenylethanol (also known as 2-phenylethanol, a characteristic rose smell) and 3-ethylphenol (leather, animal) (Culleré et al., 2010; Vahdatzadeh et al., 2015). These studies show that determining key volatiles that are responsible for the final aroma impression of truffles is challenging and complicated which involves both analytical and sensomics approaches as described in Section 2.1.3. Nonetheless, identifying key odours that make up a high-grade truffle is imperative for the establishment of a quality grading system based on

volatile profile (Rivera et al., 2010a; Romanazzi et al., 2015; Splivallo et al., 2011) and also if a successful aroma extraction method is to be developed.

A grading standard developed based on chemical signatures and sensory technology should be included as part of the grading process. This could help underpin the current truffle grading standards employed by the truffle industry so as to minimise errors associated with grading inconsistency which is merely based on the physical appearance of truffles (The Australian Truffle Growers Association, 2014; UNECE, 2017). Furthermore, databases which comprise characteristic volatile signatures of each species of truffles would also be helpful in evaluating the suitability of a technique for preserving truffle aroma, which is currently lacking.

2.3 Current truffle shelf life extension practices

To meet consumer demand for truffle based products throughout the year (Splivallo & Culleré, 2016), many postharvest preservation technologies such as refrigeration, freezing, freeze-drying, hot air dehydration, canning, packaging and irradiation have been studied (Campo et al., 2017; Culleré et al., 2012; Rivera et al., 2010b; Wang & Marcone, 2011). The application and impacts of these preservation techniques on truffles are discussed below and summarised in Table 2.3.

2.3.1 Preservation methods

2.3.1.1 Refrigeration

Refrigeration at 4°C is the most commonly used preservation technique (Sorrentino et al., 2018) but this technique is only suitable for short-term storage. For example, refrigeration in combination with low-pressure packaging extended the shelf life of black truffle for up to a maximum of 14 days (Savini et al., 2017). The main spoilage bacterial genus, *Pseudomonas*, was able to proliferate during cold storage (Sorrentino et al., 2018). The microbial population in fresh truffles (*T. melanosporum and T. borchii*) increased from 10^7-10^8 CFU/g to 10^{10} CFU/g over 15 days at 4 °C (Saltarelli et al., 2008). It has also been reported that the biochemical changes in truffles associated with senescence, such as enzymatic activity, protein, and sugar content occur during storage at 4 °C (Saltarelli et al., 2008). It is interesting to note that the biochemical changes varied among species. For example, Saltarelli et al. (2008) reported that during refrigerated storage white truffles (*T. magnatum* and *T. borchii*) were more sensitive to biochemical spoilage than black truffles (*T. melanosporum* and *T. aestivum)*. This could be due to variation in

microbiological and organoleptic qualities that have been observed in truffles of different species associated with different ecological niches and harvesting times (Rivera et al., 2011a). It is difficult to predict the applicability of a preservation technique to all truffle species (Rivera et al., 2011a). As refrigeration could only extend the shelf life of truffles for a relatively short time, it cannot be used as an effective method for long-term storage and long-distance export so that they can be made available throughout the year (Palacios et al., 2014).

2.3.1.2 Freezing

Freezing is one of the most important methods of preservation to maintain food quality. This long-term storage technique is frequently applied to truffles, but it has some limitations with regard to aroma quality (Campo et al., 2017; Culleré et al., 2013a). Significant changes in the volatile profile of frozen black truffle (*T. melanosporum*) were detected after freezing at −80°C for 15 days (Campo et al., 2017). Another study reported that the characteristic aroma of black truffle showed signs of change even after only 24 hours of freezing at −20°C (Culleré et al., 2013a). A sensory evaluation comprising a group of experienced truffle experts also agreed that frozen truffles did not retain the characteristic aroma of fresh truffles (Culleré et al., 2013a). In addition, the truffle texture is often negatively affected as a consequence of freezing (Zambonelli et al., 2016), which also indicates that freezing is not a viable method of making truffles available throughout the year.

2.3.1.3 Freeze-drying

Freeze-drying, also known as lyophilisation, is a process of dehydrating food samples at low temperature (below -20°C). Food is first frozen and then the ice is removed from the sample by sublimation under vacuum. This method could be a good strategy to preserve the original aroma and texture of fresh truffles because removing water at a low temperature could reduce the enzymatic activities in truffles and prevent the loss of volatile compounds (Palacios et al., 2014). Based on sensory evaluation studies, freeze-drying appeared to be the most successful method in conserving the overall original aroma of fresh truffles, although it may lead to a reduction in aroma intensity (Campo et al., 2017). Another sensory analysis study found that freeze-dried and fresh truffles (*T. melanosporum*) were similar in aroma (Marco et al., 2016). Nonetheless, the efficacy of freeze-drying varies among truffle species. The volatile profile of freeze-dried *T. aestivum*

was substantially altered as compared to the volatile profiles of freeze-dried *T. indicum* and *T. melanosporum* (Palacios et al., 2014). Another drawback of this preservation method is that the freeze-dried truffles must be kept in a vacuum-sealed package and the packaging material must be moisture and oxygen impermeable to prevent moisture reabsorption. Freeze-dried truffles must be consumed immediately once the package is unsealed, as they reabsorb moisture almost immediately (Bourdoux et al., 2016). The reabsorption of moisture could lead to the activation of microbes and enzymatic activities which could consequently cause food safety issues and the loss of shelf life (Bourdoux et al., 2016). This author has not found reported examples of a suitable pre-freeze-drying process that could inhibit microbes without the loss of volatiles.

2.3.1.4 Hot air dehydration

Truffle heat dehydration is another conventional preservation method. This method results in a reduction in water activity and microbial growth. It may also inactivate enzymes, which could extend shelf life. However, due to heat application, the aroma quality of truffles is compromised by this method, making it unsuitable as an acceptable preservation technique (Al-Ruqaie, 2006).

2.3.1.5 Canning

Canning is another simple and common long term food preservation method, sometimes employed by the industry to prolong truffles shelf life for commercial applications (Campo et al., 2017; Garvey & Cooper, 2001; Renowden, 2005). Normally, properly canned food may have a shelf life between 2 to 3 years (Rueangwatcharin & Wichienchot, 2015). However, thermal treatment during canning severely affects the organoleptic properties of truffle (Murcia et al., 2003). In addition to the loss of its volatile profile, the texture of canned truffles becomes soft. The aromatic properties of canned truffles have been described by a panel of trained sensory judges as dominated by nut-seed odours, which is different from the fresh truffle aroma (Campo et al., 2017).

2.3.1.6 Irradiation

Irradiation is a potential tool to minimize truffle spoilage through microbial inactivation (Reale et al., 2009a). The individuation of specific irradiation doses is crucial and must be monitored carefully to avoid undesirable chemical modifications to volatile compounds that can affect truffle aroma quality (Reale et al., 2009a). A study

demonstrated that gamma radiation treatments with lower doses (at 1, 1.5, and 2 kGy) were less effective in reducing the microbial contamination on mature black truffle (*T. aestivum*). Gamma radiation treatments with higher doses (at 5 and 10 kGy) gave high efficacy against contaminating microorganisms but negatively influenced the aroma of fresh truffles (Reale et al., 2009a). Similarly, based on a sensory evaluation performed by Rivera et al. (2011a) using trained panels, irradiated truffles were rated lower for aroma than the non-irradiated truffles (control), meaning that the irradiated truffle aroma was unacceptable. It was suggested that a 1.5 kGy dose represented the maximum level of irradiation to maintain the sensorial features of truffles (Reale et al., 2009a). Nonetheless, irradiation treatments did not extend the shelf life of black truffle (*T. melanosporum*) beyond 28 days (Rivera et al., 2011a), which is insufficient to fulfil the continuous demand for truffles throughout the year. Another drawback of irradiation is that it could lead to oxidative degradation of fatty acids (lipidic oxidation), protein degradation, changes in chitin and total phenol level, and the development of undesirable off-flavours if the irradiation dose and type are applied inappropriately (Culleré et al., 2012; Tejedor-Calvo et al., 2019; Zambonelli et al., 2016). In a study performed by Tejedor-Calvo et al. (2019), *T.melanosporum* irradiated with electron-beam and gamma-rays (both applied at 1.5 kGy and 2.5 kGy) showed no significant changes in the total carbohydrates and β-glucans levels but the chitins and total protein content (quantified by Bradford protein assay using bovine serum albumin as the protein standard) were significantly decreased as compared to the non-irradiated control. Irradiation also caused changes in the total phenol level, but the effect depended on the doses and type of irradiation used (Tejedor-Calvo et al., 2019). According to Tejedor-Calvo et al. (2019), a significant reduction in the total phenol level of *T. melanosporum* was only observed when a higher dose (2.5 kGy) of electron-beam and gamma-rays was applied. Irradiation is generally recognized as a safe and effective method to extend the shelf life of food, such as fruits and vegetables, as well as herbs and spices, by food safety authorities in various countries such as the United State, Australia, New Zealand and European. Unfortunately, truffles are not allowed to be irradiated according to the food standard regulated by EFSA, FDA, FSA, and FSANZ (EFSA, 2018; FDA, 2010; FSA, 2017; FSANZ, 2018). Some consumers consider irradiation as an unacceptable processing technique due to safety concerns (Akram & Kwon, 2010). A survey on the acceptance level of irradiated food was carried out in Santiago, Chile in 2011 revealed that about half of the total 497 interviewees associated irradiated food with radioactivity (Junqueira-Gonçalves et al., 2011).

2.3.1.7 Packaging

Modified atmosphere packaging (MAP) has been widely used to extend the shelf life and maintain the visual, textural, aroma and nutritional quality of fresh foods that undergo rapid spoilage (Nesic & Seslija, 2017). In MAP, the quality characteristics of a product is protected against changes caused by microbial growth, oxidation, colour, and aroma degradation by replacing the normal atmospheric gasses with an appropriate mixture of oxygen, carbon dioxide and nitrogen before being sealed within a vapour- or gas-barrier material (Wang & Marcone, 2011). The main advantage of using MAP in food preservation is that it does not require the extensive use of chemical preservatives or stabilizers to improve shelf life. Nonetheless, the ability of MAP to preserve truffles is short with only up to 14 days (Savini et al., 2017) and the effectiveness of MAP is lost once the package is unsealed or leaks. The other limitations of MAP include the high cost of gas packaging machinery and the potential growth of microbes due to inappropriate storage temperatures used by retailers or consumers (Nesic & Seslija, 2017; Savini et al., 2017).

The combination of MAP with microperforated films and refrigeration (4°C) has been used in postharvest storage of truffles (Rivera et al., 2010b). This technique prolonged postharvest shelf life and maintained the organoleptic characteristics of *T. aestivum* and *T. melanosporum* up to 21 days and 28 days, respectively (Rivera et al., 2010b). The typical hard and turgid texture was maintained with minimum weight loss (Rivera et al., 2010b). Although MAP has been able to extend shelf life (Rivera et al., 2010b), this method may cause the accumulation of water on the truffle surfaces, promoting sliminess and microbial growth (Savini et al., 2017) which impairs the purpose of MAP and would jeopardize consumer acceptability (Wang & Marcone, 2011). Furthermore, the shelf life could only be extended up to 28 days which is far from making truffle aroma available throughout the year.

The effect of different packaging conditions on the quality of *T. melanosporum* has been investigated using antioxidant properties and volatile compounds as natural chemical quality markers (Savini et al., 2017). Truffles were packaged under varying gas compositions and refrigerated at 4 °C for 28 days. The quality of truffles packed under partial vacuum, MAP (with a mixture of 1% O_2/99% N_2) and MAP (with a mixture of 40% CO_2/60% N_2) were compared with truffles packed in ambient atmosphere, which served as a control. Based on the volatile profile and antioxidant properties of the samples

measured for up to 14 days, it appeared that vacuum technology and MAP could retain the freshness of truffle better than that of the atmosphere packaging (control) (Savini et al., 2017). Both vacuum technology and MAP showed a similar performance with respect to truffle preservation, but the former technique is less costly than the latter one (Savini et al., 2017). Vacuum technology was suggested to be a cost-effective option for preserving the freshness of *T. melanosporum* (Savini et al., 2017). Compared to normal atmosphere packaging, this method is only suitable for short term preservation as the shelf life of truffles were only be extended from a few days to a maximum of 14 days.

2.3.2 Overall limitations of current preservation techniques

Experimental results investigating different preservation methods demonstrate that the volatile profile and organoleptic quality of truffles can vary considerably depending on the type of preservation technique used (Campo et al., 2017; Culleré et al., 2012; Rivera et al., 2010b; Wang & Marcone, 2011). It can be seen that each preservation method has its own shortcomings. It is still uncertain which preservation method is the most effective to improve the shelf life of truffles whilst maintaining their original texture and organoleptic properties (Wang & Marcone, 2011). Apart from the canning process which allows truffles to last for years, the shelf life extension achieved by most preservation methods is too short to cover the off-season. Even though the shelf life of truffles can be extended for a long period via canning, unfortunately, this technique cannot preserve the aroma quality for any length of time (Campo et al., 2017; Murcia et al., 2003).

All of the above-mentioned limitations beg for a long-term and sustainable solution (Campo et al., 2017) so that the natural truffle aroma can be made available throughout the year to replace artificial truffle aroma mixtures. Innovative techniques for the production of truffle aroma to be used as a flavouring ingredient are required to meet this need, which could add value to lower-priced truffles and contribute to the sustainable growth of the industry.

Table 2.3: Effects of different preservation methods on truffles

Method	Features	Limitations	References
Refrigeration	• most commonly used • slow down spoilage • minimal impact on volatile profile but it cannot successfully extent shelf life beyond two weeks	• in combination with packaging could only extend the shelf life of truffles for up to 14 days • *Pseudomonas* still able to proliferate • cause changes to the biochemical properties • not suitable for all truffle species	(Saltarelli et al., 2008; Savini et al., 2017; Sorrentino et al., 2018)
Freezing	• frequently applied to truffles	• significant changes in the sensory profile of frozen black truffle (*T. melanosporum*) after 15 days of storage at −80°C • showed changes after 24 hours of storage at −20°C • affect texture	(Campo et al., 2017; Culleré et al., 2013a; Zambonelli et al., 2016)
Freeze-drying/ lyophilisation	• dehydration of food by sublimation (below -20°C) • the aroma closely resemble fresh truffle, although with a lower intensity	• may not be applicable to all truffle species • freeze-dried truffles must be kept in a vacuum-sealed package (to prevent moisture absorption) • must be consumed immediately after the package is unsealed as exposure to air would reactivate microbes and cause food poisoning	(Campo et al., 2017; Marco et al., 2016; Murcia et al., 2014; Palacios et al., 2014)

Hot air drying or dehydration	• conventional • reduce water content • reduce microbial growth • cause enzymes inactivation	• affect the aroma quality of truffles	(Al-Ruqaie, 2006)
Canning	• simple and common long term preservation method • employed by companies • thermal treatment • flavour, texture, colour alterations	• severely affect the organoleptic properties of truffles • canned truffles smell like a totally different product	(Campo et al., 2017; Murcia et al., 2003)
Irradiation	• minimize spoilage via microbial inactivation • to maintain the sensorial features of truffles, the recommended maximum dose of gamma irradiation was 1.5 kGy	• the individuation of specific irradiation doses is crucial • did not improve the shelf life of black truffle (*T. melanosporum*) beyond 28 days • might cause changes in chitin and total phenol level as well as protein and fatty acids decomposition that would generate off-flavours in truffles • not allowed to be irradiated by FSANZ	(Akram & Kwon, 2010; Culleré et al., 2012; Reale et al., 2009a; Tejedor-Calvo et al., 2019; Zambonelli et al., 2016)

		• raise some health concerns among some consumers	
Packaging	• improve the shelf life of fresh truffles by slowing down respiration • does not require the addition of chemical preservatives or stabilizers	• costly • the effectiveness of MAP are lost once the package is unsealed or leaks • sliminess and microbial growth might happen due to the accumulation of water on the surface of truffles	(Nesic & Seslija, 2017; Rivera et al., 2010b; Wang & Marcone, 2011)

2.4 Aroma extraction as a potential alternative solution

Increasing demand for natural truffle aroma (Culleré et al., 2013b; Splivallo & Maier, 2012) has resulted in a search for alternative methods to extend its availability. Fresh truffles spoil rapidly along with a significant shift in their volatile profile was observed as a result of senescence and microbial growth (Rivera et al., 2010a). Such spoilage and change in aroma could be avoided by extracting and stabilising the volatile compounds from the fruiting bodies of truffles. The natural truffle extract is expected to have a longer shelf life which may allow natural truffle aroma to be made available all year round for food applications. Up to now, this approach has not been given due attention in scientific literature.

2.4.1 Advantages of truffle aroma extraction

Extraction could be a promising alternative technology for the preservation of truffle aroma to overcome current industry issues. The idea of adopting a truffle extraction strategy for the production of a truffle flavouring ingredient seems to be possible with a few potential advantages this strategy may offer (summarised in Figure 2.2).

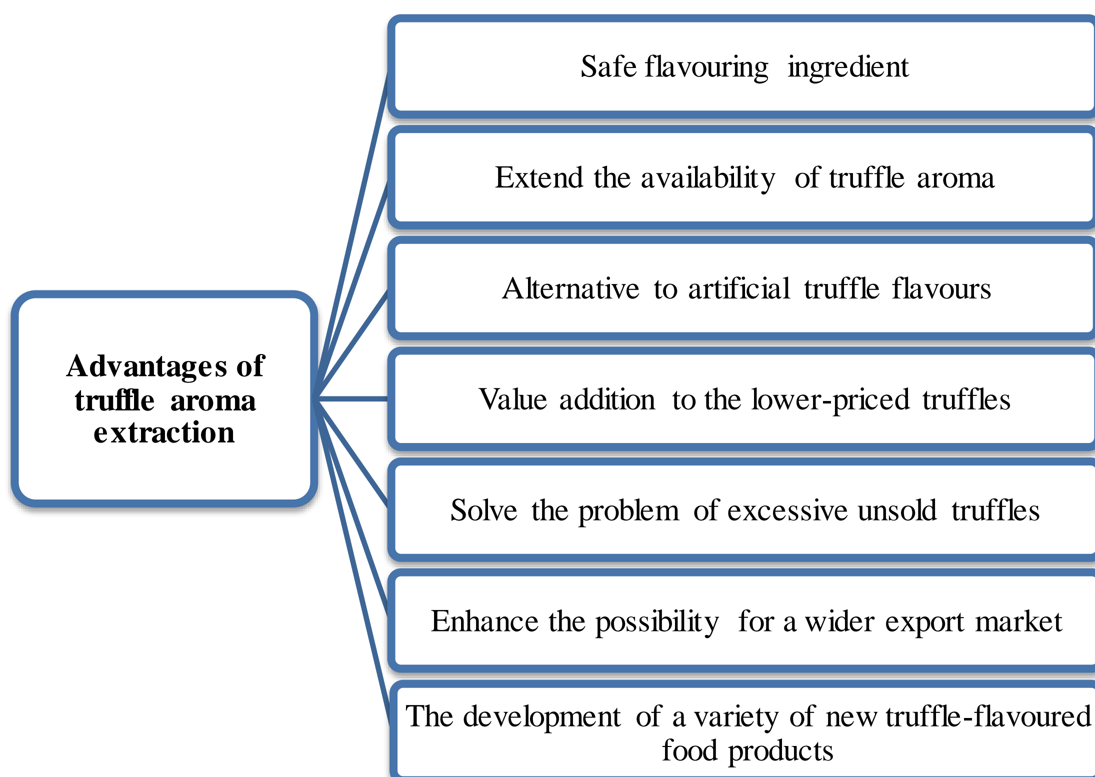


Figure 2.2: List of the advantages of truffle aroma extraction as the potential alternative to preservation methods

2.4.1.1 Truffle aroma extract as a safe flavouring ingredient

Microbial contamination is a major concern of the ready-to-eat food industry, including truffles, since they are usually consumed without additional cooking (Jaroni et al., 2010). It has been reported that extraction techniques, such as supercritical carbon dioxide extraction, can yield microbial-free plant extracts besides retaining their original flavour profile (Martinez, 2007). In view of this, extraction could serve as an incisive decontamination step for truffles, which may allow the removal of potentially contaminating pathogens from truffles and improve the safety of a truffle-based ingredient.

2.4.1.2 Extend the availability of truffle aroma

By removing microbes that are responsible for truffle spoilage via extraction, the resulting truffle aroma extract is expected to have a longer shelf life than the extremely perishable fresh truffles or preserved truffles using current preservation technologies (Ibanez & Cifuentes, 2017). Encapsulation is a technique in which sensitive compounds are surrounded or entrapped by protective wall materials or carriers (Shrestha et al., 2017). As some key odourants in truffles are known to be unstable (Buettner, 2017; Reineccius, 2017), the extract obtained can then be stabilised via encapsulation and made available all year round. The readily available aroma extract could then be conveniently incorporated into food products.

2.4.1.3 Alternative to artificial truffle flavours

Food flavourings are considered one of the important ingredients in any food product development in which a small quantity of flavours incorporated into food products could significantly enhance product quality and consumer satisfaction (Sowbhagya & Chitra, 2010).

As mentioned in Section 2.2.5, currently available artificial truffle flavours are of poor quality (Culleré et al., 2010; Splivallo & Maier, 2012; Splivallo et al., 2011). In view of the increasing consumer demand for natural food ingredients (Sagar et al., 2018; Sowbhagya & Chitra, 2010), the production of a natural truffle aroma via extraction is of high interest to the food sector.

2.4.1.4 Value addition to lower-priced or excessive unsold truffles

Smaller and offcut truffles fetch much less at market regardless of their aroma quality and therein lies an opportunity for value addition to these undervalued products. Numerous studies aimed at turning lower-priced food materials or food processing by-products into value-added food ingredients have been reported (Barretto et al., 2013; Leão et al., 2014; Sagar et al., 2018). For example, producing high-value ingredients such as protein, omega-3 fatty acids, and β-carotene from microalgae has attracted considerable interest due to low-cost biomass production (Adarme-Vega et al., 2012; Phong et al., 2017; Phong et al., 2018). Leão et al. (2014) suggested that the production of natural aromatizing oil from passion fruit waste is feasible as the extracted oil presented a volatile profile similar to that of fresh passion fruit. Likewise, Barretto et al. (2013) reported about 35 different volatile compounds in the pineapple by-product material could be processed into flavouring ingredients. Essential oil from orange waste has been extracted for use by the food industry (Dewi et al., 2018). Similarly, truffle aroma extraction could be an ideal approach that would add value to these lower-priced or unsold products.

2.4.1.5 Export market

The availability of extracted truffle aroma for food applications could increase the possibilities for export to the rising Southeast Asian market which demands a continuous supply (Lee, 2008). Truffle aroma extract produced by Australia could enable counter-seasonal export to countries in the northern hemisphere during their off-season (Garvey & Cooper, 2001). The extraction of volatile compounds from truffles would open an opportunity for the Australian truffle industry to compete with other international suppliers. Turning fresh truffles into natural truffle aroma extract that has a longer shelf life will enable truffle producing countries in the northern hemisphere like France, Italy, Spain, and the United States (Meadows et al., 2020; Zambonelli et al., 2016) to boost counter-seasonal export from Australia and New Zealand during their summer period. Besides that, this approach will also allow the development of sustainable export business to Asia. Overall, this would contribute to the commercial viability of the truffle industry by helping the emerging truffle industry to thrive and expand (Lee, 2008).

2.4.1.6 The development of a variety of new truffle-flavoured food products

Consumer acceptance is the key to successful new food product development (Gil-Chávez et al., 2013). Sensory attributes of a food product such as taste and flavour will

have an important impact on the consumer food choice and acceptance. Consumer expectations and perceptions of food product attributes are that a food product should be able to satisfy the appetite in addition to fulfilling sensory expectations (Gil-Chávez et al., 2013).

The availability of natural truffle aroma could lead to the production of a wide range of innovative truffle-flavoured food products (Pacioni et al., 2014a). The availability of such a pleasant aroma throughout the year could contribute to the sustainable development of a truffle food processing industry by creating new profitable business opportunities.

2.4.2 Commonly used methods for aroma extraction

Extraction methods that are commonly used for extracting volatiles from plant material include supercritical carbon dioxide extraction, Soxhlet extraction, distillation and cold pressing (Capuzzo et al., 2013; Kasim et al., 2014). Using these methods, numerous volatile compounds have been extracted from various plant materials to be used as food flavouring ingredients (Conde-Hernández et al., 2017; El‐Ghorab et al., 2004; Suetsugu et al., 2013). Nevertheless, as far as we know, the extraction of truffle volatiles using these techniques has not been well studied.

The recovery of volatile compounds from various plant materials using supercritical carbon dioxide extraction, Soxhlet extraction, distillation and cold pressing are described below. This provides a basis for developing an extraction process to obtain a natural truffle aroma. For easy reference, the pros and cons of employing these extraction methods for volatile extraction are summarised in Table 2.4.

Table 2.4: Advantages and disadvantages of the extraction methods

Extraction methods	Advantages	Disadvantages	References
Supercritical carbon dioxide extraction	rapidity, high product quality, no solvent residue, tuneable selectivity and solvent power, require low solvent volumes, an excellent technique for heat-sensitive volatile compounds, CO_2 is non-flammable, inexpensive and abundant than organic solvents	high initial capital cost	(Capuzzo et al., 2013; Herrero et al., 2006; Machado et al., 2013; Ormeno et al., 2011)
Steam distillation	common, economic viability, simple, flexible, versatile, scalable	heat applied during the extraction process, time-consuming, may not be efficient in recovering aromas that accumulate inside the cell or be membrane-bound	(Božović et al., 2017; Malaka et al., 2017; Shaaban et al., 2012; Sun et al., 2017)
Molecular distillation /short path distillation	an advanced vacuum distillation method, the distance between evaporator and condenser is reduced, thermal degradation of heat liable components can be minimised due to short	more expensive than the traditional distillation equipment, may not be efficient in recovering aromas that	(Ketenoglu & Tekin, 2015; Lukin et al., 2018)

	residence time and moderate temperature used under high vacuum	accumulate inside the cell or be membrane-bound	
Vacuum distillation	the temperature of vapour phase generation can be reduced by applying vacuum	may not be efficient in recovering aromas that accumulate inside the cell or be membrane-bound	(Lukin et al., 2018)
Spinning cone column distillation	a simple chemical-free process, thermal degradation of heat liable components can be minimised due to short residence time and moderate temperature used, are capable of handling slurries containing high levels of suspended solids	the raw materials being processed may cause fouling and blocking of the equipment	(Flavourtech; Graber et al., 2010; Lukin et al., 2018; Saffarionpour & Ottens, 2018)
Soxhlet extraction	simple, low-cost and requires little training no filtration is needed, a wide range of solvents can be used	heat applied during the extraction process, time-consuming, solvent evaporation after extraction may cause some volatile loss	(De Castro & Priego-Capote, 2010; Gu et al., 2009; Włodarczyk & Zarzycki, 2017)

Cold pressing	simple, technically low-cost, safe, environmentally friendly, energy-saving, no heat involved during the extraction process	usually produces high quality but low yield extract as the extraction relies solely on the pressure	(Mohammed et al., 2016; Thanonkaew et al., 2012)

2.4.2.1 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) is a technique that is an alternative to conventional extraction techniques due to several operational advantages such as rapidity, selectivity, absence of solvent residues, tuneable solvent density and a requirement for low solvent volume (Capuzzo et al., 2013). A supercritical fluid is any substance at a pressure and temperature above its critical point. Under these conditions, supercritical fluid exhibits intermediate properties of gas and liquid. Due to its gas-like character, a supercritical fluid has better penetration and solvent power than liquids, and can diffuse easily through solid materials with a rapid mass transfer rate (Khaw et al., 2017). Its selectivity or solvent power can easily be manipulated with subtle variations in the temperature and/or pressure (Khaw et al., 2017). By releasing pressure, the supercritical fluid returns to a gaseous state and evaporates, leaving behind the solvent-free extract (Ormeno et al., 2011).

Of the supercritical fluids available, carbon dioxide (CO_2) is the most preferred and widely adopted solvent in the food industry because of its practical advantages. It is inexpensive, abundant in pure form worldwide, chemically inert, odourless, colourless, non-flammable, non-explosive and recyclable (Machado et al., 2013). In addition, its critical point occurs at relatively mild conditions (nearly 31.1°C and 7400 kPa), thereby avoiding thermal degradation of the extracts, which is vital when extracting heat-labile volatile compounds (Ikeda, 2014) such as truffle volatiles (Bentley & Chasteen, 2004; Zhu & Cadwallader, 2019).

Due to its capacity to occur under relatively mild conditions, SC-CO_2 is a subject of interest in the fractionation of natural volatile compounds present in complex plant matrices (Capuzzo et al., 2013; Khaw et al., 2017; Machado et al., 2013). For example, the clove oil produced by SC-CO_2 extraction was superior in terms of quality compared to oil produced from steam distillation, hydrodistillation, and Soxhlet extraction (Guan et al., 2007). SC-CO_2 yielded excellent quality lavender (*Lavandula angustifolia* L.) essential oil that had the closest resemblance to the starting material, showing negligible thermal degradation compared to solvent extraction and hydrodistillation (Danh et al., 2013). Likewise, the SC-CO_2 extracts obtained from rosemary (*Rosmarinus officinalis*), *Chamaecyparis obtuse*, *Leptocarpha rivularis*, and kabosu (*Citrus sphaerocarpa* Tanaka) contained a chemical profile that contributes to the aromatic characteristics of the raw materials compared to conventional methods such as hydrodistillation (Conde-Hernández

et al., 2017; Jin et al., 2010; Uquiche et al., 2015; Uquiche & Garcés, 2016) and cold pressing (Suetsugu et al., 2013).

It is interesting to note that 3-(methylthio)propanal and DMDS which are believed to be one of the major contributors to the final aroma impression of truffles (Table 2.2) (Culleré et al., 2013a; Schmidberger & Schieberle, 2017) are also found in garlic (*Allium sativum*) and these two compounds were successfully extracted by SC-CO_2 from garlic (Li et al., 2010). Based on these findings, it stands to reason that 3-(methylthio)propanal and dimethyl disulfide are likely to be extracted from truffles as well.

Given that SC-CO_2 would be able to produce a representative profile of the original components present in other food materials as demonstrated in the above-mentioned studies, it is plausible to propose SC-CO_2 as a promising strategy to extract truffle volatiles for food applications. It is predicted that the heat-labile volatiles in truffles such as methanethiol and DMS with low boiling points would be retained in the extract due to the milder operating conditions of SC-CO_2.

Our hypothesis is further supported by previous literature. To the best of our knowledge, SC-CO_2 extraction of volatile compounds from truffles has been investigated but not broadly studied with only two patents and one recent paper could be found in the literature thus far. The patent filed by Mirande-David (1988) reported that a portion of the aroma could be extracted. Gao et al. (2007) claimed that SC-CO_2 is suitable for industrial application as the process was found to be highly efficient with a high extracting rate and the extract obtained was free from solvent residue. Recent research by Tejedor-Calvo et al. (2021b) also agreed that the use of SC-CO_2 to recover natural truffle aroma fraction is promising and the optimal extraction conditions were at 30 MPa pressure for 3 hours. The addition of grapeseed oil as the lipid matrix into the separators (where extracts were collected) helped trap compounds such as 2,3-butanodione, 2-methyl-1-butanol, octanal, and DMDS may be a potential improvement to the extraction method (Tejedor-Calvo et al., 2021b). As some of the compounds such as ethyl-2-methyl-butanoate, 2-acetyl-pyroline and methional were not extracted, improving the extraction method to increase the number of volatile compounds should be the focus of future study (Tejedor-Calvo et al., 2021b). All this information clearly showed that obtaining an aromatic extract from truffles by SC-CO_2 is feasible.

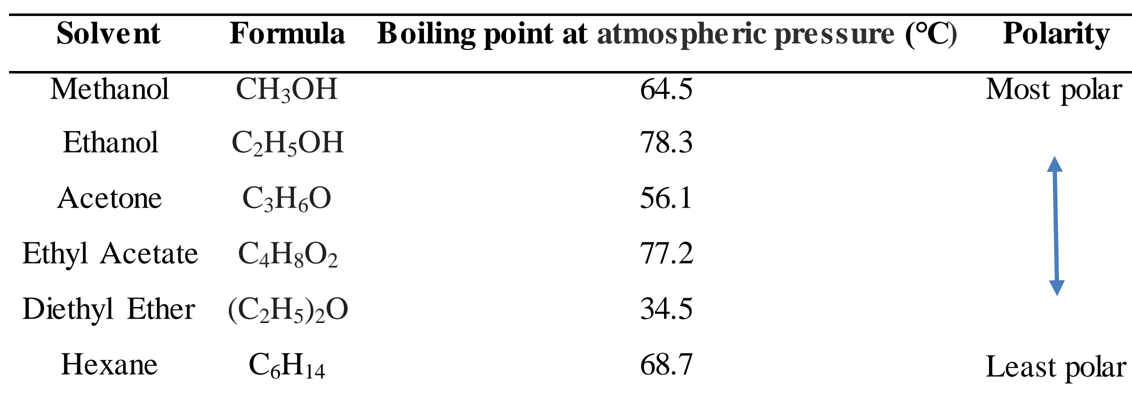
2.4.2.2 Solvent extraction

Solvent extraction is a conventional method that involves the process of separating and recovering valuable soluble components (the solute) from a solid or liquid mixture by dissolving them in a suitable solvent (Joana Gil-Chávez et al., 2013). This method has been used to obtain the volatile compounds from different plant materials such as *Citrus* spp. and *Fortunella margarita* swingle (González-Mas et al., 2019; Sicari & Poiana, 2017).

Solvent extraction is known for its simplicity, ease of operation, and low processing cost. However, there are several drawbacks of using this method. These include long processing time and large amount of solvent consumption. Additionally, there is a need for an evaporation or concentration step for target compound recovery and this would result in degradation or loss of some compounds in some cases (Joana Gil-Chávez et al., 2013; Liang et al., 2020).

The solvent choice is vital in determining an extraction performance. The compatibility between the molecular and physical properties of the solvent and target compounds is critical in selecting an appropriate solvent (Prososki et al., 2007), as it needs to be miscible with the solute in order to extract it, for which its polarity needs to be considered. Some of the most widely used solvents with varying chemical and physical properties in the extraction procedures are shown in Table 2.5. Other factors influencing the selection of solvent choice include toxicity, flammability, reactivity, cost, and stability. Understanding the potential hazards such as toxicity, flammability, and stability of a solvent during storage is essential as it requires caution in its use and handling.

Table 2.5: Chemical and physical properties of commonly used extraction solvents



Solvent	Formula	Boiling point at atmospheric pressure (°C)	Polarity
Methanol	CH_3OH	64.5	Most polar
Ethanol	C_2H_5OH	78.3	
Acetone	C_3H_6O	56.1	↕
Ethyl Acetate	$C_4H_8O_2$	77.2	
Diethyl Ether	$(C_2H_5)_2O$	34.5	
Hexane	C_6H_{14}	68.7	Least polar

Source: Adapted from (Reichardt & Welton, 2010)

2.4.2.3 Soxhlet extraction

Soxhlet extraction has been widely studied for decades in extracting flavour components from different plant materials (De Castro & Priego-Capote, 2010). In general, the extracting solvent is heated to boiling and the condensed solvent vapours are passed through a cellulose or glass thimble containing the material to be extracted. The filtrate runs back into the reservoir of boiling solvent completing the cycle (Jadhav et al., 2009; Sagar et al., 2018).

This method is simple, low-cost and no filtration is needed after extraction. A wide range of solvents can be used depending on the molecular affinity between the target compound and the choice of solvent (Włodarczyk & Zarzycki, 2017). As the samples are treated at high temperature over a long period, this method could result in the thermal degradation of some heat-labile target compounds (Gu et al., 2009; Zhang et al., 2018). In collecting the extract, the solvent needs to be evaporated, which could lead to some volatiles being lost (Wong et al., 2014).

Soxhlet extraction has been suggested to be more suitable for extracting low-volatile compounds as the recovery of high-volatile compounds has been reported to be poor (Gu et al., 2009). For example, Soxhlet extraction was less efficient in recovering high-volatile components such as benzyl alcohol, linalool oxides, and linalool from Pu-erh tea (Gao et al., 2017). In truffle aroma extraction, the recovery of high-volatile compounds is also expected to be less efficient. Possible thermal degradation of some volatiles is also likely to happen as most of the important volatile compounds in truffles are heat-labile (see Section 2.4.3 for details) (Bentley & Chasteen, 2004; Zhu & Cadwallader, 2019), suggesting the industrial applicability of this method in obtaining high quality of truffle aroma may be limited.

2.4.2.4 Cold pressing

Owing to its ability to produce authentic and unique aroma, such as in the extraction of citrus essential oils, cold pressing is commonly used to produce essential oils that contain volatile compounds (Asikin et al., 2012; Thanonkaew et al., 2012). The principle of this process is based on machine pressing the plant materials to release essential oils at room temperature (Aydeniz et al., 2014). Damage to heat-sensitive bioactive and flavour compounds are avoided as the cold-pressing process involves no heat application (Aydeniz et al., 2014; Mohammed et al., 2016).

Cold pressing has been successful in recovering a wide range of high quality essential oils from plant materials such as grapefruit (*Citrus paradisi*) (Ou et al., 2015) and orange (*Citrus sinensis* (L.) Osbeck) (Qiao et al., 2008). Another study also showed that cold-pressed Safflower (*Carthamus tinctorius*) oil contained higher phenolic, antioxidant and α-tocopherol content than their solvent extracted counterparts (Yilmaz & Güneşer, 2017). As cold pressing always results in aroma extracts that have an odour similar to that in the original raw material without causing thermal degradation to the volatile compounds, the same may hold for truffle volatile compounds that are thought to be heat-labile (Bentley & Chasteen, 2004; Zhu & Cadwallader, 2019). Nonetheless, low extraction yield and poor extraction efficiency are expected in the truffle aroma extraction as this method relies solely on mechanical pressure (Mohammed et al., 2016; Thanonkaew et al., 2012). The addition of solvent during the pressing process, which may act as a driving force in facilitating the aroma release from the truffle matrix, may be required to improve the extraction efficiency. The main problem in this process is that at the end of the extraction process, the solvent added needs to be separated and removed from the extraction mixture to obtain a solvent-free extract. But the heat and pressure applied during this step may result in the loss of some aroma volatiles and the chemical breakdown of others, altering the final flavour profile of the extract. Apart from this, the process can be laborious and time-consuming, which could be costly at an industrial level. Most of the required truffle volatile compounds would suffer chemical breakdown, volatise and be lost as the solvent is removed (Božović et al., 2017; Chemat & Boutekedjiret, 2016; Gu et al., 2009; Starmans & Nijhuis, 1996; Zhang et al., 2018). Due to these reasons, cold pressing does not seem to represent an attractive choice for aroma recovery from truffles.

2.4.2.5 Steam distillation

Steam distillation is another commonly used technique to extract natural volatile compounds from plant materials (Tongnuanchan & Benjakul, 2014). Its advantages include competitive cost, scalability, and the ability to produce high purity oil (Božović et al., 2017; Malaka et al., 2017). Nonetheless, the heat applied may cause the breakdown of any heat-labile volatile compounds (Božović et al., 2017; Chemat & Boutekedjiret, 2016; Gu et al., 2009; Starmans & Nijhuis, 1996; Zhang et al., 2018). The distillation extraction is based on the principle that, at the boiling temperature, the combined vapour pressure equals the ambient pressure. The boiling point of aromatic substances in essential oils normally range from 100 to 300°C. However, in the presence of steam or boiling water, these substances are evaporated at a temperature close to that of water boiling point

(100 °C) at atmospheric pressure (Božović et al., 2017; Chemat & Boutekedjiret, 2016; Khan & Dwivedi, 2018; Rassem & Nour, 2016). In other words, this process effectively enables essential oils to be distilled at temperatures lower than the boiling points of their individual constituents, which may minimise the loss of aroma (Božović et al., 2017).

Steam distillation has shown promise by successfully extracting a wide range of volatiles from various plant materials such as bergamot (*Citrus bergamia* Risso et Poiteau) essential oil (Belsito et al., 2007) and *Cuminum cyminum* essential oil (Li et al., 2009). But all the volatiles studied using this method have different chemical characteristics compared to the truffle volatiles which are highly volatile and reactive (see Section 2.4.3 for details). The high temperature applied coupled with a long extraction time during the distillation process might lead to possible degradation of relatively unstable odorants and a loss of the most volatile molecules. More studies are required to determine the applicability of steam distillation on truffle aroma extraction.

2.4.3 Which extraction method is the most applicable for truffles?

As mentioned in Section 2.1.3, only some odourants (see Table 2.2 as an example) contribute to the final aroma impression of truffle (Strojnik et al., 2020; Vahdatzadeh et al., 2015) and they can be used as indicators to determine the effectiveness of an extraction process. The chemical characteristics of some key volatiles found in white and black truffles (Table 2.2) are taken as examples for discussion in this section. DMS, which is generally considered the most important volatile in truffles due to its ubiquitous presence and representative truffle smell, is a nonpolar, hydrophobic sulfur-containing compound with high susceptibility to oxidation and thermal degradation (Bentley & Chasteen, 2004; Zhu & Cadwallader, 2019). Similarly, other volatile sulfur compounds such as DMDS and DMTS are also known for their strong reactivity (Vazquez-Landaverde et al., 2006). Methional or 3-(methylthio)propanal is another sulfur-containing compound that contains both aldehyde and thioether functional groups; it is heat-labile and easily breaks down to methanethiol (Di et al., 2003; Weerawatanakorn et al., 2015). Methanethiol is readily oxidized to form DMDS and DMTS in the presence of oxygen (Vazquez-Landaverde et al., 2006). Diacetyl or 2,3-butanedione, being categorised as a small, polar, hydrophobic diketone, is highly volatile, even at low temperatures (Clark & Winter, 2015). Overall, the natural variation in the chemical properties and activity of these key volatiles suggest that the extraction of truffle aroma is rather challenging. Volatile loss or degradation that give rise to off-flavour notes have

been reported when treating truffles at high temperature (Culleré et al., 2013a), thus operating an extraction process under mild conditions such as low temperature and short extraction time is preferable to avoid such undesirable changes (Ikeda, 2014; Junior et al., 2010).

When evaluating the extraction procedures of each method based on the properties of some key volatiles as discussed above, it appears that SC-CO_2 extraction, which can be operated under mild conditions with "tuneable" properties and without requiring an additional solvent removal step, could be the most promising strategy in extracting truffle aroma compared to the other conventional methods. Several previous studies (Section 2.4.2.1) which demonstrated the capability of SC-CO_2 extraction in preserving the original extract composition and properties have further strengthened our hypothesis (Coelho et al., 2003; Conde-Hernández et al., 2017; El‐Ghorab et al., 2004; Guan et al., 2007; Suetsugu et al., 2013). A more specific example where the SC-CO_2 extraction is deemed as a potential method for truffle aroma recovery is supported by the successful recovery of two key volatiles (3-(methylthio)propanal and DMDS) that are also present in truffles (Table 2.2) from garlic (Section 2.4.2.1). Additionally, SC-CO_2 extraction offers features that could overcome some limitations that exist with the conventional methods (Khaw et al., 2017) as listed in Table 2.4, making it an attractive option for truffle aroma extraction.

In addition to SC-CO_2 extraction, the use of conventional methods for truffle aroma extraction should also be given due attention as their processing conditions can be manipulated or further improved in combination with other technologies. For example, the operating temperature in distillation can be lowered by using a low-boiling organic solvent or by operating the extraction process under reduced pressure (Belsito et al., 2007; Li et al., 2009).

Overall, the notion of extracting volatile compounds from truffles is appealing and yet challenging. Numerous experimental trials are required before an extraction technique can be a feasible choice. Some potential challenges that may arise during volatiles extraction will be discussed in the "Challenges and future perspectives" section.

2.5 Challenges and future perspectives

Food preservation aims at conserving the organoleptic properties of food products while guaranteeing their safety and extending shelf life. However, consumer demand for natural

truffle aroma throughout the year could be not effectively satisfied due to the limitations of the current preservation methods (Splivallo & Culleré, 2016; Wang & Marcone, 2011). In view of numerous potential advantages that an extraction approach could deliver, the notion of extracting aroma from truffles appears to be a promising alternative to address the issues faced by the truffle industry. Nonetheless, extracting volatile compounds from truffles without compromising the aroma quality is challenging as some of the key odourants are known to be highly volatile (Buettner, 2017; Reineccius, 2017). Furthermore, due to natural variation in the volatile profile (Strojnik et al., 2020; Wang & Marcone, 2011), different truffle species may yield different extraction results. Hence it is difficult to estimate the extraction quality without the research being conducted first.

All processing stages, from pre-extraction to extraction are equally crucial as each step might significantly affect the quality of an extract. Hence, operating parameters such as solvent system, solvent-to-feed ratio, particle sizes of the starting material, temperature, pressure, treatment duration, and agitation rate should be optimised carefully according to strict scientific methods (Putnik et al., 2018). Appropriate storage and packaging conditions are the key to aroma stability and should be handled carefully (Kemp et al., 2011; Malcolmson et al., 2000). Selecting a suitable extraction process to produce a truffle flavouring ingredient that has the potential to be commercialized can be tricky. In general, an ideal extraction method should be characterised by simplicity, rapidity (Sinha et al., 2008), high selectivity, no solvent residue, high aroma retention, and high processing efficiency (Ibanez & Cifuentes, 2017).

Processing procedures must comply with food safety standards to ensure that the end product is safe for human consumption. As none of the existing extraction methods are perfect, the limitations of each method need to be taken into consideration when selecting a suitable extraction process. Some extraction methods such as solvent and Soxhlet extraction require the use of solvents (Włodarczyk & Zarzycki, 2017). Selecting an appropriate solvent that is approved for food use with the greatest affinity for the target volatile compounds is vital as it may affect the extract quality (Wong et al., 2014). At the end of the extraction process, the solvent removal step must be monitored carefully to minimise the loss of volatiles from the extract. This is pertinent to truffle aroma extraction as most of the key odourants in truffles are heat-labile (Bentley & Chasteen, 2004; Zhu & Cadwallader, 2019). The lack of technological advancements in instrumentation and software as well as adequate knowledge about truffle volatile compounds would hinder the successful design of a suitable extraction method. More fundamental studies such as

understanding the behaviour of key volatile constituents in truffles (Wang & Marcone, 2011) and identifying key factors that influence truffle aroma quality (Rivera et al., 2010a; Romanazzi et al., 2015) may be useful to improve the extraction process.

The increasing consumer demand for natural flavouring ingredients puts pressure on the commercial production of natural truffle aroma (Cataldo et al., 2016; Lukin et al., 2018; Taylor & Hort, 2007). The industrial interest lies in the cost-effective extraction of volatile compounds from fresh truffles that are of second or offcut grade for food application. With the development of more sophisticated extraction methods in the near future, it is believed that the extraction of truffle volatiles can be achieved with minimal loss. Extraction can be considered a potential alternative to the current methods in preserving truffle aroma as this approach would provide sustainable growth to the truffle industry and create a new business opportunity in the food industry.

2.6 Encapsulation could potentially be used to preserve and stabilise truffle aroma

Encapsulation is defined as a process in which an active material (guest) is entrapped or surrounded by an inert material, also known as coating or carrier material (host) (Shrestha et al., 2017; Wang et al., 2015). This approach has been widely used in the food industry to protect sensitive substances (guest) against adverse environmental conditions during processing or storage, such as light, pH, oxygen, humidity, high temperature, high shear mixer, and variations in pH (Marcillo-Parra et al., 2021; Shishir et al., 2018). With the capability to minimise degradation or loss of chemically unstable compounds during processing and storage, encapsulation has shown promise in improving the storage stability and increasing the shelf life of the final product (Wang et al., 2015).

2.6.1 Encapsulating materials

In the food industry, a carrier material (host) used for encapsulation must be food-grade, able to confer maximal protection to active substances (guest) against various environmental conditions without reacting with the guest compound, and possesses good rheological properties at high concentration (Nedovic et al., 2011). A wide range of encapsulating carrier materials such as hydrocolloids (pectin, gum Arabic, chitosan, alginate), lipids (phospholipids, hydrogenated vegetable oils, monoglyceride and triglycerides), proteins (gelatine, casein, soy protein, whey protein), and carbohydrates (modified starch, cyclodextrins (CDs), maltodextrin) can be used to encapsulate

flavouring ingredients (Vila et al., 2015; Zuidam & Heinrich, 2010). Among these encapsulating materials, CDs are commonly used as carriers and stabilisers by the food industry to protect sensitive ingredients such as flavours, aromatising agents and others from unfavourable conditions during processing and storage (Marques, 2010; Zuidam & Heinrich, 2010).

CDs are a family of macrocyclic oligosaccharides consisting of several glucose entities. They are synthesised by the enzymatic digestion of starch using glycosyl transferases (Duchêne & Bochot, 2016; Wang et al., 2015). CDs have a truncated cone structure with a hydrophobic inner cavity and a hydrophilic exterior. The unique structure and steric arrangement of CDs allow CDs to form complexes with a wide range of compounds (Duchêne & Bochot, 2016). CDs which are comprised of α-glucose units connected by α-1,4-glycosidic bonds, are categorised based on the number of glucose residues in their structure. They are available in three forms, namely α-, β-, and γ-CD, which composed of six, seven, and eight D-glucose repeating units, respectively. β-CD is the most commonly used variant in the food industry as compared to other CDs, owing to its simple production and low-cost (Reineccius et al., 2002; Wang et al., 2015).

As most flavour compounds are highly volatile and chemically unstable in the presence of light, air, heat and moisture, β-CD has been widely used to encapsulate and stabilise a wide range of flavouring ingredients for food application against undesirable changes by processing or on storage (Marques, 2010; Shrestha et al., 2017). These include oregano (*Origanum onites* L.) essential oil (Kotronia et al., 2017), sweet orange flavour (Zhu et al., 2014), and strawberry flavour (Balci-Torun & Ozdemir, 2021). To the best of our knowledge, capturing the aroma volatiles of truffles by encapsulation has yet to be reported in the literature. Sulfur-containing volatiles such as DMS, DMDS, and DMTS represent the most important group of odorants in truffles (Strojnik et al., 2020; Vahdatzadeh et al., 2015) and these compounds are known for their strong reactivity (Bentley & Chasteen, 2004; Vazquez-Landaverde et al., 2006; Zhu & Cadwallader, 2019). Given that CDs have been proven to protect and stabilise DMS and DMDS (Reineccius et al., 2002), it stands to reason that these key volatiles that are present in truffle are likely to be encapsulated by CDs.

2.6.2 Encapsulation of volatile compounds by CDs

Apart from the characteristics of the encapsulating carrier material (host) and the type and concentration of the guest compound, the encapsulation efficiency also depends on the method parameters such as the adding order of components, the solvent used, and the drying process (Da Rocha Neto et al., 2018; Maes et al., 2019). The encapsulation of volatile compounds using CDs to produce a powdered flavouring ingredient can be achieved by three methods (Furuta et al., 1994). The most popular method involves the formation of an inclusion complex in an aqueous solution of CD. In this method, the guest compound is dissolved into a CD solution to form a crystalline complex (Furuta et al., 1994; Shrestha et al., 2017). Following the complex formation, the crystalline complex is separated and dried. The second method is to blend the guest compound into a heavy suspension of CD and subsequently the complex is separated and dried (Furuta et al., 1994). The third method is called kneading, also known as slurry complexation. In this method, the guest compound can form a complex with β-CD upon direct kneading with a small amount of solvent and subsequent drying of the resulting paste (Santos et al., 2015; Tao et al., 2014). The kneading method is superior and has been preferred compared to the former two methods as there is no separation of the crystalline complex from the dilute aqueous solution and therefore it requires less energy for dehydration (Furuta et al., 1994; Shrestha et al., 2017). Studies show that kneading complexes of β-CD and natural antimicrobial agents such as carvacrol and thymol demonstrated a high entrapment efficiency, enhanced antimicrobial activity, and good storage stability (Santos et al., 2015; Tao et al., 2014).

The above-mentioned encapsulation methods have been studied to entrap tea tree oil (Shrestha et al., 2017). Changes in the volatile profiles of the encapsulated products produced by different methods were insignificant and appeared similar to the original tea tree oil (Shrestha et al., 2017). As no such encapsulation study has been reported using fresh truffles as the source of guest molecules, it would be interesting to investigate the possibility of encapsulating truffle volatiles before fresh truffles deteriorate and produce off-flavour. Through encapsulation, the production of a natural flavouring ingredient from smaller, offcut or excessive unsold truffles may allow value addition to these lower-priced truffles, benefiting the truffle industry.

2.6.3 Advantages and limitations of capturing truffle volatiles using CDs

The advantages of processing fresh truffles into a dried flavouring ingredient through encapsulation include typically stable and consistent compositions, improved flow properties, allow easy handling of dry powders with the consequent reductions in packing, transportation and storage costs that lead to economic and technological advances, labour savings, and an extended period of storage. The encapsulated product which are processed in a dried powder form may allow simple dosing and can be ready for industry application which provides convenience for consumers (Marques, 2010).

By adopting encapsulation, the loss of aroma components can be minimised, the truffle aroma can be stabilised, the storage period can be prolonged, and the encapsulated truffle powder can be conveniently transported and made readily available for use as a flavouring ingredient in food application throughout the year. The production of a natural truffle flavouring ingredient is highly desirable for the food industry as this would promote the development of a variety of new truffle-flavoured food products, thereby improving the food purchase interest among consumers (Madene et al., 2006; Yin et al., 2017).

Despite the advantages, the encapsulation of guest compounds using CDs has some limitations. These include the cost of CDs, limited loading capacity, may be restricted to low-molecular-weight guest compounds, the encapsulation efficiency can be influenced by the polarity and size of the core material, and the production of particles with irregular forms (Estevinho & Rocha, 2017; Tavares et al., 2021). The segregation problem associated with the encapsulation of garlic essential oil using CDs has been reported, in which some compounds are preferably encapsulated over others, depending on the size and polarity of the compounds found in garlic essential oil (Tavares et al., 2021).

In view of the advantages of encapsulation, which outweigh its limitations, encapsulation could be a promising technique to provide a barrier to the volatilisation of truffle aroma and achieve its storage stability. Up to now, however, there are no published work investigating the potential of encapsulating fresh truffles volatiles using CDs as the encapsulating agent.

2.7 Consumer and industry expectations

Understanding consumer behaviour and perception of food purchasing and consumption (Siegrist, 2008) is a prerequisite for the successful introduction of a natural truffle

flavouring ingredient into the food market. Consumer demand for healthy, safe, diverse and innovative food products is growing (Doyle & Glass, 2010; Oliach et al., 2021). Truffle flavouring with excellent organoleptic quality would always be the consumers' top preference. Hence, the selection of a cost-effective processing strategy without compromising the aroma quality is essential for the successful production of a natural truffle flavouring ingredient, which has not yet been demonstrated, as the use of current preservation techniques lead to the loss of truffle aroma. To ensure the sustainable and constant supply of natural truffle aroma during the off-season, the production of truffle flavouring, which would have a long shelf life and good aroma quality (Splivallo & Culleré, 2016) is highly desirable. The safety of the product has to be established before it can be made commercially available. The natural truffle flavouring should be free from microbial and solvent residues or these contaminants should be at least below the safety limit as per the relevant food safety standards. Given that there is a constant demand by the restaurant and food service industries throughout the year for truffle aroma as a flavouring ingredient (Pacioni et al., 2014a), the production of a natural truffle flavouring ingredient that closely resembles the aroma of fresh truffle holds great commercial potential.

CHAPTER 3: Microbiological evaluation of whole, sliced, and freeze-dried black truffles (*Tuber melanosporum*) under vacuum packaging and refrigerator storage

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Abstract

Black truffle (*T. melanosporum*) is usually available in the form of whole, offcut pieces (sliced to remove defects or broken from larger truffles), or freeze-dried (one of the preservation methods) but there is insufficient knowledge about microbial spoilage of these products. The changes in the microbiology of whole, sliced, and freeze-dried black truffles (*Tuber melanosporum*) were determined in this study. All truffle samples were vacuum-packaged and stored at 4 ± 2°C for 30 days and evaluated on day 0, 4, 8, 15 and 30. The total plate count, *Pseudomonas* spp. count, yeast and mould count as well as the presence of *Listeria* spp., *Salmonella* spp., and *Bacillus* spp. were examined. The main finding of this study was that the total microbial count, *Pseudomonas* spp. count, and yeasts count associated with the freeze-dried truffles were generally lower than that of the whole and sliced truffles. Whilst mould, *Listeria* spp., and *Salmonella* spp. were not detected (< 2.00 log CFU/g), *Bacillus* spp. were detected at a very low count in all sample types (< 3.48 log CFU/g). Overall, the results suggest the need to establish an effective decontamination treatment before packaging and storage to delay microbial spoilage.

Keywords: Black truffles, freeze-drying, microbiological evaluation, sliced truffles, storage studies, vacuum packaging

3.1 Introduction

Truffles (*Tuber* spp.) are considered one of the most expensive and sought-after food materials globally due to their excellent attributes and unique organoleptic features (Reale et al., 2009b; Romanazzi et al., 2015). Of all the edible truffles, Black Périgord truffle (*Tuber melanosporum* Vittad. 1831) is one of the most important commercial species owing to its high organoleptic quality (Culleré et al., 2010).

As truffles grow in the ground, they are typically colonised with diverse microbial communities such as bacteria, viruses, yeasts, and guest filamentous fungi, some of which may cause loss of shelf life (Rivera et al., 2010a; Splivallo et al., 2015; Vahdatzadeh et al., 2015). Because of the aroma strength and high price, truffles are normally used as a flavour enhancer to add to food once plated and are eaten uncooked after cleaning (Pacioni et al., 2014a). Simple surface cleaning is a common practice used in the industry to remove soil and other contaminants from fresh truffles after harvesting (Rivera et al., 2010a). This procedure, however, is not an effective microbial decontamination step as a significant microbial load could remain on and in the truffles (Rivera et al., 2010a). Yet, there is no clear established microbiological standard specifically for truffles by food legislation bodies such as Food Standards Australia New Zealand (FSANZ), European Food Safety Authority (EFSA), Food Standards Agency (FSA) as well as U.S. Food and Drug Administration (FDA) (Rivera et al., 2010a; Sorrentino et al., 2018).

The shelf life of truffles has been closely associated with the microbial communities that colonise them (Campo et al., 2017). Microbial growth on truffles leads to the deterioration of organoleptic quality through off-flavour development, loss of aroma, and texture changes (Leyva Salas et al., 2017). To extend shelf life, several preservation methods have been established to control microbial growth (Campo et al., 2017; Culleré et al., 2012; Rivera et al., 2010b; Wang & Marcone, 2011). Nonetheless, each method has one or more limitations where the truffle organoleptic quality is negatively impacted.

Refrigeration combined with packaging is the most frequently used preservation method, but this technique could only extend shelf life for a relatively short-term (Savini et al., 2017; Sorrentino et al., 2018). Freezing truffles is not considered a viable strategy as it alters their organoleptic quality (Campo et al., 2017; Culleré et al., 2013a; Zambonelli et al., 2016). The application of high temperature such as in canning and hot air drying have been shown to alter the organoleptic properties of truffles (Al-Ruqaie, 2006; Campo et al., 2017; Murcia et al., 2003), furthermore, whilst irradiation could reduce spoilage microorganism numbers (Reale et al., 2009b), irradiated foods have frequently raised safety concern among consumers (Akram & Kwon, 2010) and truffles are not permitted to be irradiated in certain countries such as Australia and New Zealand (FSANZ, 2018).

Freeze-drying, although it results in a decrease in the aroma intensity, has potential to be a preferred approach in preserving the overall original aroma of truffle compared to other techniques (Campo et al., 2017). Water removal by sublimation could mitigate the loss

of volatile compounds from truffles (Palacios et al., 2014). Close sensorial proximity between freeze-dried and fresh truffles has been demonstrated in a previous study (Marco et al., 2016). Furthermore, microbial growth inhibition in truffles could be achieved by freeze-drying as most microorganisms cannot grow under low water activity conditions; however, these organisms can re-commence growth if the moisture level increase (Bourdoux et al., 2016). The microbial population on truffles could include organisms such as *Salmonella, Listeria*, and *Bacillus* species that are most commonly associated microbial risks with low-moisture foods (Bourdoux et al., 2016; Sánchez-Maldonado et al., 2018).

Truffles are graded mainly based on physical appearances such as shape, size, and weight (The Australian Truffle Growers Association, 2014; UNECE, 2017). Whilst some truffle pieces could have been broken from larger truffles, during processing some truffles may have some parts sliced to remove defects such as surface imperfections and damage marks resulting in truffle pieces. These offcut truffle pieces are generally classified as second-grade truffles according to the truffle grading standard (The Australian Truffle Growers Association, 2014; UNECE, 2017).

There is no published literature about the viability of microorganisms such as total aerobic microorganism, *Pseudomonas* spp., yeast and mould in *T. melanosporum* that have been sliced (offcut truffle pieces) and freeze-dried compared to the whole truffles throughout storage. There is also a lack of information on the occurrence of *Listeria* spp., *Salmonella* spp., and *Bacillus* spp. in the whole, sliced, and freeze-dried truffles during storage. For these reasons, the occurrence of microbes in three differently processed truffles (whole, sliced, and freeze-dried truffles) throughout 30-day storage was evaluated in the present study. All samples were vacuum-packaged and stored at 4 ± 2°C and evaluated on day 0, 4, 8, 15, and 30.

3.2 Materials and methods

3.2.1 Fresh truffles

Truffles were harvested in February 2019 from a farm in the Catalonia region of Spain at the standard commercial harvesting stage. All the freshly collected samples were brushed gently with a soft brush to remove soil as per standard commercial practice. After rinsing with water, they were air-dried in a laminar cabinet to remove surface moisture. All samples were individually wrapped in paper towels to prevent cross-contamination before

being packed into insulated containers with ice packs held at 4 ± 2°C (Rivera et al., 2010a) and couriered to the Food Science laboratory at Curtin University within one week of harvest. The microbial analysis was initiated immediately (day 0) upon arrival of the truffles.

3.2.2 Sample preparation and storage conditions

Upon receiving the samples, under aseptic conditions, paper towels were removed and a qualitative selection of truffles was made by carefully inspecting their visual appearance. Those free of defects with a similar appearance were selected; those that were infested with parasites, spoiled or with softened texture were rejected (Culleré et al., 2012).

3.2.2.1 Whole truffles

Three randomly selected fresh truffles were individually packaged into vacuum package bags (65 µm Polyamide/Polyethylene, FPA Australia Pty Ltd, Australia) using a vacuum packaging machine (easyPACK-mk2, Webomatic, Bochum, Germany). They were stored at 4 ± 2°C for 30 days. Due to the availability and the cost of truffles (Pacioni et al., 2014a; Romanazzi et al., 2015), at each analysis day, 1 g of sample from each bag was aseptically cut for microbial analysis and the remaining truffle was vacuum-packaged and stored at 4 ± 2°C until the next analysis. The sample preparation and analysis were done in such a way because it is industrial/common practice that truffles are usually used as a flavour enhancer rather than as a food product owing to their strong scent and high price (Pacioni et al., 2014a; Romanazzi et al., 2015). In general, only a few thin slices of truffles are shaved from a whole single truffle which are sufficient to add flavour to a meal. After slicing, the remaining whole truffle will be stored in a package until required for the next preparation (Pacioni et al., 2014a).

3.2.2.2 Sliced truffles

Knowledge about microbial growth in offcut truffle pieces is limited. Microbial contamination may be higher in the offcut truffle pieces than the whole truffles due to more exposed surface area and higher moisture and nutrients release when sliced. In this study, truffles were sliced to represent the offcut truffle pieces (the second-grade truffles). All materials used for truffle cutting and handling were continually disinfected. Knife and forceps were flamed at regular intervals. About 20 fresh truffles were randomly selected and aseptically cut into thin slices (5mm) (Díaz et al., 2002). All sliced truffles were

combined and mixed well before being divided into two portions, one for the sliced and the other for freeze-dried truffles (Section 3.2.2.3). A total of 15 bags with each containing 5 g of sliced truffles were vacuum packaged immediately and stored at 4 ± 2°C for 30 days. At each sampling day, three bags were randomly selected and 1 g of sample from each bag was aseptically withdrawn for microbial analysis.

3.2.2.3 Freeze-dried truffles

About 400 g of sliced truffles prepared in Section 3.2.2.2 were freeze-dried for 48 h at -30°C, 0.37 mbar using a freeze dryer (Christ Alpha 1-2 LD plus, Germany). Once freeze-dried, a total of 15 bags with each containing 5 g of freeze-dried truffles were vacuum-sealed separately and stored at 4 ± 2°C for 30 days. At each sampling day, 1 g of sample was aseptically withdrawn from a randomly selected bag for microbial analysis.

3.2.3 Microbiological analysis

The microbial growth in the whole, sliced, and freeze-dried truffles were analysed separately on day 0, 4, 8, 15 and 30 of storage in triplicate. For each analysis, 1 g of each sample was aseptically withdrawn and mixed with 9 ml of sterile buffered peptone water (Oxoid Ltd, Victoria, Australia) and homogenised for 2 min (PRO250, PRO Scientific Inc., Oxford, USA). Serial dilutions were performed in sterile phosphate-buffered saline, pH 7.4. Subsequently, aliquots of 0.1 mL from each dilution were spread plated onto the surface of the respective culture medium and incubated accordingly.

Total plate counts were determined after spread plating on tryptone soya agar (TSA) (Oxoid Ltd, Victoria, Australia) and incubated at 37 ± 1°C for 48 h. Based on the procedures described by the manufacturer's manual, *Pseudomonas* spp. isolation agar (Becton Dickinson Pty Ltd, NSW, Australia) incubated at 37 ± 1°C for 48 h was used to culture *Pseudomonas* spp. Yeasts and moulds were cultured on Dichloran Rose-Bengal Chloramphenicol (DRBC) agar (Merck Pty Ltd, Victoria, Australia) incubated at 25 ± 1°C for 5 days. The presence of *Bacillus* spp. was determined on Polymyxin Egg Yolk Mannitol Bromothymol Blue Agar (PEMBA) (Oxoid Ltd, Victoria, Australia) incubated at 37 ± 1°C for 24 h.

To detect *Salmonella* spp., each sample from the first dilution was enriched by inoculating 1 mL of pre-enrichment culture into 9 mL of Mannitol Selenite Cystine (MSC) and Rappaport-Vassiliadis (RV) enrichment broths (Oxoid Ltd, Victoria, Australia),

respectively. The MSC broth was incubated for 24 h at 37 ± 1°C, while the RV broth was incubated at 42 ± 1°C for 24 h. After incubation, a loopful of culture from each enriched broth was streaked onto Xylose-Lysine-Desoxycholate (XLD) agar (Oxoid Ltd, Victoria, Australia) and incubated for 24 h at 37 ± 1°C. *Listeria* selective agar (LSA) (Oxoid Ltd, Victoria, Australia) was used to detect *Listeria* spp. Each sample was enriched by inoculating 1 mL of pre-enrichment culture into 9 mL of *Listeria* enrichment broth (Oxoid Ltd, Victoria, Australia) and incubated for 24-48 h at 37 ± 1°C. A loopful of the enriched bacterial culture was then streaked onto the LSA and incubated at 37 ± 1°C for 24-48 h.

Microbial colonies on TSA, *Pseudomonas* spp., DRBC and PEMBA medium were counted after incubation. Only counts of 30 to 300 colony forming units (CFU) were considered. Plates with fewer than 30 colonies are considered below the method quantification limit and are designated < 3.48 log CFU/g. The method detection limit was set at one colony from the first dilution (10^{-1}) and is designated < 2.00 log CFU/g. Each microbial count (expressed as log CFU/g) was calculated based on the mean of three samples (Miao et al., 2014). Ten bacterial colonies on the respective agar (except *Bacillus* colonies which were below the quantification limit) were isolated and tested for oxidase and catalase reactions (Bullock & Aslanzadeh, 2013; Harvey et al., 2007) before they were Gram-stained (Fluka, Australia) (Becerra et al., 2016; Coico, 2006; Rivera et al., 2010a). Spore staining was performed for *Bacillus* spp. isolates using malachite green stain solution (Hussey & Zayaitz, 2007; Sanz et al., 2008). Once stained, the morphological characteristics of each isolate were observed using a fluorescence microscope (Olympus BX51, Japan).

3.2.4 Data analysis

All experiments were performed in triplicate and all values were expressed as mean ± standard error. All statistical analyses were performed using IBM SPSS statistics 26 software. One-way analysis of variance (ANOVA) followed by post-hoc Tukey’s test was performed to compare the means of more than two independent groups. Independent samples t-test was used to compare the means of two different groups. Repeated measures ANOVA was used to compare the sample means of the whole truffles across different time points as the same truffle samples were measured over and over again. Differences with $p < 0.05$ (95 % confidence level) were considered to be statistically significant.

3.3 Results and discussion

3.3.1 Total plate counts

In this study, the initial total microbial counts of the whole and sliced truffles were 7.94 ± 0.28 log CFU/g and 8.33 ± 0.17 log CFU/g, respectively (Table 3.1). The results correspond well with the previous studies that reported the total microbial population to be 10^7 to 10^8 CFU/g in the whole ascocarps (gleba and peridium) and in the glebas of *T. melanosporum* harvested from Sarrión, Spain (Rivera et al., 2010a) and Marche, Italy (Saltarelli et al., 2008), respectively.

The size of the total microbial populations in all truffle samples remained almost constant in all treatments throughout the entire storage period in this study. When comparing the treatment types, there was no statistical difference in the total microbial counts among all treatments on day 0 and 4. But the total microbial count in freeze-dried truffles was statistically lower than that of the whole and sliced truffles on day 8, 15 and 30 (Table 3.1), indicating that some microbes could not survive in a dry environment.

Table 3.1: Total plate counts (log CFU/g) in the fresh, sliced, and freeze-dried black truffles (*Tuber melanosporum*) under vacuum packaging and refrigerator storage.

Storage duration (Day)	Total plate counts (log CFU/g)		
	Fresh truffles	**Sliced truffles**	**Freeze-dried truffles**
0	7.94±0.28 Aa	8.33±0.17 Aa	7.58±0.24 Aa
4	8.46±0.36 Aa	8.41±0.38 Aa	7.27±0.10 Aa
8	8.06±0.31 Aa	8.29±0.08 Aa	7.04±0.16 Ab
15	8.25±0.19 Aa	8.39±0.02 Aa	7.17±0.08 Ab
30	8.19±0.11 Aa	8.32±0.03 Aa	6.95±0.30 Ab

Data are expressed as mean ± standard error of three replicate samples.

Means within the same column with different uppercase letters differ significantly ($p < 0.05$) across different time points.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$) among different sample types.

3.3.2 *Pseudomonas* spp.

Pseudomonas spp., being the most abundant microbial group in *Tuber* spp., is believed to be involved in their postharvest spoilage (Rivera et al., 2010a). The initial *Pseudomonas* spp. counts in both whole and sliced truffles were 6.26 ± 0.28 log CFU/g and 6.87 ± 0.23 log CFU/g, respectively (Table 3.2). A previous study reported a higher *Pseudomonas* spp. count of 7.9 log CFU/g in the same truffle species collected from Sarrión, Spain (Rivera et al., 2010a). The variations in the microbial counts between studies could be due to differences in collection season, maturity, origin, geographical location, soil microbiology, and postharvest packaging (Chen et al., 2019; Miao et al., 2014; Monaco et al., 2020).

Pseudomonadaceae are psychrotrophic (cold-tolerant) bacteria that can proliferate even at 2°C to 5°C (Møretrø & Langsrud, 2017; Munsch-Alatossava & Alatossava, 2006; Ragaert et al., 2007) and can contribute to food spoilage particularly in food with a high water activity (Raposo et al., 2017; Sterniša et al., 2019). Sorrentino et al. (2018) reported that *Pseudomonas* spp. were still able to proliferate in truffles that were kept at 4°C. *Pseudomonas* spp. are aerobic and carbon dioxide sensitive in which their growth can be inhibited under reduced-oxygen condition and this was demonstrated by a reduction in the *Pseudomonas* spp. count in a past study when truffles were packaged in an oxygen-limited atmosphere for 28 days (Rivera et al., 2010b). The *Pseudomonas* spp. populations in all truffle samples were expected to decrease in the present study as well as they were vacuum-packaged. The *Pseudomonas* spp. populations in the sliced truffle treatment decreased by 1.22-log between day 8 and 15 the thereafter remained stable to day 30. Conversely the *Pseudomonas* spp. count in samples from the whole truffle treatment remained relatively constant for the duration of the experiment (Table 3.2). The reason for this is unclear and it is plausible that the inherent biological variation such as fluctuation among replicate biological samples could have influenced the results obtained. More studies with a larger sample size are required to confirm this finding. As described in Section 3.2.2, to compare with the sliced truffles, truffle pieces of a similar weight and size to the sliced truffles were aseptically cut from the whole truffles at each sampling point for microbial analysis. There were no statistical differences in the *Pseudomonas* spp. counts between the sliced and the whole truffles throughout storage (Table 3.2). Our finding contradicts previous literature that has associated sliced produce with a higher microbial load as compared to the intact produces (Qadri et al., 2015; Ragaert et al., 2007). A recent study observed a high microbial population in the sliced

mushrooms (*Agaricus bisporus*) as slicing provides more exposed surface area on which microbes can grow and the release of nutrients from the damaged tissues provide a complete nourishing environment for microbial growth (Jiang et al., 2018). In contrast, the presence of a cuticle on the surface of the uncut mushrooms could function as a natural protective barrier and limits the availability of nutrients and moisture for microbial proliferation (Jiang et al., 2018). The reason for the contradictory finding was not clear but we speculate that it could be due to differences in the texture, chemical composition, water content and microbial communities between truffles and mushrooms (Cheung, 2013; Djekic et al., 2017; Kalač, 2013). In general, truffles are typically colonised by a higher load of diverse bacterial communities than mushrooms as truffles grow underground (Rivera et al., 2010a; Splivallo et al., 2015; Vahdatzadeh et al., 2015) while mushrooms mostly grow above ground (Kalač, 2013; Kertesz & Thai, 2018).

Reducing water activity could limit microbial growth in food products (Erkmen & Bozoglu, 2016). Under the same storage condition, the count of *Pseudomonas* spp. in the freeze-dried truffles were below the method quantification limit (< 3.48 log CFU/g) during the initial 15 days of storage and was not detected (< 2.00 log CFU/g) on day 30. In contrast, the *Pseudomonas* spp. counts in the whole and sliced truffles were above 5 log CFU/g on day 30 (Table 3.2). This could be explained by the fact that freeze-dried products do not contain sufficient moisture to support the survivability of *Pseudomonas* spp. (Erkmen & Bozoglu, 2016; Lund et al., 2000; Møretrø & Langsrud, 2017). Our findings indicate that freeze-drying along with vacuum packaging and refrigerator storage provided a synergistic effect to reducing aerobic microbial populations in truffles. However, freeze-drying produces a highly porous product with a high sorption capacity, due to which freeze-dried products are characterised by high hygroscopicity and rapid rehydration (Tsami et al., 1998; Varghese et al., 2014). As *Pseudomonas* spp. were still detected (< 3.48 log CFU/g) during the initial 15 days of storage (Table 3.2), there is a high risk of microbial reactivation in the freeze-dried truffles as a result of moisture absorption. Thus, freeze-dried truffles must be consumed immediately once the vacuum package is opened (Chitrakar et al., 2018).

Table 3.2: ***Pseudomonas*** **spp. counts (log CFU/g) in the fresh, sliced, and freeze-dried black truffles (*Tuber melanosporum*) under vacuum packaging and refrigerator storage.**

Storage duration (Day)	*Pseudomonas* spp. counts (log CFU/g)		
	Fresh truffles	Sliced truffles	Freeze-dried truffles
0	6.26±0.28 [Aa]	6.87±0.23 [Aa]	<3.48
4	6.45±0.38 [Aa]	6.88±0.05 [Aa]	<3.48
8	6.33±0.43 [Aa]	6.79±0.08 [Aa]	<3.48
15	6.10±0.45 [Aa]	5.68±0.13 [Ba]	<3.48
30	5.42±0.29 [Aa]	5.65±0.15 [Ba]	< 2.00

Data are expressed as mean ± standard error of three replicate samples.

Means within the same column with different uppercase letters differ significantly ($p < 0.05$) across different time points.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$) between fresh and sliced truffles.

3.3.3 Yeasts and moulds

Yeasts and moulds are naturally present on truffles and are thought to cause spoilage (Rivera et al., 2010a). The presence of moulds in truffles is undesirable as they would adversely affect the visual appearance and the loss of aroma quality (Rivera et al., 2010a). Fresh truffles have a relatively low pH of 4.9 (Saidi et al., 2015), in which the acidic environment may be suitable for the growth of moulds, but surprisingly, moulds were not detected (< 2.00 log CFU/g) in all truffle samples during storage (Table 3.3). This could be due to the qualitative selection of truffles wherein only healthy produce with no visible defect was chosen for our study (Section 3.2.2). A similar observation was reported by Rivera et al. (2010a) who examined the presence of moulds in different parts of healthy and parasitised truffles. In their study, truffles without visible damage as observed under a microscope were considered healthy and the glebas of healthy truffles were found to be free from moulds (Rivera et al., 2010a).

Many yeasts, on the other hand, can grow in the presence or absence of oxygen (Walker, 2003), but their populations could be reduced under low oxygen conditions (Lund et al.,

2000). A previous study found that *Candida sake* yeast could grow in the irradiated *T. melanosporum*, affecting its organoleptic quality and shelf life (Rivera et al., 2011a).

There were no significant differences in the yeast counts between sliced and whole truffles during storage, similar to that observed for the *Pseudomonas* spp. When evaluating the yeast counts of each sample over different time points, we found that a significant reduction in the yeast populations occurred in the sliced truffles but not in the whole truffles throughout storage (Table 3.3). A further study with larger sample size is required to determine if the finding could have been due to the chance alone or an actual difference. Although many yeast species, including *C. sake* are capable of growth under low water condition (Howell, 2016; Teixidó et al., 1998), the yeast colonies in the freeze-dried truffles were below the method quantification limit (< 3.48 log CFU/g) and were not detected (< 2.00 log CFU/g) from day 15 onwards (Table 3.3). More studies are required to confirm this. Besides the low moisture content, other factors like temperature, acidity, pH, and nutrient availability and concentration may synergistically affect the overall viability of yeasts in the freeze-dried truffles (Howell, 2016). Hence, it is rather challenging to determine the exact factor that influences the growth pattern of yeasts in this case.

Table 3.3: Yeasts counts (log CFU/g) in the fresh, sliced, and freeze-dried black truffles (*Tuber melanosporum*) under vacuum packaging and refrigerator storage.

Storage duration	Yeasts counts (log CFU/g)		
(Day)	Fresh truffles	Sliced truffles	Freeze-dried truffles
0	5.01±0.38 Aa	5.41±0.06 ABa	<3.48
4	5.25±0.39 Aa	5.55±0.15 Aa	<3.48
8	4.81±0.46 Aa	5.55±0.14 Aa	<3.48
15	4.58±0.41 Aa	4.87±0.21ABa	<2.00
30	4.30±0.25 Aa	4.61±0.35 Ba	<2.00

Data are expressed as mean ± standard error of three replicate samples.

Means within the same column with different uppercase letters differ significantly ($p < 0.05$) across different time points.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$) between fresh and sliced truffles.

3.3.4 *Listeria* spp., *Salmonella* spp., and *Bacillus* spp.

Listeria colonies were not detected (< 2.00 log CFU/g) in the current study (Table 3.4). Similarly, a previous study showed that although *T. aestivum* was heavily contaminated by different microbial groups, *L. monocytogenes* was not detected in the truffles (Reale et al., 2009b). By contrast, the presence of members of the *Listeria* genus were detected on *T. aestivum* and *T. melanosporum* from Teruel, Spain (Rivera et al., 2010a). Further microbial identification at the species level showed that *L. monocytogenes* was undetectable in *T. melanosporum*, but present at a low incidence in *T. aestivum* (Rivera et al., 2010a). The low prevalence of *L. monocytogenes* in truffles as demonstrated in previous studies (Reale et al., 2009b; Rivera et al., 2010a) may be due to the inhibitory effects exerted by *Pseudomonas* spp., the dominating bacteria (Rivera et al., 2010a). Some *Pseudomonas* strains may have a negative effect on the biofilm formation by *L. monocytogenes* (Møretrø & Langsrud, 2017). Another possible reason for the rare occurrence of *L. monocytogenes* in truffles is that their population in the soil is generally low although the soil is an environmental niche for this microorganism (Vivant et al., 2013).

Salmonella was not detected (< 2.00 log CFU/g) in all truffle samples in the present study (Table 3.4). Similarly, neither Reale et al. (2009b) nor Rivera et al. (2010a) detected this bacteria in the fresh *T. melanosporum* and *T. aestivum.* In contrast to the fresh truffles, truffle-related food products such as truffle oil have been implicated as the likely source of a *Salmonella enteritidis* outbreak in Columbia in 2015 (Kuramoto-Crawford et al., 2017). The implicated product contained other food ingredients that could have been the source of contamination. For example, a multistate listeriosis outbreak that implicated truffle-containing cheese products was believed to be due to poor food hygiene practices and sanitation during the cheese-making process (Choi et al., 2014) but not from the truffle itself.

Bacillus spp. occur ubiquitously in soil (Saidi et al., 2015). Fresh truffles that grow underground have been reported to be contaminated with this genus (Saidi et al., 2015). The discovery of *Bacillus* spp. in the brûlé zone has provided evidence that *Bacillus* spp. may have originated from the soil around the truffle ascocarps (Mello et al., 2013; Saidi et al., 2015). Due to their ability to survive under unfavourable environments (Saidi et al., 2015), it was not surprising to detect *Bacillus* species in all truffle samples. They were, however, present in a very low number (< 3.48 log CFU/g) (Table 3.4). The prevalence

of this bacterial species in a wide range of food items such as pasta, rice, meat, eggs, and milk has also been reported (Desai & Varadaraj, 2010; Shin et al., 2010). According to the previous literature, counts more than 5 log CFU/g of *Bacillus cereus* in food is required to produce sufficient emetic toxin to cause disease (Finlay et al., 2002; Stenfors Arnesen et al., 2008). Based on the results from this study, it suggests that the risk of foodborne illness caused by truffles is low due to the low *Bacillus* species counts.

Nonetheless, the sampling size in the present study is short of taking any robust conclusion on the occurrence of these pathogens in the truffles. There are studies, although still scarcely explored, reported that truffles contain bioactive chemicals such as phenolic compounds that could inhibit the growth of some pathogenic microbes (Beara et al., 2014; Neggaz et al., 2018; Palacios et al., 2011; Schillaci et al., 2017; Tejedor-Calvo et al., 2021a) but the antimicrobial activity of truffles may vary depending on the truffle species and extraction methods (Neggaz et al., 2018; Tejedor-Calvo et al., 2021a). In a recent study, the antimicrobial activities of aqueous, methanol, hexane, and ethyl acetate extracts from ten different truffle species against 16 microbial species were investigated. Of all the extracts from the ten truffle species of *Tuber* and *Terfezia* genera, only five extracts from four truffle species (*Terfezia magnusii, T. aestivum, T. gennadii, and T. melanosporum*) showed a positive antimicrobial activity against pathogenic microbes such as *L. monocytogenes, Shigella flexneri,* and *Staphylococcus aureus* (Tejedor-Calvo et al., 2021a). Likewise, the Soxhlet extract of *Terfezia claveryi* showed a greater antimicrobial activity than that of the maceration extract (Neggaz et al., 2018). The abundant microbiota on the surface or peridium of truffles was believed to inhibit the antimicrobial activity of truffles (Tejedor-Calvo et al., 2021a) but we speculate that the relationship between the antimicrobial compounds and the microbiota can be two ways; in which the antimicrobial compounds in truffles may control the occurrence and survival of pathogenic microbes, explaining why the truffles are safe for consumption. However, more studies are required to confirm our speculation.

Table 3.4: The presence of *Listeria* spp., *Salmonella* spp., *Bacillus* spp., *Bacillus* spp. spore in the fresh, sliced, and freeze-dried black truffles (*Tuber melanosporum*) under vacuum packaging and refrigerator storage.

Sample types	*Listeria* spp.	*Salmonella* spp.	*Bacillus* spp.	*Bacillus* spp. spore
Fresh truffles	<2.00	<2.00	<3.48	<3.48
Sliced truffles	<2.00	<2.00	<3.48	<3.48
Freeze-dried truffles	<2.00	<2.00	<3.48	<3.48

Each experiment was performed in triplicate.

3.4 Conclusion

This study provides a general overview of the microbiological quality of the whole, sliced, and freeze-dried truffles. As the numbers of *Pseudomonas* spp. and yeasts were negligible in the freeze-dried truffles, it can be concluded that low water activity would provide a less favourable condition for the survivability of microbes. Based on the results of the current study, the risk of getting foodborne illness associated with *Bacillus* species is low as the number present in the truffles was too low to cause disease. More studies are required to confirm if truffles contain antimicrobial compounds that may react against the pathogens in truffles and render the safety of consuming truffles. The microbial loads in the truffles studied indicate the need to develop an effective processing step before packaging and storage to delay microbial spoilage. Integrating synergistic antimicrobial treatment with the preservation method could be a new focus to improve the microbiological quality of truffles without losing the aroma quality.

CHAPTER 4: Comparison of different extraction techniques on the key volatile profile and microbial quality of Black Périgord truffle (*Tuber melanosporum*) extracts

The content in this chapter has been revised and resubmitted for publication as follows:

Phong, W. N., Beh, C. C., Gibberd, M., Dykes, G. A., Payne, A. D., & Coorey, R. Impacts of different extraction techniques on the key volatile profile and microbial quality of Black Périgord truffle (*Tuber melanosporum*) extracts. *Journal of Food Science.* (under review)

Abstract

Black truffle is highly prized mainly for its unique aroma and rarity but it is seasonal and highly perishable. Current preservation methods like canning and hot air dehydration could significantly improve shelf life but a loss of aroma is observed. A natural extract that closely resembles actual truffle aroma could potentially extend the supply of truffle aroma all year round. Extracting the key volatiles of truffle using different solvents (ethanol, acetone, hexane, liquefied butane) was first evaluated. Having determined that ethanol was the preferred solvent for Soxhlet extraction, a comparison was made between Soxhlet and SC-CO_2 extraction. Soxhlet extract (6.62 ± 0.33%) showed a significantly higher mass recovery than SC-CO_2 extract (2.49 ± 0.38%). Of the ten key volatiles, six (methanethiol, dimethyl disulfide, isoamyl alcohol, dimethyl trisulfide, 1-octen-3-ol, *p*-cresol) were detected in both extracts, suggesting that the aroma of natural extracts had a higher complexity than the synthetic aroma. All the tested microbes were reduced to the point where none were detected (< 2.00 log CFU/g) in both the extracts. The overall finding suggests that the production of a natural flavouring ingredient from truffles by volatile extraction is promising.

Keywords: Black Périgord truffle volatiles, microbial quality, solvent extraction, Soxhlet extraction, supercritical carbon dioxide (SC-CO_2) extraction

4.1 Introduction

Black Périgord truffle (*Tuber melanosporum* Vittad. 1831) is a highly valued edible fungus owing to its unique aroma, favoured organoleptic properties, short season of supply, rarity, and high perishability (Culleré et al., 2010; Krauß & Vetter, 2020; Segelke

et al., 2020). Due to its high price, black truffle is commonly used as the signature ingredient to enhance flavour (Pacioni et al., 2014a). Despite its high commercial value, there is limited opportunity for a wider market as black truffle is seasonal and has a short shelf life, making it unavailable all year round (Campo et al., 2017).

Long-term preservation of truffles without losing their unique aroma is important to extend the supply, especially during the off-season. But improving the shelf life of fresh truffles for long-term storage without compromising their organoleptic qualities remain challenging. Several preservation technologies (refrigeration, freezing, hot air drying, canning, and irradiation) have been studied in the past to maintain the sensory characteristics of truffles (Campo et al., 2017; Culleré et al., 2012). But these preservation techniques have their shortcomings and challenges with none of them able to significantly improve truffle shelf life with minimal loss of aroma (Phong et al., 2022c).

A synthetic flavouring that resembles black truffle aroma has been created and used extensively in the food industry (Splivallo & Maier, 2012). The synthetic flavouring, however, lacks the complexity of actual truffle aroma (Wernig et al., 2018). Furthermore, there is growing consumer demand for natural ingredients in food products. Subsequently, any advance in food processing that allows for the extraction and stabilisation of the compounds contributing to the aroma of truffles may develop opportunities to add value to lower grade truffles during the production season and to enable more options in food production (Phong et al., 2022c). Based on previous studies, in developing such a natural food ingredient, it is only ten key volatiles are important, as it is these that humans perceive as the aroma in black truffle (Culleré et al., 2010; Culleré et al., 2013a). For this reason, such work needs to concentrate on the extraction of these ten key volatiles.

An alternative approach that may allow truffle aroma available during the off-season could be achieved through the extraction and stabilisation of the aroma volatile compounds from truffles. Truffles are usually consumed without cooking, but their microbial load may be high (Phong et al., 2022b; Rivera et al., 2010a). Extraction may remove microorganisms, producing a microbial-free ingredient that meets legislative requirements and can readily be consumed with an extended shelf life (Phong et al., 2022c). Current truffle grading standards are based on size and physical appearance (The Australian Truffle Growers Association, 2014; UNECE, 2017) and have resulted in smaller and off-cut truffles, despite having a high aroma quality, to be graded as poor

quality and of lesser value than larger truffles. The manufacture of a flavouring ingredient from excess truffles may contribute to the sustainable growth of the truffle industry (Phong et al., 2022c).

Techniques such as solvent extraction, Soxhlet extraction, and supercritical fluid extraction are commonly used to recover volatiles from food materials (El‐Ghorab et al., 2004; Suetsugu et al., 2013) such as Pu-erh tea (Gao et al., 2017), *Citrus* spp. (González-Mas et al., 2019), garlic (Li et al., 2010), lavender (Danh et al., 2013), and rosemary (Conde-Hernández et al., 2017). Solvent and Soxhlet extraction methods are simple and low-cost but require a solvent removal step (De Castro & Priego-Capote, 2010). A wide range of solvents can be used in both methods depending on the molecular affinity between the target compounds and the solvent. To ensure a high extraction efficiency, the selected solvent should be able to penetrate the target material and make good physical contact (Phong et al., 2018). To the best of our knowledge, the extraction of truffle volatiles to produce a food flavouring ingredient using solvent extraction and Soxhlet extraction have not been reported in the literature. On the other hand, supercritical fluid extraction is a promising alternative compared to conventional extraction as it could replace or reduce organic solvent usage during extraction and produce extracts with no solvent residues (Ormeno et al., 2011). Carbon dioxide (CO_2) is a widely adopted solvent in supercritical fluid extraction due to its low-cost and abundance. CO_2 is also chemically inert, odourless, colourless, non-flammable, non-explosive, and recyclable, making it ideal to extract foodstuffs (Machado et al., 2013). Previous studies showed that SC-CO_2 could produce a range of extracts with good quality; for example, SC-CO_2 extracts derived from rosemary, lavender, *Chamaecyparis obtuse*, and *Leptocarpha rivularis* contained chemical compounds that were responsible for the aromatic characteristics of their starting material (Conde-Hernández et al., 2017; Danh et al., 2013; Jin et al., 2010; Uquiche et al., 2015; Uquiche & Garcés, 2016). As far as we are aware, the extraction of volatile compounds from truffles using supercritical carbon dioxide (SC-CO_2) extraction has been reported but not well studied with only two patents and one recent paper could be found in the literature up to this point. The patent filed by Mirande-David (1988) claimed that a portion of the truffle aroma could be extracted but there is a lack of information about which volatile compounds were successfully recovered from the process. Another patent reported that SC-CO_2 could produce a solvent-free extract at a high extracting rate, making SC-CO_2 suitable for industrial application (Gao et al., 2007). Most recently, Tejedor-Calvo et al. (2021b) also demonstrated the potential application

of $SC\text{-}CO_2$ in recovering aroma fraction from truffles, as evidenced by the detection of some of the key volatile compounds in the extracts obtained. The extraction was carried out at 40°C and the optimal extraction conditions reported were 30 MPa for 3 h (Tejedor-Calvo et al., 2021b). Nonetheless, a comparison between volatiles extracted by $SC\text{-}CO_2$ and other extraction methods have not been reported, which needs to be understood before food industry applications can be developed and optimised. Furthermore, there is a lack of information about the physical properties and microbial quality of the truffle extract derived from $SC\text{-}CO_2$ and none of which were reported by Tejedor-Calvo et al. (2021b). We hypothesised that extraction may allow the removal of generally occurring microbial contamination from truffles, allowing the development of a safe flavouring ingredient for food application (Phong et al., 2022c). To fill this gap in knowledge, investigation on these aspects should be considered to provide a comprehensive profile of the truffle extracts obtained. In this study, a comparison of a panel of differing solvents was first undertaken to determine the most suitable solvent for truffle aroma extraction. The volatile profile and mass of the extracts were compared. Having determined that ethanol was the preferred solvent for Soxhlet extraction, we compared the mass recovery, chemical composition, and microbial counts of truffle extracts produced by $SC\text{-}CO_2$ and Soxhlet extraction with ethanol as the extractant. The ultimate goal of this study was to produce truffle extract containing key volatiles that are known to be important in fresh truffle aroma (Culleré et al., 2010; Culleré et al., 2013a) for use as a flavouring ingredient in the food industry. To the best of our knowledge, there is no similar published study making such a comparison in terms of the physical properties, key volatile profile, and microbiological quality of the truffle extracts derived from different extraction methods and this study was performed to close that gap.

4.2 Materials and methods

4.2.1 Raw material

Matured truffles were harvested from the Manjimup region of Western Australia. All freshly collected samples were brushed to remove soil, rinsed with tap water, and air-dried in a laminar flow cabinet to remove surface moisture as per industrial practice (Culleré et al., 2013a; Phong et al., 2022b). All truffles were packed in an ice-cooled insulated container held at 4 ± 2°C and transported to our laboratory within 24 h of harvest.

4.2.2 Starting material preparation

Upon receiving the samples, a visual qualitative assessment of truffles was made. Those free of defects with a similar appearance were selected; those that showed signs of insect damage or spoilage were rejected (Phong et al., 2022b).

Without undergoing any drying process, several randomly selected fresh truffles from the remaining population were crushed into smaller particle size using a mortar and pestle to allow sufficient mixing and increase surface contact with the corresponding solvent during extraction (Phong et al., 2018). The crushed samples were combined and mixed well and then immediately packaged into vacuum bags (65 μm Polyamide/Polyethylene, FPA Australia Pty Ltd, Malaga, WA, Australia) using a vacuum packaging machine (easyPACK-mk2, Webomatic, Hansastrasse, Bochum, Germany) (Culleré et al., 2013a) and stored at 4 ± 2°C until required for extraction or analysis. As fresh truffles are highly perishable, the crushed samples were processed immediately after sample preparation and therefore they were only able to use in Experiment 1 (solvent extraction).

To ensure Experiment 2 can be continued during the off-season using truffles from the same harvesting season, frozen crushed truffles were used in the SC-CO_2 and Soxhlet extraction. The frozen crushed samples were prepared by following the same procedure as fresh samples, except that they were stored at -80 ± 2°C until required for extraction or analysis. No direct comparison of results between Experiment 1 and 2 were conducted.

4.2.3 Extraction of the volatile fraction from truffles

An overview of the experimental procedures is illustrated in Figure 4.1.

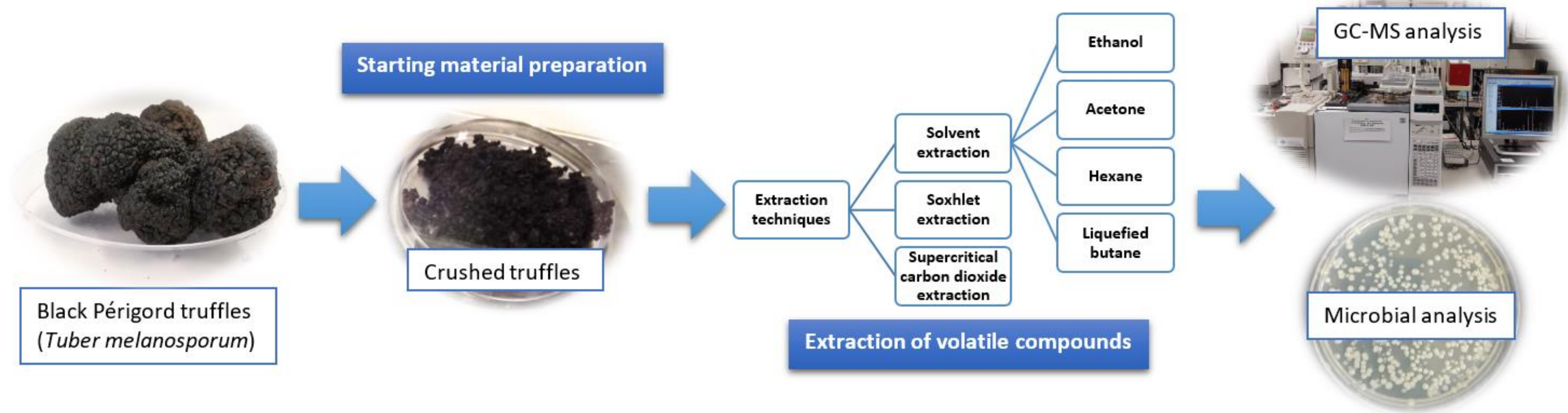

Figure 4.1: An overview of the entire experimental procedure

4.2.3.1 Experiment 1 - Solvent extraction (ethanol, acetone, hexane, and liquefied butane)

Crushed truffles (50 g) were placed in a 250 mL conical flask containing 100 mL of 99.5% ethanol (LabServ, Thermo Fisher Scientific, Scoresby, Victoria, Australia). The mixture was stirred for 1 h at room temperature (20 ± 2 °C) with a magnetic stirrer. After extraction, the solvent-extract mixture was filtered using Whatman Grade 54 filter paper (Gao et al., 2017). The same procedure was repeated with 99.8% acetone and 95% hexane (LabServ, Thermo Fisher Scientific, Scoresby, Victoria, Australia), respectively, as an extractant. Ethanol and acetone were removed in a Büchi rotary evaporator Rotavapor R-300 (Büchi Labortechnik AG, Postfach, Flawil, Switzerland) at 40°C, 400 Pa for approximately 4 h. The removal of hexane by rotary evaporator was performed at room temperature for about 3 h. The extracts were immediately analysed by gas chromatography-mass spectrometry (GC-MS) after weighing.

As for the butane extraction, 73% butane (ca. 10 ml) (Tradeflame, Bunnings, Australia) was condensed in a pressure vessel at -80 ± 2°C before adding 4 g of crushed truffles into the pressure vial. The tube was sealed and allowed to stir at room temperature for 2 h behind a blast shield. The vial was cooled to -80 ± 2°C and the butane extract was decanted into a GC vial at -80 ± 2°C. Thereafter the vial was placed in an ice-water bath (0 °C) to allow the butane to evaporate. The extract was analysed by GC-MS.

4.2.3.2 Experiment 2 - Comparison of Soxhlet and SC-CO_2 extraction

(a) **Soxhlet extraction**

Based on the volatile profile of the extracts from Experiment 1, ethanol was chosen as the solvent for Soxhlet extraction. Soxhlet extraction was performed as per Ciarlini et al. (2017), with modification. About 25 g of frozen crushed truffles were placed in an extraction thimble and then transferred to a Soxhlet extractor Büchi extraction unit E-816 (Büchi Labortechnik AG, Postfach, Flawil, Switzerland). The extractor was filled with 100 mL of ethanol (LabServ, Thermo Fisher Scientific, Scoresby, Victoria, Australia) and heated to reflux for 3 h.

After extraction, the exhausted material in the thimble was discarded, whilst the solvent-extract mixtures in the flask were collected and the solvent was removed in a Büchi rotary evaporator Rotavapor R-300 (Büchi Labortechnik AG) at 40°C, 400 Pa. The extract was collected and weighed before analysis.

(b) **SC-CO_2 extraction**

SC-CO_2 extraction was conducted according to the procedures described by Danh et al. (2013), with modification. The extraction system is comprised of high-pressure vessels, fittings, and valves (Swagelok, Perth, Australia), CO_2 syringe pumps (Teledyne ISCO, NE, USA), pressure gauge (Swagelok), and a customised water bath with a heater circulator (Thermoline, NSW, Australia). Briefly, about 18 g of frozen crushed truffles were placed in a stainless-steel extraction vessel. The extraction system was pressurised with CO_2 up to 2.05 x 10^7 Pa at 60°C and the system was isolated for 120 min. The operating condition was optimised to achieve a sterility level of 10^{-6} (Beh et al., 2019; Dillow et al., 1999). Subsequently, CO_2 was passed through the system at an average flow rate of 9 mL/min for another 120 min to extract the volatiles. The extract was collected in a cooled trap during the process and weighed before analysis.

4.2.4 Headspace solid-phase microextraction (SPME)

SPME was automated using the GERSTEL MultiPurpose Sampler (MPS). A flexible fused silica fibre coated with a 50/30 µm layer of divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) (57298-U, Supelco, Bellefonte, Pennsylvania, USA) was chosen to sample the volatiles (Díaz et al., 2009; Díaz et al., 2002). The fibre was conditioned according to the manufacturer's instructions before its use (Supelco) (Díaz et al., 2009; Phong et al., 2022a; Phong et al., 2022d).

To establish a baseline for comparison with the extract obtained from each extraction method, the same analysis procedures were also applied to the starting materials as a reference. About 1 g of crushed truffles (reference material) or 0.1 g of truffle extracts (except hexane extract in which only 0.05 g were analysed due to the low amount of extract obtained) were transferred into a 20 mL headspace glass vial. 1,2-Dichlorobenzene (Sigma Aldrich, Sydney, NSW, Australia), which was not present in truffles or the extracts and separated well from other compounds in the chromatogram (Liu et al., 2017b), was added to all samples as an internal standard and the vials were closed with a magnetic screw cap (Thermo Fisher Scientific). The vials were allowed to equilibrate for 5 min at 53 °C before the fibre was introduced into the headspace vial for 13.6 min. The volatiles bound to the fibre were then desorbed in a splitless injector for 2 min at 200 °C (Díaz et al., 2009).

4.2.5 GC-MS analysis

GC-MS analysis was performed as per previous literature (Choo et al., 2021; Díaz et al., 2003), with some modifications. An Agilent-6890 GC system coupled to a GC 5973 mass spectrometer (Agilent, Santa Clara, California, USA) was used to carry out the analysis. The GC oven temperature program was increased from 35 °C (held 5 min) to 240 °C at 5 °C min^{-1} (held 3 min). Mass spectra were recorded in electron impact (EI) ionisation mode, scanning the 40-250 m/z range at 0.46 sec/scan (3 µ-scans) after 1.5 min of solvent delay. Helium (at 6.74 psi, 1.0 mL/min flow rate) was used as the carrier gas. The capillary GC column used was a 30 m (length) x 250 µm (diameter) x 0.25 µm (film thickness) i.d. HP-INNOWax GC column (Agilent) fused silica capillary column (Model Number: Agilent 19091N-133).

The chromatographic data obtained from MSD ChemStation F.01.03.2357 (1989-2015 Agilent Technologies, Inc.) was analysed using National Institute of Standards Technology mass spectral database program (NIST, Version 2.3, 2017, USA). Volatile compound identification was based on the comparison between the mass spectra for each compound with those in the NIST 2017 Mass Spectral library (a minimum mass spectral match quality of 700 was used as the criterion) (Watson & Sparkman, 2007) as well as with published mass spectra, in addition to the GC retention time of each compound and Kovats retention indices (calculated based on n-alkanes) (Wernig et al., 2018).

The peak area of the individual compound was determined based on area normalisation by integration. Each identified compound was expressed in a relative percentage (%) by calculating the proportion of its peak area to the total peak areas of all the compounds of interest (Díaz et al., 2003). For some compounds such as methanethiol, dimethyl sulfide (DMS), dimethyl disulfide (DMDS), and dimethyl trisulfide (DMTS) that did not produce any fully resolved peaks in the chromatogram due to their low concentration and/or co-elution with other peaks with the similar retention time that make the integration difficult, the extraction of a single ion chromatogram was utilised instead of total ion chromatogram to determine the relative percentage of these compounds. The selective mass chosen (m/z) for identifying methanethiol, DMS, DMDS, and DMTS was 47, 62, 94, and 126, respectively.

4.2.6 Microbiological analysis

For the Soxhlet and SC-CO_2 extractions, a sample of starting material or truffle extract (0.1 g) was homogenised with 0.9 mL sterile buffered peptone water (Oxoid Ltd, Melbourne, Victoria, Australia) for 2 min in a homogeniser (PRO250, PRO Scientific Inc., Oxford, Connecticut, USA). Subsequently, serial dilutions were performed in sterile phosphate-buffered saline. A 0.1 mL aliquot from each dilution was spread-plated onto the culture media and incubated. *Pseudomonas* isolation agar (Becton Dickinson Pty Ltd, Sydney, NSW, Australia) was used as the selective media to culture *Pseudomonas* species and incubated at 37 ± 1°C for 48 h (Phong et al., 2022b). Dichloran Rose-Bengal Chloramphenicol (DRBC) agar (Merck Pty Ltd, Melbourne, Victoria, Australia) was used to determine yeast and mould counts, incubated at 25 ± 1°C for 5 days. Polymyxin Egg Yolk Mannitol Bromothymol Blue Agar (PEMBA) (Oxoid Ltd) was used to determine the presence of *Bacillus* spp. and incubated at 37 ± 1°C for 24 h (Phong et al., 2022b).

To detect *Salmonella* spp., the homogenate was enriched by inoculating 0.1 mL of pre-enrichment culture into 0.9 mL of Rappaport-Vassiliadis (RV) and Mannitol Selenite Cystine (MSC) enrichment broths (Oxoid Ltd), respectively. The RV broth was incubated at 42 ± 1°C for 24 h whereas MSC broth was incubated for 24 h at 37 ± 1°C. After incubation, a loopful of culture from each enriched broth was streaked onto Xylose-Lysine-Desoxycholate (XLD) agar (Oxoid Ltd) and incubated at 37 ± 1°C for 24 h. To detect *Listeria* spp. the homogenate was enriched by inoculating 0.1 mL into 0.9 mL of *Listeria* enrichment broth (Oxoid Ltd) and incubated at 37 ± 1°C for 24-48 h. Subsequently, a loopful of enriched culture was streaked onto *Listeria* selective agar (LSA) (Oxoid Ltd) plate and incubated for 24-48 h at 37 ± 1°C (Phong et al., 2022b).

The numbers of microbial colonies (expressed as log CFU/g) that grew on *Pseudomonas*, DRBC, and PEMBA agar were counted after incubation. Only counts of 30 to 300 colony forming units (CFU) were considered. Each count was enumerated in log CFU/g based on the mean of triplicates. Plates with fewer than 30 colonies are considered below the method quantification limit and are denoted as < 3.48 log CFU/g. The method detection limit was set at one colony from the first dilution (10^{-1}) and is denoted as < 2.00 log CFU/g. Ten colonies on the respective agar (except *Bacillus* colonies which were below the method quantification limit) were isolated and examined (Phong et al., 2022b). Preliminary characterisation of microbial isolates was performed by catalase and oxidase tests. Isolates that presented typical biochemical reactions were Gram-stained (Fluka,

Castle Hill, NSW, Australia) and observed under an Olympus BX51 microscope (Shinjuku-ku, Tokyo, Japan) (Phong et al., 2022b).

4.2.7 Mass recovery

The mass recovery of truffle extract obtained from each extraction method was determined as follows (Li et al., 2010):

Mass recovery (%) = weight of collected extract (g) / weight of raw material used in the extraction (g) X 100%

4.2.8 Data analysis

All experimentation was conducted in triplicate and all values were expressed as mean ± standard error. All statistical analyses were performed using IBM SPSS statistics version 26 software. One-way analysis of variance (ANOVA) followed by post-hoc Tukey's test was performed to compare the means of more than two independent groups. Independent samples t-test was used to compare the means of two different groups. Differences with $p < 0.05$ (95% confidence level) were considered to be statistically significant (Baldi & Moore, 2017). No direct statistical comparison was made between Experiment 1 and Experiment 2 (they were treated as independent studies) to avoid sample bias as a result of different starting materials used that showed a variation in their volatile profiles.

4.3 Results and discussion

This study was divided into two experiments. Experiment 1 was to determine solvent choice (ethanol, acetone, hexane, and liquefied butane) for the extraction of key volatiles that have been identified as important in proving the final aroma impression of *T. melanosporum* (Culleré et al., 2010; Culleré et al., 2013a), which could then be applied as a safe food ingredient in developing new products. Due to the limited amount of hexane extracts obtained and the fact that the butane extracts were too low to accurately measure, it was impossible to conduct a microbial analysis in Experiment 1. Overall, the findings demonstrated that ethanol extracted most of the key volatiles with the highest mass recovery (Table 4.2 and 4.3) hence it was selected as the preferred solvent for Soxhlet extraction (Section 4.3.3).

In Experiment 2, a comparison was made between the two extraction methods (SC-CO_2 and Soxhlet extraction). To allow the study to be continued during the truffle off-season,

frozen truffles which were stored at -80 ± 2°C were used in Experiment 2. The fact that freezing may affect the aroma and microbial population in truffles shall be normalised by comparing the truffle extracts with the frozen samples as the starting materials. As such, our results were strictly based on the differential extraction treatments for comparison, similar to the work by Phong et al. (2017). SC-CO_2 and Soxhlet extraction demonstrated the ability to extract six out of the ten key volatiles. The microbial analysis showed that extracting volatile fractions using these methods could reduce microbes to a point below the detection limit (< 2.00 log CFU/g) (Section 4.3.3.3). The results provide a positive indication that the extraction of volatiles from truffles to produce a natural and safe flavouring ingredient is possible.

4.3.1 Important volatile compounds in *T. melanosporum*

A total of 75 volatiles in truffles with various functional groups were identified in the present study (Table S1). It is widely accepted that not all volatiles are responsible for what humans perceive as aroma in truffles (Culleré et al., 2010; Culleré et al., 2013a; Schmidberger & Schieberle, 2017). For this reason, ten key volatiles, which are believed to be the major contributors to the final aroma impression of *T. melanosporum* (Table 4.1), were selected based on previous studies (Culleré et al., 2010; Culleré et al., 2013a). These key volatiles were used as key indicators to evaluate and compare the volatile profile of the extracts obtained in the present study.

Table 4.1: List of potentially important volatile compounds in *T. melanosporum*

Key volatiles	Boiling point (°C)	Aromatic descriptor
Methanethiol	5.95	Cooked cabbage, vegetable
Dimethyl sulfide (DMS)	37.34	Truffle, sulphur
Ethyl-3-methylbutanoate	133	Fruit, anise
Dimethyl disulfide (DMDS)	110	Truffle, sulphur
Isoamyl alcohol	131	Cheese
1-Octen-3-one	166-167	Mushroom
Dimethyl trisulfide (DMTS)	165-170	Pungent
1-Octen-3-ol	173-175	Mushroom, earth, mould
Methional	165	Boiled potatoes
***p*-Cresol**	202	Phenolic/ leather

Partially adapted from Culleré et al. (2010) and Culleré et al. (2013a).

4.3.2 Experiment 1 - Solvent extraction (ethanol, acetone, hexane, and liquefied butane)

The impacts of different solvents (ethanol, acetone, hexane, and liquefied butane) on the key volatile profile and mass recovery of the extracts were examined. Ethanol, acetone, and hexane were selected as they are some of the commonly used extraction solvents in the food sector (Chemat et al., 2019). Butane has also been used in the food sector as a processing aid and liquefied butane extraction was selected in this study due to its low vapour pressure that allows butane to be easily separated from the extracted raw materials at low temperature and this would enable more volatiles to be successfully extracted (Rapinel et al., 2017). The extraction conditions, mass recovery, and physical appearance of the extracts are illustrated in Table 4.2. Both ethanol and acetone extraction produced a similar brownish paste-like extract but the mass recovery of ethanol extract (5.67 ± 0.35%) was significantly higher than acetone extract (3.77 ± 0.32%). It was most likely that the ethanol could have extracted a wider proportion of the dry matter and retained more water besides the volatile fraction. Through our observation, ethanol and acetone demonstrated good physical contact with the truffle samples during the extraction process. In contrast, as evidenced by a very low mass recovery obtained by hexane (0.16 ± 0.02%), there was no apparent observable mixing between the truffle samples and hexane; instead, they formed two distinct layers despite continuous stirring being applied throughout the extraction process, making hexane unsuitable as a solvent for extracting truffle volatiles. A similar problem was reported previously when extracting lipids from wet microalgae (*Chlorella pyrenoidosa*) using hexane which led to a much lower mass recovery than the dry biomass (de Jesus et al., 2019). This was because the moisture in the wet biomass prevented penetration of hexane into the microalgal cell as hexane cannot form hydrogen bonds with water, thereby hindering physical contact between hexane and lipid material (de Jesus et al., 2019).

The key volatiles of the truffle extracts produced by ethanol, acetone, hexane, and liquefied butane are presented in Table 4.3. None of the solvents were able to extract all of the ten key volatiles present in the original truffles. Some key volatiles were not detected in any of the extracts. Ethanol and butane extraction exhibited a better extractive performance (with seven key volatiles present) than acetone and hexane which extracted only five and three key volatiles, respectively (Table 4.3). It is interesting to note that the key volatile profile of ethanol and butane extracts varied significantly. For example, butane extract contained a significantly high amount of isoamyl alcohol than the ethanol

extract. While a significantly high amount of *p*-cresol can be found in the ethanol extract, this volatile was not detected in the butane extract. Methional was successfully recovered by butane extraction but not ethanol extraction. The variation in the key volatile profile between ethanol and butane extracts could be due to differences in the solubility, selectivity, and affinity of the solvent types. More studies are required to confirm our speculation. Given that butane gas needs to be compressed into a liquid during extraction for an efficient mass transfer between solvent and samples, the setup for a butane extraction is more complicated and this may be a major limiting factor from the point of view of energy efficiency and industrial applicability in addition to its safety risk coupled with its low yield, it was thus excluded from further consideration. As acetone and hexane extracted fewer key volatiles than ethanol, ethanol was therefore considered the preferred solvent for extracting truffle volatiles in terms of its amount of mass recovery and volatile profile, in addition to its availability, biodegradability, and relative safety (Wong et al., 2014).

Table 4.2: Comparison of extraction conditions, mass recovery, and physical properties of truffle extracts obtained by solvent extraction using ethanol, acetone, hexane, and liquefied butane

Extraction solvent	Extraction conditions			Mass recovery (%)	Physical properties	
	Time (h)	Pressure (bar)	Temperature (°C)		Form at ambient condition	Colour
Ethanol	1	1	RT	5.67 ± 0.35 [a]	Paste	Brownish
Acetone	1	1	RT	3.77 ± 0.32 [b]	Paste	Brownish
Hexane	1	1	RT	0.16 ± 0.02 [c]	Liquid	Clear
Liquefied butane	2	-	RT	N/A [#]	Liquid	Colourless

Data are presented as mean ± standard error of replicates.

N/A: not available, cannot be measured

[#] The mass recovery was too low to accurately measure in this study.

RT: room temperature

Means within the same column with different lowercase letters differ significantly ($p < 0.05$).

Table 4.3: Relative percentages of the potential key volatiles in crushed truffle and truffle extracts obtained by solvent extraction using ethanol, acetone, hexane, and liquefied butane

Key volatiles	Crushed truffles (%)	Solvent extraction (%)			
		Ethanol	Acetone	Hexane	Liquefied Butane
Methanethiol $^{\$}$	0.0038 ± 0.0004 ^{a}	0.0147 ± 0.0029 ^{a}	0.0085 ± 0.0055 ^{a}	0.0007 ± 0.0003 ^{a}	0.0004 ± 0.0004 ^{a}
Dimethyl sulfide (DMS) $^{\$}$	0.1269 ± 0.0457 ^{a}	0.0115 ± 0.0046 ^{ab}	0.0042 ± 0.0023 ^{b}	ND	0.0292 ± 0.0100 ^{ab}
Ethyl-3-methylbutanoate	0.0904 ± 0.0163	ND	ND	ND	ND
Dimethyl disulfide (DMDS) $^{\$}$	0.0087 ± 0.0011 ^{a}	0.0344 ± 0.0157 ^{a}	0.0273±0.0052 ^{a}	ND	0.0057 ± 0.0014 ^{a}
Isoamyl alcohol	20.1256 ± 1.6432 ^{a}	0.1585 ± 0.0385 ^{c}	ND	1.0425 ± 0.9459 ^{c}	39.1363 ± 1.8080 ^{b}
1-Octen-3-one	0.0926 ± 0.0496	ND	ND	ND	ND
Dimethyl trisulfide (DMTS) $^{\$}$	0.0021 ± 0.0001 ^{a}	0.0996 ± 0.0604 ^{a}	0.0568 ± 0.0313 ^{a}	ND	0.0147 ± 0.0087 ^{a}
1-Octen-3-ol	4.6304 ± 0.9122 ^{a}	0.9435 ± 0.1490 ^{a}	ND	4.5325 ± 2.4355 ^{a}	1.0743 ± 0.1795 ^{a}
Methional	0.8753 ± 0.4496 ^{a}	ND	1.2831 ± 1.0755 ^{a}	ND	0.1371 ± 0.1371 ^{a}
***p*-Cresol**	0.0148 ± 0.0042 ^{b}	4.5652 ± 1.7502 ^{a}	ND	ND	ND

Data are reported as the mean ± standard error of replicates.

ND: not detected

Relative percentage (%) of each identified volatile: peak area of an individual component / total peak area of all components X 100%

Footnote [$]: These compounds did not produce any fully resolved peaks in the chromatogram due to their low concentration and/or co-elution with other peak with the similar retention time. Extraction of a single ion chromatogram (the selective mass (m/z) chosen for methanethiol, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide was 47, 62, 94, and 126, respectively) was utilised to determine the presence and the relative amount of these compounds.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$).

4.3.3 Experiment 2 - Comparison of Soxhlet and SC-CO_2 extraction

4.3.3.1 Physical appearance

The extraction conditions, mass recovery, and physical properties of the extracts achieved by Soxhlet and SC-CO_2 extraction are presented in Table 4.4. The truffle extracts obtained by these two methods were physically different. The SC-CO_2 extraction gave a clear liquid, whereas the Soxhlet extraction gave a brownish paste. The mass recovery of the Soxhlet extraction was 6.62 ± 0.33% which was at least double than that of the SC-CO_2 extract (2.49 ± 0.38%). A possible explanation for the difference in the mass recovery could be that the polar nature of ethanol and its miscibility with water (Pena-Pereira & Tobiszewski, 2017) may allow efficient physical contact with truffles that generally comprise about 75% water (Palacios et al., 2014). As a result, a broader range of fungal metabolites or other components such as protein, carbohydrate, fat, and water could have been co-extracted by Soxhlet extraction compared to SC-CO_2 extraction (Danh et al., 2013; Wu et al., 2021), resulting in a higher mass recovery in the Soxhlet extract (Table 4.4).

Table 4.4: Comparison of extraction conditions, mass recovery and physical properties of truffle extracts obtained by SC-CO_2 and Soxhlet extraction

Extraction method	Extraction conditions			Mass recovery (%)	Physical properties	
	Time (h)	Pressure (bar)	Temperature (°C)		Form at ambient condition	Colour
SC-CO_2	2	205	60	2.49 ± 0.38^{b}	Liquid	Clear
Soxhlet (ethanol)	3	1	80	6.62 ± 0.33^{a}	Paste	Brownish

Data are reported as the mean of triplicate extractions ± standard error.

Means within the same column with different lowercase letters differ significantly ($p < 0.05$).

4.3.3.2 Volatile profile

When comparing the key volatiles in the extracts and the starting material, not all ten important volatiles were recovered by both techniques (Table 4.5). Of the ten important volatiles (Table 4.1), six (methanethiol, DMDS, isoamyl alcohol, DMTS, 1-octen-3-ol, and *p*-cresol) were successfully extracted by Soxhlet and SC-CO_2 extraction. Our results were supported by Tejedor-Calvo et al. (2021b) who also reported that not all key volatile compounds identified in the truffles such as methional and ethyl-2-methylbutanoate were successfully extracted by SC-CO_2 extraction, indicating that the loss of some reactive volatiles during an extraction process is inevitable. Nevertheless, the successful recovery of the six key volatiles by both Soxhlet and SC-CO_2 extraction methods clearly demonstrated that the natural extracts contained more key volatiles with a higher aroma complexity than the synthetic truffle flavours which typically contain only two to five molecules and do not represent the actual truffle aroma (Wernig et al., 2018). The finding suggests that the extraction of volatile compounds from truffles for the production of a natural flavouring ingredient is promising.

SC-CO_2 extraction can be operated under milder processing conditions than Soxhlet extraction, minimising chemical breakdown and loss at recovery. This is because CO_2 is gaseous at ambient temperature and pressure; thus isolation of the extract from the liquid CO_2 can be achieved by releasing pressure (Machado et al., 2013) with minimum impact to volatiles. As pressure decreases, the liquid CO_2 returns to its gaseous state and evaporates, leaving behind the solvent-free extract (Ormeno et al., 2011). It is expected that the SC-CO_2 extract would retain more volatiles than the Soxhlet extract. However, there was no significant difference in the relative percentages in four of the six key volatiles (methanethiol, DMDS, isoamyl alcohol, and DMTS) between SC-CO_2 extract and Soxhlet extract in the present study. It was observed that *p*-cresol was the only compound that showed a significantly higher amount in the SC-CO_2 extract (7.4492 ± 0.3334%) compared to the Soxhlet extract (6.1727 ± 0.2602%). Whereas 1-octen-3-ol was significantly lower in the SC-CO_2 extract (2.1588 ± 0.1414%) than that of the Soxhlet extract (5.2962 ± 0.5220%).

The Soxhlet and SC-CO_2 extracts did not contain DMS (Table 4.5). Similarly, in Experiment 1, DMS was not detected in the hexane extract and a significant loss was observed in the acetone extract (Table 4.3). The reason for the loss or absence of DMS in the extracts obtained by each method could be due to its co-evaporation during solvent

elimination step at the end of the extraction process (Sun et al., 2017). The same phenomena were evident in an experiment that involved the extraction of volatiles from *Exocarpium Citri Grandi* using solvent extraction (Xie et al., 2013). Only 39.98% of terpenes (the main volatile components of *Exocarpium Citri Grandi*) was retained in the Soxhlet extract. The major loss of terpenes was believed to have occurred during evaporation step (Xie et al., 2013).

Methanethiol has a much lower boiling point (bp) (5.95°C) than DMS (37.34°C), which is likely to escape during solvent removal. But it is interesting to note that methanethiol was detected in the Soxhlet extract (0.0180 ± 0.0009%) and SC-CO_2 extract (0.0145 ± 0.0017%) (Table 4.5). Although methanethiol is very sensitive to evaporation, this compound was seen in both extracts, likely due to the breakdown of DMDS (Kiragosyan et al., 2020). The interconversion between DMDS and methanethiol has been reported in previous literature (Lyimo et al., 2009; Mashkina, 2000; Splivallo et al., 2011). There is no doubt that DMDS, which has a high boiling point (110°C), was successfully recovered by Soxhlet and SC-CO_2 extraction at 0.0312 ± 0.0021% and 0.0513 ± 0.0100%, respectively (Table 4.5). Our findings were in agreement with Tejedor-Calvo et al. (2021b) who observed the trapping of DMDS in the truffle extract derived from SC-CO_2 extraction operating at 40°C with the addition of grapeseed oil as a fat matrix in the separator before depressurization of the extraction process. Similarly, DMTS was detected in the extracts, most likely due to its thermally stable characteristic (bp 165-170°C) (Table 4.5).

Ethyl-3-methylbutanoate, which belongs to the ester group, is another important compound that contributes to the fruity odour in black truffle (Table 4.1). Unfortunately, this compound was not detected in either extract in the present study (Table 4.5). Ethyl-3-methylbutanoate is known to be susceptible to hydrolysis (Theodorou et al., 2007), but more studies are required to confirm if this was the reason that led to the loss of this compound in the extracts. Both extraction processes could not retain 1-octen-3-one in the extracts as well. The possible co-evaporation of this thermally stable compound (bp 166-167°C) in ethanol or CO_2 during the removal step can be ruled out as this step was performed at a low temperature in both methods. As the truffle extracts consisted of a blend of volatiles mixture with diverse functional groups (Buzzini et al., 2005; Splivallo et al., 2011), one possibility could be that 1-octen-3-one reacted with other compounds in the extract. Thiols were present in the extracts as good nucleophiles in conjugate addition reactions. More studies are needed to confirm this. Similarly, the reason for the loss of

methional (bp 165°C) despite having a high boiling point could be that this compound was more reactive than compounds such as isoamyl alcohol (bp 131°C), 1-octen-3-ol (bp 173-175°C), and *p*-cresol (bp 202°C) that were retained in the extracts (Table 4.5). Our observation was aligned with a SC-CO_2 extraction study done by Tejedor-Calvo et al. (2021b), in which 2-methyl-1-butanol (also known as amyl alcohol) was detected in the truffle extract but methional, despite having a higher boiling point, was not successfully extracted.

Table 4.5: Relative percentages of the potential key volatiles in crushed truffles and truffle extracts

Key volatiles	Relative percentage (%)		
	Crushed truffles	Soxhlet extraction (ethanol)	$SC\text{-}CO_2$ extraction
Methanethiol $^{\$}$	0.0059 ± 0.0007 ^{b}	0.0180 ± 0.0009 ^{a}	0.0145 ± 0.0017 ^{a}
Dimethyl sulfide (DMS) $^{\$}$	0.0116 ± 0.0004	ND	ND
Ethyl-3-methylbutanoate	0.1172 ± 0.0132	ND	ND
Dimethyl disulfide (DMDS) $^{\$}$	0.0056 ± 0.0009 ^{a}	0.0312 ± 0.0021 ^{a}	0.0513 ± 0.0100 ^{a}
Isoamyl alcohol	15.0171 ± 0.0641 ^{a}	1.4160 ± 0.3168 ^{b}	2.1965 ± 0.2184 ^{b}
1-Octen-3-one	0.1627 ± 0.0286	ND	ND
Dimethyl trisulfide (DMTS) $^{\$}$	0.0045 ± 0.0004 ^{b}	0.0487 ± 0.0096 ^{a}	0.0254 ± 0.0049 ^{a}
1-Octen-3-ol	6.2194 ± 0.6424 ^{a}	5.2962 ± 0.5220 ^{a}	2.1588 ± 0.1414 ^{b}
Methional	0.8424 ± 0.0511	ND	ND
***p*-Cresol**	0.5192 ± 0.0173 ^{c}	6.1727 ± 0.2602 ^{b}	7.4492 ± 0.3334 ^{a}

Data are reported as the mean of triplicate extractions ± standard error.

ND: not detected

Relative percentage (%) of each identified volatile: peak area of an individual component ⁄ total peak area of all components X 100%

Footnote [$]: These compounds did not produce any fully resolved peaks in the chromatogram due to their low concentration and/or co-elution with other peak with the similar retention time. Extraction of a single ion chromatogram (the selective mass (m/z) chosen for methanethiol, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide was 47, 62, 94, and 126, respectively) was utilised to determine the presence and the relative amount of these compounds.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$).

4.3.3.3 Microbial analysis

The microbial counts in the starting material and the extracts obtained from Soxhlet and SC-CO_2 extraction were determined and compared (Table 4.6). Before extraction, *Pseudomonas* spp. and yeasts which may spoil truffles, were detected at 6.46 ± 0.07 log CFU/g and 5.95 ± 0.09 log CFU/g, respectively. *Bacillus* spp. are most commonly found in soil (Saidi et al., 2015), but a relatively low level (< 3.48 log CFU/g) of *Bacillus* spp. was detected in the starting material. *Listeria* spp. and *Salmonella* spp. were selected for investigation in this study because they are the most common foodborne pathogens of concern in ready-to-eat products (Bourdoux et al., 2016). Neither genus was detected in the starting materials of the current study, which is consistent with previous studies (Phong et al., 2022b; Rivera et al., 2010a).

SC-CO_2 extraction is considered one of the most promising extraction techniques because in addition to being used as an extraction tool, this method is capable of inactivating a wide range of microorganisms (Beh et al., 2019). In this study, SC-CO_2 extraction was able to effectively control all microorganisms studied, including *Bacillus* spp., which were below the detection limit (< 2.00 log CFU/g) in the truffle extract. A previous study showed that under the same operating condition (60°C, 2.05 x 10^7 Pa for 120 min), spore-forming *Bacillus* spp. was reduced by 5-log (Dillow et al., 1999). The occurrence of SC-CO_2 sterilisation was believed to be due to the unique mass transfer properties of CO_2 which can diffuse readily into the cell and lead to pH alteration within the cell resulting in death (Dillow et al., 1999).

Similar to the SC-CO_2 extract, all the microorganisms tested in the Soxhlet extracts were also below the detection limit (< 2.00 log CFU/g). Ethanol is a general bactericide (Arjmand et al., 2015; Thomas, 2012); however, ethanol alone is insufficient to inactivate bacterial spores (Arjmand et al., 2015). A possible explanation for the reduction of *Bacillus* spp. to below the detection limit (< 2.00 log CFU/g) in the extract may be that Soxhlet extraction was operated at 80°C and in combination with ethanol could have caused a synergistic sporicidal effect against *Bacillus* spores. A previous study showed that ethanol-heat treatment (70°C) could alter the inner membrane properties of *Bacillus* spores leading to its inactivation (Loison et al., 2016). The result of this study indicates that it is possible to produce a truffle volatile extract that is microbiologically safe to be used as a food ingredient.

Table 4.6: Microbial counts in frozen crushed truffle and truffle extracts

Microbial counts (log CFU/g)	Crushed truffles	Extraction methods	
		Supercritical CO_2	Soxhlet
***Pseudomonas* spp.**	6.46 ± 0.07	< 2.00	< 2.00
Yeasts	5.95 ± 0.09	< 2.00	< 2.00
***Listeria* spp.**	< 2.00	< 2.00	< 2.00
***Salmonella* spp.**	< 2.00	< 2.00	< 2.00
***Bacillus* spp.**	< 3.48	< 2.00	< 2.00

Data reported as the mean of triplicate measurements ± standard error.

4.4 Conclusion

This study demonstrated that the physical and chemical properties of truffle extract may vary depending on the type of extraction technique used, as evidenced by a significant difference in the mass recovery between the SC-CO_2 and Soxhlet extraction. Not all of the key volatiles were successfully extracted under the current operating conditions in the present study. Nonetheless, six out of ten key volatiles were successfully recovered by both the SC-CO_2 and Soxhlet extraction methods, indicating that natural extracts had a higher aroma complexity than the synthetic truffle flavours which contain less aroma volatile molecules and would therefore be less desirable. An interesting finding of this study is that the elimination of microbes from truffle extracts to the point where none were detected (< 2.00 log CFU/g) can be achieved via both Soxhlet and SC-CO_2 extraction methods meaning a safe food ingredient can be developed. Taken together, the outcome showcases the potential to extract volatiles from black truffles to produce a natural flavouring ingredient that could be made available for food application throughout the year. Overall, this study is the first step towards advancing our understanding of the impacts of extraction on the mass recovery, microbial quality, and key volatile profile of truffle extract and could provide the framework for future volatiles extraction from truffles. Future research needs to optimise the extraction technique to overcome the technical challenges and associated limitations to obtain truffle extract that has close proximity to black truffle aroma.

CHAPTER 5: Comparative evaluation of encapsulation using β-cyclodextrin versus freeze-drying for better retention and stabilising of Black Périgord truffle (*Tuber melanosporum*) aroma

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Abstract

This study aimed to develop a novel technique to retain and stabilise compounds contributing to truffle aroma by encapsulation using β-cyclodextrin. Two experiments were conducted. In the first, truffle products resulting from three different encapsulation methods: direct mixing method (M1), direct mixing followed by ethanol addition method (M2), and paste method (M3) were compared with untreated truffles (positive control) in terms of their key volatile profile and the change in microbial population over a 90 day period. M2-derived product was the least optimal in retaining key volatile compounds despite showing the lowest microbial population. There was no significant difference in the volatile profile of products derived from M1 and M3 on day 0. However, it was observed that M3-derived product could retain its volatile profile better than M1-derived product by day 90. M3 was compared with freeze-drying in the second experiment. Freeze-dried truffles showed an overall higher relative percentage of volatiles than M3-derived product on day 0. However, by day 90, some volatile changes occurred in the freeze-dried truffles but not in the M3-derived product. The findings indicate that while freeze-drying could adequately conserve truffle volatiles, the encapsulation of volatile compounds in β-cyclodextrin could improve volatile stability of truffle product and allow for longer storage. Microbes were found in all encapsulated truffle products and freeze-dried truffles on day 0 and 90, suggesting the need to explore the possibility of incorporating a decontamination step in the process prior to either encapsulation or freeze-drying.

Keywords: Black Périgord truffle, β-cyclodextrin, Encapsulation, Truffle aroma retention and stability, Volatile compounds

5.1 Introduction

Black Périgord truffle (*Tuber melanosporum* Vittad. 1831) is a sought-after delicacy worldwide (Romanazzi et al., 2015), well known for its distinctive aroma notes (Culleré et al., 2010). Black truffle has a short harvest season and deteriorates rapidly after harvest (Pacioni et al., 2014b; Zambonelli et al., 2016). Artificial truffle aroma has been used extensively in the food industry (Cataldo et al., 2016; Lukin et al., 2018) but there are consumer concerns over the consumption of artificially flavoured food products. For this reason, therein lies a value in producing a more complex and naturally derived product (Phong et al., 2022c; Torregiani et al., 2017).

Due to seasonality (truffles are harvested mostly in winter from June to August in the Southern Hemisphere and from December to February in the Northern Hemisphere) and its short shelf life, there is a period when natural truffle aroma is not available for food applications anywhere in the world (Phong et al., 2022c). Long-term preservation of truffles is essential to extend the supply. However, improving the shelf life of fresh truffles for long-term storage whilst safeguarding their sensory qualities is challenging (Rivera et al., 2011a). Many aroma compounds that have been identified as significant contributors to the overall sensory impression of black truffle (Culleré et al., 2010) are volatile and reactive (Buettner, 2017). For example, dimethyl sulfide (DMS) has a low boiling point (37°C) and is susceptible to oxidation (Bentley & Chasteen, 2004; Buettner, 2017; Zhu & Cadwallader, 2019). Developing a mild processing method to retain and stabilise truffle aroma before fresh truffles deteriorate and produce off-flavour is therefore an imperative (Campo et al., 2017; Culleré et al., 2013a; Phong et al., 2022c). Furthermore, the current truffle grading standards are based on size and physical appearance rather than aroma quality (The Australian Truffle Growers Association, 2014; UNECE, 2017). Despite having a high aroma quality, smaller and offcut truffles are graded poor quality than larger truffles and therein lies an opportunity for value addition to these lower-priced truffles (Hall et al., 2017; Phong et al., 2022c).

Freeze-drying is among the methods used to preserve the aroma of truffles by removing moisture from truffles at temperatures below -20°C (Campo et al., 2017; Marco et al., 2016; Palacios et al., 2014). Freeze-drying appears to be the preferred method to preserve the original aroma of truffles compared to canning, hot air drying, and freezing at -80°C (Campo et al., 2017; Marco et al., 2016). However, some volatile changes and a reduction

in the aroma intensity were still detected in the freeze-dried truffles (Campo et al., 2017; Palacios et al., 2014).

On the other hand, encapsulation has shown promise in retaining the aroma and increasing the shelf life of food products by inhibiting volatilisation of aroma compounds and preventing off-flavour formation caused by oxidation or degradation during processing and storage (Wang et al., 2015). Encapsulation is a technique in which active or sensitive compounds such as those responsible for aroma (guest) are surrounded or entrapped by protective wall materials or carriers (host) (Shrestha et al., 2017; Wang et al., 2015). A wide range of materials such as starches, proteins, lipids, gums or combinations thereof can be used to encapsulate flavouring ingredients (Zuidam & Heinrich, 2010). Among these materials, cyclodextrins (CDs) are commonly used as carriers and stabilisers for flavours in the food industry (Marques, 2010; Zuidam & Heinrich, 2010). Numerous investigations have demonstrated the potential of using CD as a vehicle to encapsulate various types of flavours and volatile compounds for use as a food ingredient such as oregano (*Origanum onites* L.) essential oil (Kotronia et al., 2017), sweet orange flavour (Zhu et al., 2014), and strawberry flavour (Balci-Torun & Ozdemir, 2021). However, research on capturing the aroma volatiles of truffles via encapsulation has yet to be reported in the literature. We hypothesised that encapsulation could minimise degradation or loss of chemically unstable truffle volatiles during processing and storage compared to the current preferred preservation method for truffles, namely the freeze-drying method. Encapsulation can be a promising technique to capture truffle aroma before fresh truffles deteriorate, thereby achieving longer shelf stability over that of freeze drying and allowing natural truffle aroma to be readily available for food application throughout the year. The production of a natural flavouring ingredient from smaller and offcut truffles via encapsulation could add value to these lower-priced truffles, which will benefit the truffle industry.

In a study by Feng et al. (2019), three species (*Tuber sinensis*, *Tuber sinoalbidum*, and *Tuber sinoexcavatum*) of ripe truffles were crushed into truffle purees and their volatile profiles were determined. Torregiani et al. (2017) conducted a similar study in which the volatile profiles of different commercial truffle products such as truffle oils and truffle sauces were assessed, which indicated that different products may display differences in their aroma profiles. Neither of these two studies examined encapsulation of the aroma compounds for application into food products.

The current study aimed to develop a novel strategy of encapsulating the volatile compounds of fresh black truffle using β-cyclodextrin (β-CD) as an encapsulating material to produce a natural flavouring ingredient that can be stored for the long term. This study was divided into two experiments. In the first experiment, three different encapsulation methods of preparation, namely direct mixing method (M1), direct mixing followed by ethanol addition method (M2), and paste method (M3) were compared. In the second experiment, a comparison was made between the preferred encapsulation technique that retained the volatile compounds best based on the first experiment's findings and freeze-drying.

5.2 Materials and methods

5.2.1 Raw material: fresh truffles

Mature truffles harvested from a farm situated in the Pyrenees Mountains in the Catalonia region of Spain in February 2020 were used in Experiment 1. Experiment 2 used matured truffles harvested from the Manjimup region of Western Australia in August 2020. Apart from the limited availability of fresh truffles restricted by seasonality and short harvesting season, the fresh truffles needed to be processed immediately upon arrival due to their high perishability. Due to which fresh truffles from different harvests had to be used for Experiment 1 and 2. No direct comparison was made between Experiment 1 and 2 due to the different origins of truffles. All freshly collected truffles were brushed, rinsed with tap water to remove any soil and air-dried in a laminar air flow cabinet as per industrial practice (Culleré et al., 2013a). The cleaned truffles were packed in an insulated container held at 4 ± 2°C (Rivera et al., 2010a) and immediately shipped to our laboratory (approximately seven days for Experiment 1 and three days for Experiment 2).

5.2.2 Starting material preparation: crushed truffles for Experiment 1 and 2

Qualitative selection of truffles was made by carefully inspecting their visual appearance upon sample arrival. Truffles that were free of defects and with a similar appearance were selected, whereas those that showed signs of invertebrate infestation, spoilage with soft texture or damage were rejected (Culleré et al., 2012). Without undergoing any drying process, the selected truffles were crushed in a mortar and pestle and mixed well to reduce possible natural variation in volatile profile and microbial community (interspecies) which may be due to the growing condition and natural habitat (Strojnik et al., 2020; Torregiani et al., 2017). The homogenised mix of truffles was then divided into 12 portions (untreated truffles as the positive control and three different encapsulation

methods (M1, M2, and M3) were assessed in triplicate) for Experiment 1 and nine portions (untreated truffles as the positive control, freeze-drying, and M3 were assessed in triplicate) for Experiment 2. There was 100 g in each portion and all of them were separately vacuum-packaged into individual 210 X 300 mm vacuum bags (65 µm Polyamide/Polyethylene, FPA Australia Pty Ltd, Malaga, WA, Australia) using a vacuum packaging machine (easyPACK-mk2, Webomatic, Hansastrasse, Bochum, Germany) and stored at 4 ± 2°C until required (experiments were conducted within the same day of starting material preparation).

5.2.3 Experiment 1 - Comparison of three encapsulation methods

Three different methods (M1, M2, and M3) similar to the procedures described by Shrestha et al. (2017), with some modification, were studied using crushed truffles as the starting material (Section 5.2.2). Untreated truffles (starting material) were used as the positive control. Three sets of negative control (C1, C2, and C3) corresponding to each method were prepared in the same manner, except no truffles were added. Following processing, the volatile profile and microbial quality of these control sets and encapsulated products were determined (Section 5.2.5 and 5.2.6). Then, the control sets and encapsulated products were separately vacuum-packed and stored at 4 ± 2°C until required, and the same analyses (Section 5.2.5 and 5.2.6) were repeated after storage for 90 days. The formulation of the three encapsulation methods is summarised in Table 5.1.

5.2.3.1 Direct mixing method (M1)

Food-grade β-CD (100 g) (CAVAMAX® W7 Food) (Wacker Chemie AG, Victoria, Australia) was mixed with 100 g of crushed truffles in a beaker using a stick blender (Kambrook KSB7, Breville Group Ltd, NSW, Australia) for 2 min at the minimum speed of the blender. The mixture was freeze-dried (Christ Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at -30°C, 0.37 mbar until constant weight. The dried complex was then ground into a fine powder using a mortar and pestle. The controls (C1) were prepared in the same manner, except no truffles were added.

5.2.3.2 Direct mixing followed by ethanol addition method (M2)

Ethanol (100 mL) (LabServ, Thermo Fisher Scientific, Scoresby, Victoria, Australia) was added to the mixture of β-CD (100 g) and crushed truffles (100 g). The mixture was homogenised using a stick blender (Kambrook KSB7, Breville Group Ltd) for 2 min at

the minimum speed of the blender. The complexed paste was freeze-dried (Christ Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH) at -30°C, 0.37 mbar until constant weight. The dried complex was then powdered using a mortar and pestle. The controls (C2) were prepared by following the same procedure but without adding truffles into the process.

5.2.3.3 Paste method (M3)

Into 100 g of β-CD, 100 mL of deionised water was added and mixed to form a paste before adding 100 g of crushed truffles. Next, the mixture was homogenised using a stick blender (Kambrook KSB7, Breville Group Ltd) for 2 min at the blender's minimum speed. The complexed paste was freeze-dried (Christ Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH) at -30°C, 0.37 mbar until constant weight. The dried complex was then powdered using a mortar and pestle. The same procedures were repeated for this method's controls (C3) without the addition of truffles.

Table 5.1: Truffle-β-CD complexes derived from M1, M2, and M3.

Encapsulation method	Truffles (g)	β-CD (g)	Deionised water (mL)	Ethanol (mL)	Freeze-drying	Truffle-CD ratio
C1*	-	100	-	-	48 h at -30°C	-
M1	100	100	-	-	48 h at -30°C	1:1
C2*	-	100	-	100	48 h at -30°C	-
M2	100	100	-	100	48 h at -30°C	1:1
C3*	-	100	100	-	48 h at -30°C	-
M3	100	100	100	-	48 h at -30°C	1:1

C1*, C2*, C3*: negative control for each method (without adding truffles)

5.2.4 Experiment 2 - Comparison of encapsulation and freeze-drying

Due to its perceived stability based on the outcome of experiment 1, M3 was considered the preferred method for further study. Whereas according to previous studies, freeze-drying is regarded as the preferred method in preserving the overall original aroma of fresh truffles compared to canning, hot air drying, and freezing (Campo et al., 2017; Marco et al., 2016). For this reason, a comparison was made between M3 and freeze-drying. Following processing, the volatile profile and microbial quality of the products from each method were determined (Section 5.2.5 and 5.2.6). The balance of each product was separately vacuum-packed and stored at 4 ± 2°C until subsampled for volatile and microbial analyses (Section 5.2.5 and 5.2.6) after 90 days of storage.

5.2.4.1 Paste method (M3)

The same procedures as per Section 5.2.3.3 were performed to obtain the powdered complexes (M3) and their corresponding controls (C3).

5.2.4.2 Freeze-drying

Fresh truffles that had been crushed were freeze-dried at 0.37 mbar, -30°C using a freeze drier (Christ Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH) until constant weight (about 48 h).

5.2.5 Volatiles analysis

As per (Díaz et al., 2009) with some modification, headspace solid-phase microextraction (HS-SPME) with a SPME holder (GERSTEL MultiPurpose Sampler for automated sampling) combined with gas chromatography-mass spectrometry (GC-MS) was used to analyse the volatile compounds of all samples on day 0 and 90.

5.2.5.1 Headspace sampling by SPME

A flexible fused silica fibre coated with a 50/30 µm layer of divinylbenzene/ Carboxen/ Polydimethylsiloxane (DVB/CAR/PDMS) (57298-U, Supelco, Bellefonte, Pennsylvania, USA) was used to sample the volatiles from the headspace above samples. The SPME fibre was conditioned based on the manufacturer's instructions prior to use (Supelco) (Díaz et al., 2009). For the untreated truffles (positive control) analysis, about 1 g of crushed truffles (containing about 25% dry matter) was weighed into a 20 mL headspace glass vial. The β-CD only negative control, truffle-β-CD complexes, and freeze-dried truffles were weighed into a 20 mL headspace glass and rehydrated to the similar moisture

content as the untreated truffles (positive control). 1,2-Dichlorobenzene (Sigma Aldrich, Sydney, NSW, Australia) was added to each vial as an internal standard (Liu et al., 2017b) before the vials were sealed with a magnetic screw cap (Thermo Fisher Scientific). The rehydrated samples were incubated for 10 min at room temperature before headspace sampling of volatile compounds (Palacios et al., 2014). All vials were equilibrated for 5 min at 53°C. The extractor (SPME fibre) was then introduced into the vials through the cap and was exposed in the headspace of the vial for 13.6 min to absorb a sample of the volatiles. After that, the fibre was pulled into the needle assembly and the SPME device was removed from the vial before inserting into the injection port of GC for thermal desorption of the analysts at 200 °C for 2 min using the splitless injection method (Díaz et al., 2009). Blank runs using empty vials were performed regularly between samples to ensure no carryover occurred during chromatography analysis (Splivallo et al., 2012).

5.2.5.2 GC-MS analysis

The volatile compounds were analysed by GC-MS according to the method described by Díaz et al. (2003), with some modification. An Agilent-6890 GC system coupled to a GC 5973 mass spectrometer (Agilent Technologies Inc., Santa Clara, California, USA) equipped with a 30 m (length) x 250 µm (diameter) x 0.25 µm (film thickness) i.d. HP-INNOWax GC column (Agilent Technologies Inc.) fused silica capillary column (Model Number: Agilent 19091N-133) was used to separate the volatile compounds. The oven temperature was programmed from 35°C (held for 5 min) to 240°C at 5°C min^{-1} increments (held for 3 min). The mass spectrometer was operated in the electron ionisation (EI) mode. Data acquisition was performed in a scanning mode in the range of 40-250 m/z at 0.46 sec/scan (3 µ-scans) after a 1.5 min of solvent delay. Helium was used as the carrier gas at 6.74 psi with a flow rate of 1.0 mL/min.

Chromatographic data were processed using MSD ChemStation F.01.03.2357 (1989-2015 Agilent Technologies, Inc.). Volatile compounds were identified by comparing their mass spectral fragmentation patterns with entries in the National Institute of Standards and Technology (NIST) mass spectral database (Version 2.3, 2017, USA) using spectral match quality ≥ 700 as the criterion (Watson & Sparkman, 2007), previously reported mass spectra in the literature, GC retention time of each compound as well as the Kovats retention indices (RI) were calculated based on a series of n-alkanes under the same chromatographic condition (Wernig et al., 2018).

The percentage composition of each identified compound was determined based on peak area normalisation by integration. The relative amount of individual compounds were expressed in relative percentage (%) by calculating the proportion of each individual peak area to the total peak area of all the compounds of interest (Ruiz-Hernández et al., 2018). Extraction of a single ion chromatogram was used to determine the relative amounts of compounds that did not produce any fully resolved peaks in the chromatogram owing to their low concentration and/or co-elution with other peaks with similar retention time, making the integration rather difficult.

5.2.5.3 Important volatile compounds in *T. melanosporum*.

According to the previous literature, only a small part of the volatile components are responsible for what humans perceive as aroma active compounds in truffles (Culleré et al., 2010; Culleré et al., 2013a; Feng et al., 2019; Mustafa et al., 2020; Schmidberger & Schieberle, 2017; Strojnik et al., 2020). Therefore, ten key volatile constituents (Table 5.2) that have been described to be responsible for the final aroma impression of *T. melanosporum* were selected based on previous studies (Culleré et al., 2010; Culleré et al., 2013a) and used as indicators to evaluate the volatile profile of each truffle product processed by different methods in the current study.

Table 5.2: Boiling point (°C), functional group, and aromatic descriptor corresponding to each of the potentially important volatiles in *T. melanosporum*.

Volatile compounds	Boiling point (°C)	Functional group	Aromatic descriptor
Methanethiol	5.95	Thiol	Cooked cabbage
Dimethyl sulfide (DMS)	37.34	Sulfur	Truffle, sulfur
Ethyl-3-methylbutanoate	133	Ester	Fruit, anise
Dimethyl disulfide (DMDS)	110	Sulfur	Truffle, sulfur
Isoamyl alcohol	131	Alcohol	Cheese
1-Octen-3-one	166-167	Ketone	Mushroom
Dimethyl trisulfide (DMTS)	165-170	Sulfur	Pungent
1-Octen-3-ol	173-175	Alcohol	Mushroom, earthy, fungal
Methional	165	Both aldehyde and thioether	Boiled potatoes
***p*-Cresol**	202	Phenol	Phenolic/ leather

Adapted from Culleré et al. (2013) with modification.

5.2.6 Microbiological analysis

Each sample (1 g) was homogenised in 9 ml of sterile buffered peptone water for 2 min using a homogeniser (PRO250, PRO Scientific Inc., Oxford, USA). Decimal dilutions were performed using sterile phosphate-buffered saline. Serially diluted samples (0.1 mL) were pour-plated onto the respective culture medium and incubated. Total plate counts were determined on tryptone soya agar (TSA) (Oxoid Ltd, Melbourne, Victoria, Australia) after incubation for 48 h at 37 ± 1°C. *Pseudomonas* species were determined on *Pseudomonas* isolation agar (Becton Dickinson Pty Ltd, Sydney, NSW, Australia) after incubation for 48 h at 37 ± 1°C. *Bacillus* spp. were determined on Polymyxin Egg Yolk Mannitol Bromothymol Blue Agar (PEMBA) (Oxoid Ltd) after incubation for 24 h at 37 ± 1°C (Phong et al., 2022b).

To detect the presence of *Salmonella* spp., 1 mL of each sample from the first decimal dilution (10^{-1}) were inoculated into 9 mL of Mannitol Selenite Cystine (MSC) and Rappaport-Vassiliadis (RV) enrichment broths (Oxoid Ltd), respectively. The MSC and RV broths were incubated for 24 h at 37 ± 1°C and 42 ± 1°C, respectively. Following incubation, a loopful of culture from each enrichment broth was streaked onto Xylose-Lysine-Desoxycholate agar (Oxoid Ltd) and incubated for 24 h at 37 ± 1°C. For *Listeria* spp. detection, 1 mL of pre-enrichment culture from each sample were inoculated into 9 mL of *Listeria* enrichment broth (Oxoid Ltd) and incubated at 37 ± 1°C for 24-48 h. A loopful of enriched culture was then streaked onto the *Listeria* selective agar (Oxoid Ltd) and incubated for 24-48 h at 37 ± 1°C (Phong et al., 2022b).

After incubation, microbial colonies that grew on TSA, *Pseudomonas*, and PEMBA medium were counted. Only counts of 30 to 300 colony forming units (CFU) were considered and each count (expressed as log CFU/g) was calculated based on the mean of triplicate. Plates with fewer than 30 colonies are considered below the method quantification limit and are denoted as < 3.48 log CFU/g. The method detection limit was set at one colony from the first dilution (10^{-1}) and is denoted as < 2.00 log CFU/g. A presence or absence was reported for *Salmonella* spp. and *Listeria* spp. Characterisation of microbial isolates was performed by oxidase and catalase tests. Isolates that demonstrated typical biochemical reactions were Gram-stained (Fluka, Castle Hill, NSW, Australia) and their morphological characteristics were observed under an Olympus BX51 microscope (Shinjuku-ku, Tokyo, Japan) (Phong et al., 2022b).

Once samples were withdrawn for the initial microbiological analysis on day 0, the balance was aseptically vacuum-packaged and stored at 4 ± 2°C. Similar to the volatile analysis, the same microbiological analysis as per day 0 were conducted on these samples after 90 days of storage.

5.2.7 Data analysis

All experiments were performed in triplicate and the data were reported as mean ± standard error and statistically analysed using IBM SPSS version 26 software. One-way analysis of variance (ANOVA) followed by post-hoc Tukey's test was performed to compare the means of more than two independent groups. Independent samples t-test was performed to compare the means of two different groups. Paired samples t-test was used to compare two means that are from the same group at two different time points (day 0 and 90). Differences with $p < 0.05$ (95% confidence level) were considered to be statistically significant.

5.3 Results and discussion

5.3.1 Key volatile compound analysis

As mentioned in Section 5.2.5.3, ten key volatile compounds were selected as indicators to evaluate the encapsulation methods (Table 5.2) (Culleré et al., 2010; Culleré et al., 2013a). In the present study, nine key volatiles except 1-octen-3-one were detected in the untreated truffles (Table 5.3 and Table 5.4). A possible reason for the undetectable 1-octen-3-one could be that it was below the detection limit or its absence was due to the impacts of the growing location, environmental factor, and maturation stage that lead to natural variation in the volatile profile (Strojnik et al., 2020; Torregiani et al., 2017).

5.3.2 Experiment 1 - Comparison of three encapsulation methods

Three different encapsulation methods, namely M1, M2, and M3, as described in Section 5.2.3 were compared with regards to their volatile profile and microbial quality. A storage study for a period of 90 days was conducted to evaluate if the truffle aroma can be encapsulated and retained over time. This is the first step to determine the success of encapsulating the truffle aroma over time, which has not been reported before and needed to be performed before further investigation on the physicochemical characteristics of the encapsulated products. The results of volatile and microbial analyses are shown in Table 5.3 and Table 5.5, respectively.

5.3.2.1 Aroma volatiles on day 0

M1 is the complexation of guest molecules in a dry CD powder without solvent. It is a simple method of mixing truffles directly with CD powder at an appropriate proportion so that volatile compounds are potentially entrapped within its structure but the complex produced by this method may result in a low inclusion efficiency (Shrestha et al., 2017). The addition of solvent during encapsulation (M2 and M3) was expected to not only assist the guest molecules in distributing evenly in the CD powder but also increase the molecular mobility of CD, facilitating the diffusion of the volatile compounds into the CD cavity (Shrestha et al., 2017). In the present study, however, the M1-derived product retained a similar relative percentage of key volatiles as the M3-derived product on day 0 with most volatiles retained compared to the untreated truffles (positive control) (Table 5.3). Nonetheless, a comparison between M2 and M3 indicates that the truffle volatiles' inclusion efficiency may depend on the type of solvent used. We found that while *p*-cresol was not detected, most of the volatile compounds (DMS, isoamyl alcohol, 1-octen-3-ol, and methional) in the M2-derived product presented at a lower relative percentage compared to the products derived from M1 and M3 (Table 5.3). This suggests that water (M3) may be a better option than ethanol (M2) to be added during truffle aroma encapsulation. A possible explanation for this could be that water might have encouraged the hydrophobic volatile molecules to go into the cavity of the β-CD due to the hydrophobic effect. As ethanol dissolves most hydrophobic molecules, this solvent may likely compete with β-CD for the volatiles. As for the undetectable *p*-cresol in the M2-derived product (Table 5.3), we speculate that *p*-cresol would form a weaker bond with β-CD than ethanol as *p*-cresol is soluble in ethanol (Kunsagi-Mate et al., 2009). Thus, *p*-cresol might not have been encapsulated.

5.3.2.2 Comparison of the volatile stability at the end of storage

The key volatile composition of the untreated truffles changed significantly compared to the encapsulated complexes (M1, M2, and M3) over the 90 day period (Table 5.3). This implies that the volatile molecules could have been protected by β-CD during processing and storage. This was in agreement with previous literature claiming that encapsulated volatile compounds can be stabilised in CD (Shrestha et al., 2017).

Among all encapsulated complexes (M1, M2, and M3), the M3-derived product was the most stable with no significant changes observed in all the key volatiles after 90 days of storage. The M1-derived product appeared to be more stable than the M2-derived product

after 90 days of storage. For example, methional and DMTS were the two volatiles that showed significant changes in M1-derived product, whereas three volatiles (ethyl-3-methylbutanoate, isoamyl alcohol, and 1-octen-3-ol) were significantly changed in the M2-derived product after storage (Table 5.3). We speculate that the addition of water in M3 would have facilitated the absorption of aroma volatiles into the β-CD cavity, thereby preventing volatile loss and conferring better stability than M1. The results suggest that M3 could be the preferred technique to achieve ingredient stability during processing and storage.

Table 5.3: Relative percentages of the key volatiles in the untreated truffles and truffle-β-CD complexes derived from M1, M2, and M3 on day 0 and 90.

Volatiles	Relative percentage (%)			
	Untreated truffles	M1-derived product	M2-derived product	M3-derived product
Day 0				
Methanethiol $	0.0051±0.0002 Aa	0.0053±0.0004 Aa	0.0023±0.0005 Aa	0.0052±0.0014 Aa
Dimethyl sulfide (DMS) $	0.0606±0.0019 Aa	0.0582±0.0040 Aa	0.0152±0.0018 Ab	0.0490±0.0141 Aa
Ethyl-3-methylbutanoate	0.5055±0.0389 Ba	0.2388±0.0154 Ac	0.3553±0.0064 Bb	0.2543±0.0261 Abc
Dimethyl disulfide (DMDS) $	0.0053±0.0002 a	0.0055±0.0005 Aa	0.0036±0.0002 Aa	0.005±0.0007 Aa
Isoamyl alcohol	22.8363±0.3652 Aa	20.1843±0.9419 Aab	12.8422±0.3569 Ac	17.5052±0.8933 Ab
1-Octen-3-one	ND	ND	ND	ND
Dimethyl trisulfide (DMTS) $	0.0047±0.0008 a	0.0039±0.0009 Aa	0.0030±0.0002 Aa	0.0022±0.0004 Aa
1-Octen-3-ol	2.0340±0.4431 a	0.9901±0.2799 Aa	0.2227±0.0047 Bb	0.9426±0.0332 Aa
Methional	1.1130±0.0591 a	1.204±0.1143 Ba	0.5286±0.0495 Ab	1.2526±0.0401 Aa
***p*-Cresol**	0.0548±0.0036 Aa	0.0202±0.0061 Ab	ND	0.0156±0.0027 Ab
Day 90				
Methanethiol $	0.0032±0.0002 Bb	0.0099±0.0016 Aa	0.0032±0.0001 Ab	0.0057±0.0023 Aab
Dimethyl sulfide (DMS) $	0.0023±0.0002 Bb	0.0556±0.0062 Aa	0.0176±0.0011 Ab	0.0598±0.0066 Aa
Ethyl-3-methylbutanoate	1.9307±0.0843 Aa	0.2220±0.0271 Ac	0.6328±0.0365 Ab	0.2071±0.0066 Ac

Dimethyl disulfide (DMDS) $	ND	0.0053±0.0009 Aa	0.0020±0.0010 Aa	0.0051±0.0003 Aa
Isoamyl alcohol	16.8561±0.5728 Ba	16.0553±2.3527 Aa	9.9866±0.0637 Bb	12.7235±0.3027 Aab
1-Octen-3-one	ND	ND	ND	ND
Dimethyl trisulfide (DMTS) $	ND	0.0005±0.0002 Ba	0.0017±0.0005 Aa	0.0012±0.00001 Aa
1-Octen-3-ol	ND	0.8826±0.1060 Aa	0.2823±0.0051 Ab	1.0241±0.1393 Aa
Methional	ND	2.0175±0.2869 Aa	0.5832±0.0224 Ab	1.1919±0.1378 Ab
***p*-Cresol**	0.0378±0.0022 Aa	0.0318±0.0041 Aab	ND	0.0132±0.0069 Ab

Data reported as the mean of triplicate measurements ± standard error.

ND: not detected

Values were expressed as relative percentages (%) with the total peak area of all analytes of interest being 100%

Relative percentage (%) of each identified volatile: peak area of an individual component / total peak area of all components X 100%.

Footnote $: The specific mass fragment (m/z) designated for methanethiol, DMS, DMDS, DMTS was 47, 62, 94, and 126, respectively.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$).

Means within the same column (compare day 0 and 90) with different uppercase letters differ significantly ($p < 0.05$).

5.3.3 Experiment 2 - Comparison of encapsulation and freeze-drying

Due to the reason as mentioned in Section 5.2.4, a comparison was made between the M3-derived product and the freeze-dried truffles regarding their volatile profile (Table 5.4) and microbial quality (Table 5.6) in Experiment 2.

5.3.3.1 Aroma volatiles on day 0

Compared to the untreated truffles, the freeze-dried truffles seemed to retain more volatiles than the M3-derived product. Whilst all key volatiles were detected in the freeze-dried truffles, ethyl-3-methylbutanoate was the only compound that was not seen in the M3-derived product. There were no significant differences in the relative percentages of methanethiol, DMS, and DMTS in the freeze-dried truffles and the M3-derived product. Three volatiles (isoamyl alcohol, 1-octen-3-ol, and *p*-cresol) in the freeze-dried truffles were statistically significant higher than that of the M3-derived product, except DMDS and methional which were in a lower relative percentage in the freeze-dried truffles than that of the M3-derived product (Table 5.4). The overall result suggests that the freeze-dried truffles exhibited an overall better volatile profile than the M3-derived product on day 0.

5.3.3.2 Comparison of the volatile stability at the end of storage

Similar to Experiment 1, the volatile profile of the untreated truffles significantly changed after storage. It was observed that methanethiol had significantly reduced, while the other five volatiles (DMDM, DMTS, 1-octen-3-ol, methional, and *p*-cresol) were not detected on day 90. Ethyl-3-methylbutanoate was the only volatile compound that showed a significant increase in the untreated truffles after 90 days of storage (Table 5.4). A previous study by Savini et al. (2020) reported an increase of ethyl-3-methylbutanoate throughout 35 days of refrigerator storage under different packaging conditions and this phenomenon was related to the decay of fresh black truffle. The decrease of 1-octen-3-ol after 90 days (Table 5.4) is most likely due to truffle degradation was also supported by Savini et al. (2020). Another study investigating the volatile changes in *T. magnatum* under accelerated storage at 23°C reported that methional did not significantly reduce with storage time (Niimi et al., 2021) which is contradictory to our observation (Table 5.4). The contradictory results suggests that not all volatiles show similar changing pattern across similar studies and the unclear trend in the volatile changes could be due to the natural variation among truffles and the complexity and changes of the truffle-bacteria interactions under different storage conditions (Niimi et al., 2021; Savini et al.,

2020). The Niimi et al. (2021) study was based on white truffles from Marche Region (central Italy), Hungary, and Croatia and the Savini et al. (2020) study was based on black truffles from Abruzzo region (Southern Italy), whereas we used black truffles harvested from Western Australia in the present study, it can be expected that besides the natural truffle to truffle variability, different growing regions will host different bacteria that can lead to differences in interactions with the truffle leading to differences in the volatile profile changes as reported by previous studies (Splivallo et al., 2015; Strojnik et al., 2020; Vahdatzadeh et al., 2015). Nonetheless, in the current study by day 90 the changes in the overall volatile profile demonstrate the occurrence of spoilage in the fresh truffles (Table 5.4).

On the other hand, most of the volatiles in the freeze-dried truffles did not change except ethyl-3-methylbutanoate, isoamyl alcohol, and *p*-cresol showed a significant reduction after 90 days of storage. As for the M3-derived product, all the detected key volatiles remained unchanged at day 90. As the freeze-dried truffles contained an initial higher percentage of isoamyl alcohol (17.4310 ± 0.6409%) than that of the M3-derived product (8.2433 ± 0.1466%) on day 0, it is interesting to note that despite a decline by about 20% in the relative percentage of isoamyl alcohol (13.2997 ± 0.4996%) in the freeze-dried truffles, this compound was still significantly higher than that of the M3-derived product (5.9454 ± 0.4578%) on day 90 (Table 5.4). Given that the volatile changes in the untreated truffles, freeze-drying, and encapsulated products are complex, more studies are required to understand the interaction between the volatile molecules and the freeze-dried truffles and encapsulated complexes.

The overall finding indicates that though freeze-drying could adequately retain and stabilise truffle aroma, the encapsulation of volatiles in β-CD could help to extend the storage period of truffle aroma further. Apart from freeze-drying, encapsulation could be another option for stabilising the truffle aroma from deterioration during storage, which would enable truffle aroma to be made available for food applications during the off-season. Given that the encapsulation of truffle aroma is promising as demonstrated in the present study, further studies to evaluate the shelf life of encapsulated products using an Accelerated Storage Stability Test are required. This method would provide kinetic data on volatile stability of the encapsulated products (Mishkin et al., 1984; Mizrahi & Karel, 1977).

Table 5.4: Relative percentages of the key volatiles in the untreated truffles, freeze-dried truffles, and truffle-β-CD complexes derived from M3 on day 0 and 90.

Volatiles	Relative percentage (%)		
	Untreated truffles	Freeze-dried truffles	M3-derived product
Day 0			
Methanethiol $^{\$}$	0.0031±0.0001 ^{Aa}	0.0038±0.0013 ^{Aa}	0.0051±0.0004 ^{Aa}
Dimethyl sulfide (DMS) $^{\$}$	0.5069±0.1335 ^{Aa}	0.1006±0.0179 ^{Ab}	0.1536±0.0227 ^{Ab}
Ethyl-3-methylbutanoate	0.1510±0.0120 ^{Ba}	0.0790±0.0046 ^{b}	ND
Dimethyl disulfide (DMDS) $^{\$}$	0.0028±0.0001 ^{b}	0.0040±0.0003 ^{Ab}	0.0062±0.0006 ^{Aa}
Isoamyl alcohol	24.4230±1.7314 ^{Aa}	17.4310±0.6409 ^{Ab}	8.2433±0.1466 ^{Ac}
1-Octen-3-one	ND	ND	ND
Dimethyl trisulfide (DMTS) $^{\$}$	0.0017±0.0004 ^{b}	0.0053±0.0007 ^{Aab}	0.0064±0.0013 ^{Aa}
1-Octen-3-ol $^{\$}$	2.3682±0.2382 ^{a}	1.9250±0.3559 ^{Aa}	0.7014±0.0587 ^{Ab}
Methional $^{\$}$	2.4096±0.2295 ^{a}	0.2272±0.0163 ^{Ac}	0.9697±0.0130 ^{Ab}
***p*-Cresol**	0.1317±0.0153 ^{a}	0.0897±0.0055 ^{ab}	0.0253±0.0018 ^{Ac}
Day 90			
Methanethiol $^{\$}$	0.0006±0.0001 ^{Ba}	0.0038±0.0006 ^{Aa}	0.0041±0.0013 ^{Aa}
Dimethyl sulfide (DMS) $^{\$}$	0.0126±0.0008 ^{Ac}	0.0891±0.0034 ^{Ab}	0.1178±0.0040 ^{Aa}
Ethyl-3-methylbutanoate	0.7265±0.0434 ^{A}	ND	ND

Dimethyl disulfide (DMDS) $	ND	0.0056±0.0004 Aa	0.0065±0.0003 Aa
Isoamyl alcohol	23.6225±1.1404 Aa	13.2997±0.4996 Bb	5.9454±0.4578 Ac
1-Octen-3-one	ND	ND	ND
Dimethyl trisulfide (DMTS) $	ND	0.0104±0.0013 Aa	0.0078±0.0005 Aa
1-Octen-3-ol $	ND	1.7058±0.2088 Aa	0.7146±0.1301 Ab
Methional $	ND	0.2119±0.0416 Ab	0.7200±0.1051 Aa
***p*-Cresol**	ND	ND	0.0284±0.0065 A

Data reported as the mean of triplicate measurements ± standard error.

ND: not detected

Values were expressed as relative percentages (%) with the total peak area of all analytes of interest being 100%

Relative percentage (%) of each identified volatile: peak area of an individual component / total peak area of all components X 100%.

Footnote $: The specific mass fragment (m/z) designated for methanethiol, DMS, DMDS, DMTS, methional, and 1-octen-3-ol was 47, 62, 94, 126, 48, and 57, respectively.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$).

Means within the same column (compare day 0 and 90) with different uppercase letters differ significantly ($p < 0.05$).

5.3.4 Microbial analysis in Experiment 1 and 2

The microbial results of all samples were summarised in Table 5.5 (Experiment 1) and Table 5.6 (Experiment 2). According to the manufacturer's specification (Wacker Chemie AG), a low level of microorganisms (max. 3 log CFU/g) would be present in the β-CD. Hence, this is within our expectation when one or two bacterial colonies (< 3.48 log CFU/g) were detected in the controls in Experiment 1 (C1 and C3 on day 0; C3 on day 90) and Experiment 2 (C3 on day 0 and 90) (Table 5.5 and 5.6).

In Experiment 1, about 7.99 ± 0.02 log CFU/g of total aerobic microbial counts and 6.06 ± 0.06 log CFU/g of *Pseudomonas* spp. were detected in the untreated truffles. In contrast, very few total aerobic microbial counts (< 3.48 log CFU/g) and no *Pseudomonas* spp. (< 2.00 log CFU/g) were detected in the M2-derived product (Table 5.5). This indicates that the addition of ethanol in M2 could act as a disinfectant in the process. Although there was no pre-treatment to remove microbes prior to encapsulation and freeze-drying, some reductions in the microbial counts were observed in the dried products compared to the untreated truffles, suggesting that some microbes could not survive the processing conditions. The total microbial count in the products derived from M1 and M3 were significantly lower than the untreated truffles, with 6.67 ± 0.22 log CFU/g and 6.44 ± 0.09 log CFU/g, respectively; the *Pseudomonas* counts in the products derived from M1 and M3 were also significantly lower than the untreated truffles, showing 3.73 ± 0.15 log CFU/g and 4.03 ± 0.13 log CFU/g, respectively on day 0 (Table 5.5). A similar observation was noted in Experiment 2 on day 0 in which the total microbial counts in the M3-derived product (5.56 ± 0.20 log CFU/g) was significantly lower than the untreated truffles (7.31 ± 0.24 log CFU/g); the *Pseudomonas* counts in the freeze-dried truffles (3.55 ± 0.05 log CFU/g) and the M3-derived product (< 3.48 log CFU/g) were significantly reduced compared to the untreated truffles (5.47 ± 0.11 log CFU/g) (Table 5.6).

The decreasing trend observed in the microbial counts by day 90 implies that dried vacuum-packaged truffle products stored at cold temperature may create an environment not hospitable for some microorganisms' survival and growth. For example, the initial total microbial counts in the freeze-dried truffles (6.42 ± 0.23 log CFU/g) and the M3-derived product (5.56 ± 0.20 log CFU/g) were significantly reduced to 5.70 ± 0.14 log CFU/g and 5.04 ± 0.15 log CFU/g, respectively after day 90 (Table 5.6). The *Pseudomonas* counts in the products derived from M1 and M3 decreased from about 3-4

log CFU/g to < 3.48 log CFU/g, respectively after 90 days of storage (Table 5.5). Similarly, the *Pseudomonas* counts in the freeze-dried truffles and the M3-derived product (from day 0 to 90) significantly decreased from 3.55 ± 0.05 log CFU/g to < 3.48 log CFU/g and from < 3.48 log CFU/g to < 2.00 log CFU/g, respectively (Table 5.6).

Salmonella spp. and *Listeria* spp. were below the detection limit (< 2.00 log CFU/g) in all samples in Experiment 1 and 2 (Table 5.5 and 5.6). Likewise, *Salmonella* spp. was not detected in *T. melanosporum* and *T. aestivum* (harvested in Spain) by Rivera et al. (2010a) as well as *T. aestivum* (harvested in Italy) by Reale et al. (2009). Reale et al. (2009) did not find *Listeria monocytogenes* in *T. aestivum* which were collected in Italy. Research conducted by Rivera et al. (2010a) claimed that *L. monocytogenes* was only detected in *T. aestivum* with low occurrence but undetected in *T. melanosporum* (both truffle species were harvested in Spain). Apart from its generally low population in the soil (Vivant et al., 2013), the low presence of *L. monocytogenes* could be due to the competitive inhibitory effect exerted by the dominance of *Pseudomonas* species that possess the ability to produce siderophores to capture essential nutrients (Harris et al., 1989; Lamikanra, 2002; Rivera et al., 2010a). Due to their ubiquity and abundance in soil, *Bacillus* spp. have frequently been found in truffles of different origins and species (Chen et al., 2019; Perlińska-Lenart et al., 2020; Saidi et al., 2015). In Experiment 1 (Table 5.5), *Bacillus* spp. were detected in some products derived from M1 and M2 (day 0 and 90) but were below the detection limit (< 2.00 log CFU/g) in the untreated truffles and the M3-derived product (day 0 and 90). It could be that *Bacillus* spp. was present in such a low number, thus it was too low to be detected in these samples. The ability to form spores enable *Bacillus* spp. to survive extreme conditions explains the detection of *Bacillus* spp. in the encapsulated complexes and the freeze-dried truffles after 90 days of storage (Table 5.5 and 5.6). As alcohols are generally ineffective against inactivating spores (Leggett et al., 2012; Nerandzic et al., 2015; Thomas, 2012), the addition of ethanol in M2 may not be able to inactivate *Bacillus* spores (Table 5.5).

Given that the microbial load on fresh truffles is generally diverse and high, incorporating a decontamination step prior to encapsulation or freeze-drying is deemed essential. More studies are required to explore the possibility of including an additional decontamination step to reduce the microbial population in the fresh truffles before processing the fresh truffles into dried products via encapsulation or freeze-drying.

Table 5.5: Microbial loads (log CFU/g) in the untreated truffles, β-CD only negative control (C1, C2, and C3), and truffle-β-CD complexes derived from M1, M2, and M3 on day 0 and 90.

Microbial counts (log CFU/g)	Samples						
	Untreated truffles	C1	M1-derived product	C2	M2-derived product	C3	M3-derived product
				Day 0			
Total plate count	7.99±0.02 ^{Aa}	<3.48	6.67±0.22 ^{Ab}	<2.00	<3.48	<3.48	6.44±0.09 ^{Ab}
***Pseudomonas* spp.**	6.06±0.06 ^{Aa}	<2.00	3.73±0.15 ^{b}	<2.00	<2.00	<2.00	4.03±0.13 ^{b}
***Listeria* spp.**	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
***Salmonella* spp.**	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
***Bacillus* spp.**	<2.00	<2.00	<3.48	<2.00	<3.48	<2.00	<2.00
				Day 90			
Total plate count	6.85±0.04 ^{Ba}	<2.00	5.58±0.29 ^{Ab}	<2.00	<3.48	<3.48	6.00±0.12 ^{Ab}
***Pseudomonas* spp.**	5.46±0.11 ^{B}	<2.00	<3.48	<2.00	<2.00	<2.00	<3.48
***Listeria* spp.**	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
***Salmonella* spp.**	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
***Bacillus* spp.**	<2.00	<2.00	<3.48	<2.00	<3.48	<2.00	<2.00

Data reported as the mean of triplicate measurements ± standard error.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$).

Means within the same column (compare day 0 and 90) with different uppercase letters differ significantly ($p < 0.05$).

Table 5.6: Microbial loads (log CFU/g) in the untreated truffles, freeze-dried truffles, β-CD only negative control (C3), and truffle-β-CD complexes (M3) on day 0 and 90.

Microbial counts (log CFU/g)	Samples			
	Untreated truffles	Freeze-dried truffles	C3	M3-derived product
Day 0				
Total plate count	7.31±0.24 ^{Aa}	6.42±0.23 ^{Aab}	<3.48	5.56±0.20 ^{Ab}
***Pseudomonas* spp.**	5.47±0.11 ^{Aa}	3.55±0.05 ^{b}	<2.00	<3.48
***Listeria* spp.**	<2.00	<2.00	<2.00	<2.00
***Salmonella* spp.**	<2.00	<2.00	<2.00	<2.00
***Bacillus* spp.**	<3.48	<3.48	<2.00	<3.48
Day 90				
Total plate count	6.46±0.20 ^{Aa}	5.70±0.14 ^{Bb}	<3.48	5.04±0.15 ^{Bb}
***Pseudomonas* spp.**	3.91±0.29 ^{B}	<3.48	<2.00	<2.00
***Listeria* spp.**	<2.00	<2.00	<2.00	<2.00
***Salmonella* spp.**	<2.00	<2.00	<2.00	<2.00
***Bacillus* spp.**	<3.48	<3.48	<2.00	<3.48

Data reported as the mean of triplicate measurements ± standard error.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$).

Means within the same column (compare day 0 and 90) with different uppercase letters differ significantly ($p < 0.05$).

5.4 Conclusion

Two experiments were conducted in this study. In Experiment 1, three different encapsulation methods (M1, M2, and M3) were compared. M2 demonstrated the greatest microbial reduction compared to M1 and M3, but it was the least optimal in retaining the key volatiles of truffles that contribute to its aroma profile. All key volatiles were detected in the products derived from M1 and M3 on day 0. However, after 90 days, some volatile changes were observed in the M1-derived product but not in the M3-derived product, suggesting M3 as the preferred method to retain and stabilise truffle aroma. In Experiment 2, M3 was compared with freeze-drying. The freeze-dried truffles retained an overall higher relative percentage of the key volatiles than that of the M3-derived product on day 0, but after 90 days, the M3-derived product retained more of the volatile compounds. This suggests that while freeze-drying could adequately preserve truffle aroma, encapsulating the volatiles in β-CD could potentially be used to further stabilise truffle aroma for an extended period of time making it available as an ingredient for food product applications during the truffle off-season. The presence of microbes in the encapsulated complexes and the freeze-dried truffles on day 0 and 90 suggests the need to incorporate a pre-processing step to reduce microbial loads, which needs to be determined in the future. The knowledge obtained from this study opens up an exciting venture for applying encapsulation of truffle aroma as a method of preservation. As far as we are aware, this is the first study to report on the possibility of encapsulating truffle aroma volatile. Before the process can be commercialised to preserve truffle aroma, some other encapsulation parameters need to be determined by future research in order to confirm the possibility of encapsulating volatiles into the β-CD cavity. These include determining the physicochemical characteristics of the encapsulated products in terms of the encapsulation efficiency, encapsulation yield, the application of an Accelerated Storage Stability Test to obtain kinetic data on volatile stability, and characterising the formation of an inclusion complex between the volatile molecules and β-CD by NMR/FTIR/DSC techniques.

CHAPTER 6: Impacts of different processing techniques on the key volatile profile, sensory, and consumer acceptance of black truffle (*Tuber melanosporum* Vittadini)

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Abstract

Fresh truffles which include black truffle (*Tuber melanosporum*) deteriorate and lose aroma rapidly after harvest, therefore postharvest processing via freeze-drying or encapsulation is an option to preserve truffle aroma for extended supply. However, the aroma profile that directly affects the truffle quality and consumer acceptance is influenced by processing and producers require processing options that balance processing feasibility with retention of a suitable aroma profile. This study aimed to determine the impacts of freeze-drying and encapsulation on the profile of key volatiles, consumer discrimination, and overall sensory impression (aroma intensity, truffle aroma rating, liking, and acceptability) of processed truffle products compared to the starting material (positive control). The study combined experimental-scale processing with GC-MS analysis and consumer sensory evaluation to compare and optimise postharvest processing options. Based on the results, some volatile changes were detected in the processed truffle products compared to the positive control which were aligned with the consumer discrimination (triangle test) and the aroma intensity score (sensory ranking test). Despite some chemical and sensory differences detected, the consumer panel did not have any preference for processed truffle products compared to the positive control. The overall finding indicates the potential value of processing truffles into a natural flavouring ingredient for food application via freeze-drying or encapsulation which should be of great interest for the truffle and food industry. According to the correlation analysis, the consumer acceptance of a truffle product may be increased by retaining 1-octen-3-ol and methional, while reducing the amount of *p*-cresol in the product. This study also highlights the importance of conducting a sensory evaluation to complement an analytical instrument.

Keywords: Black truffle, encapsulation, freeze-drying, consumer sensory evaluation, volatile compounds

6.1 Introduction

Black Périgord truffle (*Tuber melanosporum* Vittadini 1831) is the most sought-after edible fungus due to its captivating aroma, rarity, and short season of supply (Amicucci et al., 2002; Reale et al., 2009a; Sorrentino et al., 2013). Being one of the most luxurious and rarest delicacies worldwide (Romanazzi et al., 2015), black truffle is generally used as a signature ingredient to enrich food flavour (Pacioni et al., 2014a). Nonetheless, factors such as short harvest season and high perishability have restricted its opportunity for a broader market (Campo et al., 2017) and therein lies an opportunity for value addition to truffles that do not meet the grade for fresh supply.

Given that fresh truffles deteriorate and lose aroma rapidly after harvest (Campo et al., 2017), postharvest processing of fresh truffles is necessary to maintain its sensory characteristics for extended supply all year round (Rivera et al., 2011a). Several preservation techniques like refrigeration, freeze-drying, freezing, hot air drying, canning, and irradiation have been studied in the past (Campo et al., 2017; Culleré et al., 2012; Rivera et al., 2010b) but each method has its own limitations. Refrigeration is the most commonly used preservation technique (Sorrentino et al., 2018) but it is too short to cover the off-season as fresh truffles could only be last for a maximum of 2 weeks in vacuum packaging (Savini et al., 2017); whereas there is a legal ban of using irradiation to treat truffles in the United States, European countries, Australia, and New Zealand (EFSA, 2018; FDA, 2010; FSA, 2017; FSANZ, 2018) and this technique could not prolong the truffle shelf life beyond 1 month. Freeze-drying, freezing, hot air drying, and canning are considered long-term preservation techniques. Among them, freeze-drying has been described as the preferred technique (Campo et al., 2017; Marco et al., 2016). Thermal treatment during hot air drying and canning severely affect the aroma quality of truffles (Al-Ruqaie, 2006; Campo et al., 2017; Murcia et al., 2003). Although some changes to the volatile profile and reduction in the aroma intensity of truffles were observed after freeze-drying (Campo et al., 2017; Palacios et al., 2014), the overall original aroma of the freeze-dried truffles was the least affected compared to truffles that were treated with canning, freezing, and hot air drying (Campo et al., 2017; Marco et al., 2016). However, there is a lack of information about the consumers' liking and acceptability of the freeze-

dried truffles as compared to the fresh truffles and therefore a consumer sensory evaluation needs to be conducted to fill the gap.

Turning the highly perishable truffles into a flavouring ingredient is acceptable as truffles are usually used as a flavour enhancer rather than as a food product (Pacioni et al., 2014a). An alternative and/or complementary method of processing is encapsulation. Encapsulation is a process in which sensitive compounds such as volatile aroma are surrounded or entrapped by protective wall materials (Shrestha et al., 2017; Wang et al., 2015) to create products that retain high aroma potential with a broad array of volatile compounds that may otherwise be lost during processing. Through encapsulation, undesirable volatile changes caused by degradation or oxidation during processing and storage can be mitigated, leading to an extended shelf life (Wang et al., 2015). Beta-cyclodextrin (β-CD) is one of the most commonly used encapsulating agents (Reineccius et al., 2002; Wang et al., 2015) and has shown its ability in protecting the loss of various types of volatiles from different sources such as strawberry (Balci-Torun & Ozdemir, 2021), oregano (*Origanum onites* L.) essential oil (Kotronia et al., 2017), and sweet orange (Zhu et al., 2014). The volatile profile of truffles may possibly be modified during processing due to the nature and stability of some key odourants such as dimethyl sulfide (DMS), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and methional are known to be highly volatile and reactive (Culleré et al., 2013a). A previous study showed that DMS and DMDS could be protected and stabilised by β-CD (Reineccius et al., 2002) therefore it is hypothesised that the above key volatiles that are present in truffles are likely to be protected by β-CD as well. Processing fresh truffles into a dried flavouring ingredient via encapsulation is promising but as far as we are aware, preserving truffle aroma using this approach has not been reported yet. In addition to a shelf life extension, encapsulation of truffle aroma may allow easy handling, simple dosing, storage cost reduction, ease of transportation, and convenience for consumers, which would ultimately improve the purchase interest among consumers (Marques, 2010).

There are a few methods to analyse the impact of processing on truffle aroma. Gas chromatography-mass spectrometry (GC–MS) is extensively used in the identification and quantification of volatile components in truffles (Culleré et al., 2010). Even though GC-MS analysis can be pretty accurate as it provides an objective measure of chemical compounds, there are some limitations of using instrumental analysis (Chambers & Koppel, 2013). As a prior sample extraction is required before analysis (Andrewes et al., 2021) and thermally labile compounds can be changed during the heating step of the GC

(Chambers & Koppel, 2013), this would add further complexity to the instrumental analysis (Andrewes et al., 2021). To overcome the limitations of GC-MS analysis and to obtain a complete aroma profile of the truffle products, a sensory evaluation needs to be conducted to complement the GC-MS analysis. Sensory evaluation has traditionally been divided into two clearly defined areas: sensory analytical tests (with a trained panel test) and consumer sensory analysis (with a large number of untrained consumers). Analytical tests could overcome the limits of instrumental methods as this approach uses panellists that are trained to measure the sensory characteristics of products. Whereas consumer tests are used to evaluate the perception and acceptance of products (García-Gómez et al., 2022). There are also a few shortcomings of using sensory analysis such as laborious, costly, time-consuming, and inconsistent evaluation standards (Chambers & Koppel, 2013; Fan et al., 2016). In recent years, electronic senses such as e-noses and e-tongues have been developed to address challenges that exist in using traditional sensory methods that require panels of human assessors (Schlossareck & Ross, 2019). However, as human olfaction is the ultimate discriminator of aroma quality, none of these electronic senses are thought to adequately replace human sensory panels to measure human perception such as consumer preference and acceptability (Andrewes et al., 2021; Fan et al., 2016). As opposed to instrumental analysis, no prior sample extraction is required before a sensory evaluation (Andrewes et al., 2021). In view that a market success is ultimately determined by consumers, it is necessary to cross-reference the instrumental data with the human sensory response to determine the aroma quality and consumer perception of a food product (Lawless, 1991), which is important to get a better description and real-world feedback of a new product before its launch in the market.

To the best of our knowledge, no previous studies have been reported which preserve truffle aroma via encapsulation and compare it (both in terms of instrumented analysis of aroma compounds and sensory evaluation) with freeze-drying. Changes in the volatile profile can have a direct impact on the aroma quality of truffles which is the main criteria determining the value as a flavouring ingredient and thereby consumer liking and acceptance. For this reason, the present study aimed to determine which processing technique, either freeze-drying or encapsulation could better retain the volatile profile of truffles. A consumer sensory evaluation was also performed to determine the consumer discrimination among, and overall impression of, the truffle products.

6.2 Materials and methods

6.2.1 Raw material: fresh truffles

Mature truffles were harvested in the Manjimup region of Western Australia in July. All freshly collected truffles were brushed and rinsed with tap water to remove any soil followed by air-drying in a laminar air flow cabinet as per common industrial practice. The cleaned truffles were packed in an insulated container held at 4 ± 2°C (Rivera et al., 2010a) and immediately couriered to our laboratory within 24 h of harvest.

6.2.2 Starting material preparation: frozen truffles

Following Culleré et al. (2012), a qualitative selection of truffles was made by carefully inspecting their visual appearance upon sample arrival. Those free of defects and with a similar appearance were selected, whilst those that showed spoilage with soft texture, insect infestation or damage were rejected. About 1 kg of the selected truffles were crushed and mixed well to reduce possible natural variation among each truffle that was linked to the growing condition and natural habitat. The homogenised mix of truffles was then vacuum-packaged (65 µm Polyamide/Polyethylene, FPA Australia Pty Ltd, Malaga, WA, Australia) using a vacuum packaging machine (easyPACK-mk2, Webomatic, Hansastrasse, Bochum, Germany) and stored at -80 ± 2°C until required. About 200 g of frozen truffles (starting material) were used as the positive control, whereas the rest were used to prepare freeze-dried truffles and truffle-β-CD complexes. As completing the entire sensory evaluation required a week (Section 6.2.6), frozen truffles were used as a starting material throughout the study. This was to overcome the limitations of using fresh truffles as a raw material that are known to be highly perishable and rapidly losing aroma, thereby keeping the control constant.

6.2.3 Preparation of freeze-dried truffles

About 250 g of frozen truffles were freeze-dried at 0.37 mbar, -30°C using a freeze drier (Christ Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) until the weight was constant (48 h). The freeze-dried truffles were vacuum-packed and stored at 4 ± 2°C until required for analyses. Each experiment was performed in triplicate.

6.2.4 Preparation of truffle-β-CD complexes

Encapsulation of truffle aroma in β-CD using the paste method similar to the procedures described by Shrestha et al. (2017), with some modification, were prepared at two different ratios and their formulation strategies are shown in Table 6.1. For truffle-β-CD complexes 1:1, 200 mL of deionised water was mixed with 200 g of food-grade β-CD (CAVAMAX® W7 Food) (Wacker Chemie AG, Victoria, Australia) to form a paste before adding 200 g of frozen truffles. For truffle-β-CD complexes 1:2, 400 mL of deionised water was mixed with 400 g of food-grade β-CD (CAVAMAX® W7 Food) (Wacker Chemie AG) to form a paste before adding 200 g of frozen truffles. Each mixture was homogenised using a stick blender (Kambrook KSB7, Breville Group Ltd, NSW, Australia) for 2 min at the minimum speed of the blender (setting 1). The complexed paste was freeze-dried (Christ Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH) at -30°C, 0.37 mbar until constant weight. Next, the dried complexes were powdered using a mortar and pestle. A set of negative control corresponding to each method was prepared in the same manner, except no truffles were added into the process. Each control set and encapsulated products were separately vacuum-packed and stored at 4 ± 2°C until required for volatile and sensory analyses. Each experiment was performed in triplicate.

Table 6.1: The formulation strategies of truffle-β-CD complexes 1:1 and truffle-β-CD complexes 1:2

Truffle-CD ratio	Truffles (g)	β-CD (g)	Deionised water (mL)	Freeze-drying
1:1	200	200	200	48h at -30C
1:2	200	400	400	48h at -30C

6.2.5 Volatile analysis

Headspace solid-phase microextraction (HS-SPME) with a SPME holder (GERSTEL MultiPurpose Sampler SPME injection for automated sampling) combined with gas chromatography-mass spectrometry (GC-MS) was used to perform volatile analysis, as per Díaz et al. (2009), with some modification.

6.2.5.1 Headspace sampling by solid-phase microextraction (SPME)

A 1 cm length of flexible fused silica fibre coated with a 50/30 µm layer of divinylbenzene/ Carboxen/ Polydimethylsiloxane (DVB/CAR/PDMS) (57298-U, Supelco, Bellefonte, Pennsylvania, USA) was used to sample the volatiles from the headspace above samples (Duncan et al., 2017). The SPME fibre was conditioned before use according to the manufacturer's instructions (Supelco). For the positive control (starting material), about 1 g of frozen truffles (containing about 25% dry matter) was weighed into a 20 mL headspace glass vial. Dry matter content was 25% based on a moisture content of 75%, which is similar to previous literature (Palacios et al., 2014). Dried samples such as β-CD as the negative control, the truffle-β-CD complexes, and the freeze-dried truffles were weighed into glass vials and rehydrated to similar moisture content as the frozen truffles (positive control) (Campo et al., 2017; Palacios et al., 2014). To enable comparison between samples, the truffle content (containing about 0.25 g dry matter) was set as a constant variable in which every truffle samples were weighed accordingly so as to contain a similar amount of truffles. A 10 µL of 10 ppm 1,2-dichlorobenzene (Sigma Aldrich, Sydney, NSW, Australia), which was used as the internal standard (Liu et al., 2017b), was added to each vial before the vial was sealed with a magnetic screw cap (Thermo Fisher Scientific, Scoresby, Victoria, Australia). The use of an internal standard added to the matrix to be analysed before extraction can compensate for matrix effects and losses of analytes during extraction (Pati et al., 2021). However, the use of internal standard has some drawbacks related to the response of a wide range of volatiles during the chromatographic analysis (Ruiz-Hernández et al., 2018). As the chemical structure of all the 75 volatile compounds analysed in the present study were highly diverse, the internal standard cannot be expected to behave the same as all of the analytes. The conversion of the analyte/internal standard response ratio into the analyte concentration can thus be misleading (Pati et al., 2021). According to similar previous studies on truffle volatile analysis, several authors have expressed their results as a chromatographic peak area percentage for each volatile (Choo et al., 2021; Díaz et al., 2009; Díaz et al., 2003; Díaz et al., 2002). For these reasons, the internal standard was not used as a quantification tool but used to validate the performance of GC-MS instrument in the present study. The rehydrated samples were incubated for 10 min at room temperature (20 °C ± 2 °C) to favour water absorption prior to headspace sampling (Campo et al., 2017; Palacios et al., 2014). During the headspace sampling, all vials were equilibrated for 5 min at 53°C. Subsequently, SPME fibre was introduced into the vials for 13.6 min to absorb volatiles, then injected and desorbed for 2 min in a 200°C injection

port using a splitless injection method (Díaz et al., 2009). The settings were based on a previous work in which a complete optimisation of the extraction conditions was carried out (Díaz et al., 2002). To avoid carryover during chromatography analysis, SPME fibre was conditioned after each run. The fibre stayed in the autosampler for 15 minutes at the injection temperature under high split to remove all adsorbed materials. Blank runs using empty vials were performed occasionally between samples to check for carryover (Splivallo et al., 2012).

6.2.5.2 GC-MS analysis

Volatile compounds of all samples were analysed as per Díaz et al. (2003), with some modification. The compounds were separated using an Agilent-6890 GC system coupled to a GC 5973 mass spectrometer (Agilent Technologies Inc., Santa Clara, California, USA) equipped with a 30 m (length) x 250 µm (diameter) x 0.25 µm (film thickness) i.d. HP-INNOWax GC column (Agilent Technologies Inc.) fused silica capillary column (Model Number: Agilent 19091N-133). The oven temperature program began at 35°C (held for 5 min) and was ramped up to 240°C at a rate of 5°C min^{-1} (held for 3 min). Helium was used as a carrier gas at 6.74 psi with 1 mL/min constant flow rate. The mass spectrometer was run in the electron ionisation (EI) mode. Data acquisition was performed in a scanning mode in the range of 40-250 m/z at 0.46 sec/scan (3 µ-scans) after a 1.5 min of solvent delay. A solvent delay was set where the detector was temporarily turned off for 1.5 min after injection in order to reduce damage to the source filaments from the solvent (methanol) (Wang et al., 2018).

Chromatographic data were processed using MSD ChemStation F.01.03.2357 (1989-2015 Agilent Technologies, Inc.). Identification of peaks was done by comparing their mass spectral fragmentation patterns with entries in the National Institute of Standards and Technology (NIST) mass spectral database (Version 2.3, 2017, USA) using spectral match quality ≥ 700 as the criterion (Watson & Sparkman, 2007), published literature, retention time, and the Kovats retention indices (RI) which were calculated based on n-alkane series under the same chromatographic condition (Wernig et al., 2018).

Each compound was determined based on peak area normalisation by integration. The relative amount of individual compounds were expressed in relative percentage (%) by calculating the proportion of each individual peak area to the sum of peak area of all the compounds of interest (Ruiz-Hernández et al., 2018). Certain compounds (methanethiol, DMS, DMDS, DMTS, methional, and 1-octen-3-ol) were difficult to integrate as they did

not produce any fully resolved peaks in the chromatogram due to their low concentration and/or co-elution with other peaks. Because of this, extraction of a single ion chromatogram was used to determine their relative percentages.

6.2.6 Sensory evaluation of truffle aroma

A consumer sensory evaluation on the four truffle products was performed using an untrained panel, based on the methodology recommended by Australian Standard sensory analysis for triangle test: AS2542.2.2:2014 (Australia Standard, 2014) and Lawless and Heymann (2010) for consumer sensory test. This study was approved by the Curtin University Human Research Ethics Committee (Approval number: HRE2019-0374). Following previous research which consisted of around 50 untrained panellists for consumer sensory study (Baião et al., 2021; Johnson et al., 2021), a total of randomly selected 71 participants who have had truffle consumption aged above 18 years old (31 males, 30 females, and 10 prefer not to say) were recruited in the study.

Prior to the commencement of the sensory evaluation, the participants were required to complete a pre-screening questionnaire. To be eligible to take part in this sensory evaluation, they must be over 18 years old without any known food allergies or intolerances, no frequent head colds, no nasal disease, have not been taking any medication that will affect senses, and were neither currently pregnant nor currently breastfeeding for the females. Informed consent from each participant was obtained before the sensory evaluation. There was no training provided other than basic instructions for completing the evaluation. The entire sensory evaluation was completed within a week with approximately an hour per session.

6.2.6.1 Sample preparation

To ensure consistency in food preparation, all samples were prepared on the same day of sensory evaluation. Similar to the sample preparation for volatile analysis, the truffle content (containing about 0.25 g dry matter) was set as a constant among all treatments. All dried truffle samples were rehydrated to similar moisture content as the frozen truffles (positive control) (Campo et al., 2017; Palacios et al., 2014). Each sample was served in an amber glass vial and brought to room temperature. Once prepared, the lid of each vial was immediately tightened to minimise evaporation of aroma. Each sample was labelled with three-digit random numbers and the samples were allowed to equilibrate for at least 10 min before they were presented to the participants.

Participants were seated in quiet and comfortable partitioned booths illuminated with yellow light to mask visual differences of the samples during the study. On a given day of evaluation, the serving order of samples was randomised and balanced across participants so there was no fatigue or bias created by the sequence of samples (Duncan et al., 2017). Participants were asked to sniff the samples and between samples, they were offered sliced lemon to calibrate their olfactory senses. The time between sample sets was approximately 1.5 to 2 min and the participants were instructed to rest for 1.5 to 2 min before sniffing the next sample after sniffing the piece of lemon, this was to ensure that the olfactory sense of the participants have been cleared (Grosofsky et al., 2011).

6.2.6.2 Triangle test

A triangle test was conducted to determine if participants could detect aroma differences between processes (Lawless & Heymann, 2010). A total of six pairings (AB, AC, AD, BC, BD, and CD) were required to assess the four truffle products (A = frozen truffles (positive control), B = freeze-dried truffles, C = truffle-CD ratio 1:2, D = truffle-CD ratio 1:1). For AB pairing, the six possible order combinations are: AAB, ABA, BAA, BBA, BAB, and ABB and the order combinations should be randomised across participants. Participants received six triangle sets (six permutations) in total. In each permutation set (three samples), two identical samples and one odd sample were presented to each participant and they were asked to identify the odd one out. If they could not determine which sample was the odd one, they were asked to record their best guess and may note under remarks that they were guessing.

6.2.6.3 Sensory ranking test

After the triangle test, a multiple-sample sensory ranking test was conducted to compare a complete set of multiple samples on the basis of ranks of preference or attribute intensity (Pimentel et al., 2016). The participants were asked to sniff the four samples derived from different processes and rank them in terms of the aroma intensity, truffle aroma rating, overall liking, and overall acceptability by giving a score to each sample using a general labelled magnitude scale corresponding to their degree of sensation (Kalva et al., 2014). A separate scale was used for each parameter. The length of each scale was 15 cm. The score (%) for each sample was enumerated by measuring the distance (cm) from the bottom of the scale to the mark indicated by the participants and divided by the total length of the scale X 100%. Sensory scores obtained were averaged for each attribute.

6.2.7 Data analysis

All data were reported as mean ± standard error and statistically analysed using IBM SPSS version 26 software. Data of the volatile profile of each processed truffle product were analysed using one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test to compare the means of more than two independent groups. Independent samples t-test was performed to compare the means of two different groups. For sensory evaluation, the triangle test was analysed by a nonparametric binomial test and a chi-square test to determine if a difference between observed data and expected data is due to chance (≤ 33%) or a relationship between the variables. Sensory ranking test (aroma intensity, truffle aroma rating, overall liking, and acceptability) were carried out using a one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test. Differences with $p < 0.05$ (95 % confidence level) were considered to be statistically significant. The Pearson correlation coefficients (r) between the key volatiles and the consumer sensory testing scores of four truffle products were determined to investigate the strength and direction of an association between these two data sets.

6.3 Results and discussion

6.3.1 Important volatile compounds in *T. melanosporum*

In this study, a total of 75 volatiles were chosen as the compounds of interest based on previous studies (Choo et al., 2021; Culleré et al., 2010; Culleré et al., 2013a; Díaz et al., 2003). However, not all volatiles are responsible for what humans perceive as aroma in truffles (Culleré et al., 2010; Culleré et al., 2013a; Feng et al., 2019; Mustafa et al., 2020; Schmidberger & Schieberle, 2017; Strojnik et al., 2020). For this reason, we were only interested in the ten volatile compounds which are known contributors to the perceived aroma of black truffles (Table 6.2) and they were used as the indicators (Culleré et al., 2010; Culleré et al., 2013a) to evaluate the impact of different processing techniques on the key volatile profile of truffles (Section 6.3.2).

6.3.2 Key volatile profile

Three different truffle products namely freeze-dried truffles and truffle-β-CD complexes (1:2 and 1:1) were compared in terms of key volatile profile with frozen truffles as a positive control (Table 6.2). All the key volatiles were detected in the positive control except 1-octen-3-one, most likely due to its low concentration, which fell below the detection limit or its absence can also be attributed to natural variation in the volatile

profile as a result of different environmental factors, maturity of truffles (Strojnik et al., 2020; Torregiani et al., 2017) and the pre-storage condition of the starting materials. Despite the fact that keeping starting materials at -80°C prior to the experiment could alter the profile of volatile compounds (Campo et al., 2017) and being a limitation of the present study, this approach was required to ensure the continuity of supply for the experiment.

Some changes were observed in the key volatile profiles of truffle products after being freeze-dried or encapsulated, although both methods were performed at low temperatures. However, of all the nine key volatiles detected in the truffles exposed to the freeze-drying treatment, there was no significant difference in the relative percentages in five of the nine key volatiles (methanethiol, DMDS, 1-octen-3-ol, methional, and *p*-cresol) with the positive control. The relative percentages of DMS, ethyl-3-methylbutanoate, and isoamyl alcohol were significantly lower in the freeze-dried truffles than in the positive control, whereas DMTS was significantly higher. Similarly, Palacios et al. (2014) reported some volatile changes in three truffle species (*T. melanosporum*, *T. aestivum*, and *T. indicum*) that had been freeze-dried but the impact seemed to be truffle species-dependent with *T. aestivum* suffered the most impact compared to the other two. Likewise, Campo et al. (2017) observed some differences in the freeze-dried truffles as compared to the fresh truffles based on an olfactometric approach such as the loss of ethyl esters and the concentration of aldehydes. The volatile changes in truffles were believed to be caused by enzymatic and oxidative reactions (Palacios et al., 2014) as some compounds like 1-octen-3-one can be reduced by enzymatic activity to 3-octanone (Darriet et al., 2002); whereas compounds with alcohol group such as isoamyl alcohol can be oxidised to their corresponding aldehydes, ketones, and acids (Raymond E March et al., 2006).

As for the processing treatments generating truffle-β-CD complexes (1:1 and 1:2), seven out of nine key volatiles were detected. The key volatile profiles of both truffle-β-CD complexes were quite similar with no significant difference observed in all the seven key volatiles between them (Table 6.2). This is within our expectation as both products (the truffle content was set as a constant variable as described in Section 6.2.5.1) underwent the same encapsulation processing conditions except that the amount of β-CD added was double in the truffle-β-CD complexes 1:2 but the addition of more β-CD showed a negligible impact on the key volatile profile of truffles. There was no difference in the relative percentage of DMS between the truffle-β-CD complexes 1:2 and the positive control. But similar to the freeze-dried truffles, the relative percentage of DMS was

significantly lower in the truffle-β-CD complexes 1:1 as compared to the positive control. While we speculate that a higher amount of encapsulating agent may be helpful in retaining DMS in the truffle-β-CD complexes 1:2, it is interesting to note that ethyl-3-methylbutanoate, which imparts the fruity odour in black truffle, was detected in the freeze-dried truffles (0.0859 ± 0.0099%) but undetectable in the two truffle-β-CD complexes. The additional steps in the encapsulation processing treatment that may lead to the loss of volatiles compared to the simple freeze-drying technique can be ruled out as the addition of β-CD as the encapsulating agent were meant to improve the volatile retention and stability (Madene et al., 2006). The encapsulation process is the formation of inclusion complex with volatile compounds as reported in the common odour compounds like cinnamaldehyde and thymol (Ponce Cevallos et al., 2010). However, encapsulation of truffle aroma has not published as yet and this warrants further study. As an encapsulation of truffle aroma has not been found in the literature, we do not have any previous information to make a comparison with our findings. The loss of ethyl-3-methylbutanoate during the encapsulation process is unclear and further research is required to confirm this. Methanethiol, which contributes to the cooked cabbage odour in black truffle, was undetectable in the two truffle-β-CD complexes, probably due to its presence at trace level originally in the starting material which makes it difficult to retain. Another possible reason for its loss could be the conversion of methanethiol to DMTS in the presence of oxygen (Vazquez-Landaverde et al., 2006). This further supports our speculation as a higher relative percentage of DMTS was observed in the freeze-dried truffles (0.0094 ± 0.0004%), the truffle-β-CD complexes 1:1 (0.0093 ± 0.0004%), and the truffle-β-CD complexes 1:2 (0.0076 ± 0.0008%) as compared to the positive control (0.0047 ± 0.0005%), probably as a result of the oxidation products of methanethiol (Vazquez-Landaverde et al., 2006). The reduction of DMS in the freeze-dried truffles and the truffle-β-CD complexes compared to the positive control could also be due to its oxidative characteristic.

Overall, the findings suggest that the volatile changes in truffles are complex and inevitable during processing due to the chemical properties of some key volatiles which are highly reactive and volatile (Culleré et al., 2013a; Palacios et al., 2014). Nonetheless, measuring volatile changes of truffles as a result of processing merely based on an analytical instrument alone does not provide a complete interpretation of the aroma quality effect (Duncan et al., 2017). Given that consumer perception and acceptance are key to successful food products, a consumer sensory evaluation that serves to complement

analytical instruments and provides a more complete profile of a food product (Lawless, 1991) was therefore performed in the subsequent study (Section 6.3.3).

Table 6.2: Relative percentages (%) of the key volatile profile of frozen truffles (positive control), freeze-dried truffles, truffle-β-CD complexes 1:1, and truffle-β-CD complexes 1:2

Key volatiles	Aromatic descriptor*	Relative percentage (%)			
		Frozen truffles (positive control)	Freeze-dried truffles	Truffle-β-CD complexes 1:1	Truffle-β-CD complexes 1:2
Methanethiol [$]	Cooked cabbage, vegetable	0.0016±0.0004 [a]	0.0012±0.0006 [a]	ND	ND
Dimethyl sulfide (DMS) [$]	Truffle, sulphur	0.1439±0.0217 [a]	0.0657±0.0094 [b]	0.0798±0.0056 [b]	0.0965±0.0093 [ab]
Ethyl-3-methylbutanoate	Fruit, anise	0.2886±0.0477 [a]	0.0859±0.0099 [b]	ND	ND
Dimethyl disulfide (DMDS) [$]	Truffle, sulphur	0.0064±0.0004 [b]	0.0099±0.0008 [b]	0.0205±0.0011 [a]	0.0210±0.0009 [a]
Isoamyl alcohol	Cheese	22.3337±0.0704 [a]	13.2973±0.7330 [b]	5.1892±0.2403 [c]	5.9551±0.3090 [c]
1-Octen-3-one	Mushroom	ND	ND	ND	ND
Dimethyl trisulfide (DMTS) [$]	Pungent	0.0047±0.0005 [b]	0.0094±0.0004 [a]	0.0093±0.0004 [a]	0.0076±0.0008 [a]
1-Octen-3-ol	Mushroom, earth, mould	3.4789±0.3246 [a]	2.6595±0.2070 [a]	3.3439±0.1308 [a]	3.7893±0.3919 [a]
Methional	Boiled potatoes	0.0632±0.0028 [a]	0.0534±0.0091 [a]	0.0911±0.0299 [a]	0.1135±0.0270 [a]
***p*-Cresol**	Phenolic/ leather	0.2690±0.0126 [a]	0.2733±0.0233 [a]	0.1727±0.0157 [b]	0.1245±0.0098 [b]

Data are reported as the mean of triplicate extractions ± standard error.

ND: not detected

Relative percentage (%) of each identified volatile: peak area of an individual component / total peak area of all components X 100%

Footnote [$]: The specific mass fragment (m/z) designated for methanethiol, DMS, DMDS, and DMTS was 47, 62, 94, and 126, respectively.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$).

*The list of aroma descriptor is adapted from Culleré et al. (2010) and Culleré et al. (2013a).

6.3.3 Sensory evaluation

In this study, a triangle test was used to examine the consumers' ability to distinguish the differences among the truffle products. The difference between the observed data and the expected data either due to chance (≤ 33%) or above chance (> 33%) was determined by a nonparametric binomial test. The score of participants for each permutation in a triangle test is illustrated in Figure 6.1. Based on the nonparametric binomial test, 93% of participants demonstrated the ability to distinguish the positive control from the other three products which was above the required 33% ($p < 0.001$) to rule out a chance selection. In total, 86% of participants performed significantly above the chance of 33% ($p < 0.001$) in distinguishing the freeze-dried truffles from the other three products. About 97% of participants could distinguish each of the two truffle-β-CD complexes from the other three products above the chance selection of 33% ($p < 0.001$), respectively. Overall, 94% of participants could distinguish all the four truffle products above the chance of 33% ($p < 0.001$). However, when comparing each permutation based on a chi-square test, the participants could distinguish all the five pairings ($p < 0.001$) except the positive control from the freeze-dried truffles (X^2 frozen vs freeze-dried = 0.704) (Table 6.3). This indicates that although some volatile changes occurred in the freeze-dried truffles (Section 6.3.2), the sensory differences between the freeze-dried truffles and the positive control were undetected by human olfaction, suggesting that the freeze-dried truffles exhibited closer proximity with the positive control than the two truffle-β-CD complexes based on the consumer perception.

As we could not ascertain if the sensory differences based on the triangle test would reduce the perceived aroma profile of the processed truffles and negatively influence the consumer perception and acceptability. A further sensory evaluation using a sensory ranking test was used to evaluate the overall impression delivered by the truffle products (Figure 6.2). No direct statistical comparison was made between the nominal data (triangle test) with continuous data (GC-MS analysis and consumer sensory test). The results obtained from the GC-MS analysis and triangle test were merely used to support the findings of consumer sensory test. In terms of the aroma intensity score, both the positive control (73.57 ± 1.62 %) and the freeze-dried truffles (69.52 ± 1.59 %) were significantly higher than truffle-β-CD complexes 1:1 (58.62 ± 1.90 %) and truffle-β-CD complexes 1:2 (53.64 ± 1.83 %), respectively. This means that both the positive control and the freeze-dried truffles exhibited a stronger aroma than the two truffle-β-CD complexes despite all containing a similar amount of dry matter (the truffle content was

set as a constant variable as described in Section 6.2.6.1). But the aroma intensity score showed no difference between positive control and freeze-dried truffles (Figure 6.2) which aligned with the triangle test (X^2 frozen vs freeze-dried = 0.704) (Table 6.3). There was no difference in the aroma intensity score between the two truffle-β-CD complexes (Figure 6.2) which is consistent with the key volatile profile (Section 6.3.2 and Table 6.2) but interestingly consumers were able to distinguish these two products in the triangle test (X^2 truffle-β-CD complexes 1:2 vs truffle-β-CD complexes 1:1 = 6.768) (Table 6.3). In spite of containing a similar amount of truffles (the truffle content was set as a constant variable as described in Section 6.2.6.1), the two truffle-β-CD complexes scored a lower aroma intensity than the positive control and freeze-dried truffles probably due to the addition of β-CD to entrap the volatile compounds which would have suppressed the release of volatiles from the truffle-β-CD complexes (Madene et al., 2006). However, when added to a food product as an ingredient, with its dissolution the aroma volatiles could be released and this warrants further study. Given that the truffle volatiles can be encapsulated and the truffle products were accepted by consumers, the encapsulation efficiency need to be determined in future research before the process can be commercialized.

Liking does not always lead to acceptance of a product. In a liking test, some participants might dislike all products presented and choose the least offensive. In such a case, producing or selling either version of the product would clearly not be a good idea, but the liking test fails to provide such information. It is also possible to get conflicting results from liking and acceptance questions from some participants if they change their basis for decision-making between the two questions (Lawless & Heymann, 2010). For these reasons, both liking and acceptance questions were asked in the present study. Despite some sensory differences were observed between truffle products as shown in the triangle test and aroma intensity score, there was no significant difference in terms of the overall liking ($F = 0.262$, $p = 0.853$), acceptability ($F = 0.195$, $p = 0.899$), and truffle aroma rating ($F = 0.056$, $p = 0.983$) across all the four truffle products (Figure 6.2), this suggests that the sensory differences and intensity of the truffle aroma did not influence the consumers' liking and acceptability. It was also found that both the liking and acceptance questions did not conflict with each other. As none of the products was preferable or acceptable over the positive control, this indicates that there is a potential value of processing truffles into a natural flavouring ingredient either by freeze-drying or encapsulation.

It was interesting to determine which key volatiles have a greater influence, either positive or negative, on the sensory perception (aroma intensity, overall liking, and overall acceptability) of each truffle product. The relationships between the key volatiles and sensory perception were identified by Pearson correlation coefficients (Table 6.4). The correlation coefficient values lie between -1 and 1, where -1 would show an inverse relationship and 1 a direct linear relationship (Chambers & Koppel, 2013). It was observed that while isoamyl alcohol and p-cresol had a strong positive impact on the aroma intensity, some volatile compounds such as DMDS and methional were negatively correlated with the aroma intensity. The overall liking was positively correlated with the presence of DMS and 1-octen-3-ol but negatively correlated with the presence of DMTS. The presence of p-cresol appeared to have a negative impact on the overall acceptability, whereas 1-octen-3-ol and methional showed significant positive correlations with the overall acceptability. The overall results suggest that the type and amount of key volatile compounds that are present may significantly affect the aroma intensity, overall liking, and overall acceptability of a truffle product. Given that 1-octen-3-ol, methional, and p-cresol showed significant correlations with overall acceptability of the truffle products, this infers that the consumer acceptance of a truffle product may increase as the amount of 1-octen-3-ol and methional can be retained in the process product, whilst the amount of p-cresol in a truffle product reduces.

Table 6.3: Comparison of each permutation in a triangle test analysed by a chi-square test

Permutation	Chi-square	p value
Frozen (positive control) vs freeze-dried truffles	0.704	0.401
Positive control vs truffle-β-CD complexes 1:2	66.261	< 0.001
Positive control vs truffle-β-CD complexes 1:1	14.901	< 0.001
freeze-dried vs truffle-β-CD complexes 1:2	28.845	< 0.001
freeze-dried vs truffle-β-CD complexes 1:1	21.303	< 0.001
truffle-β-CD complexes 1:2 vs truffle-β-CD complexes 1:1	6.768	< 0.001

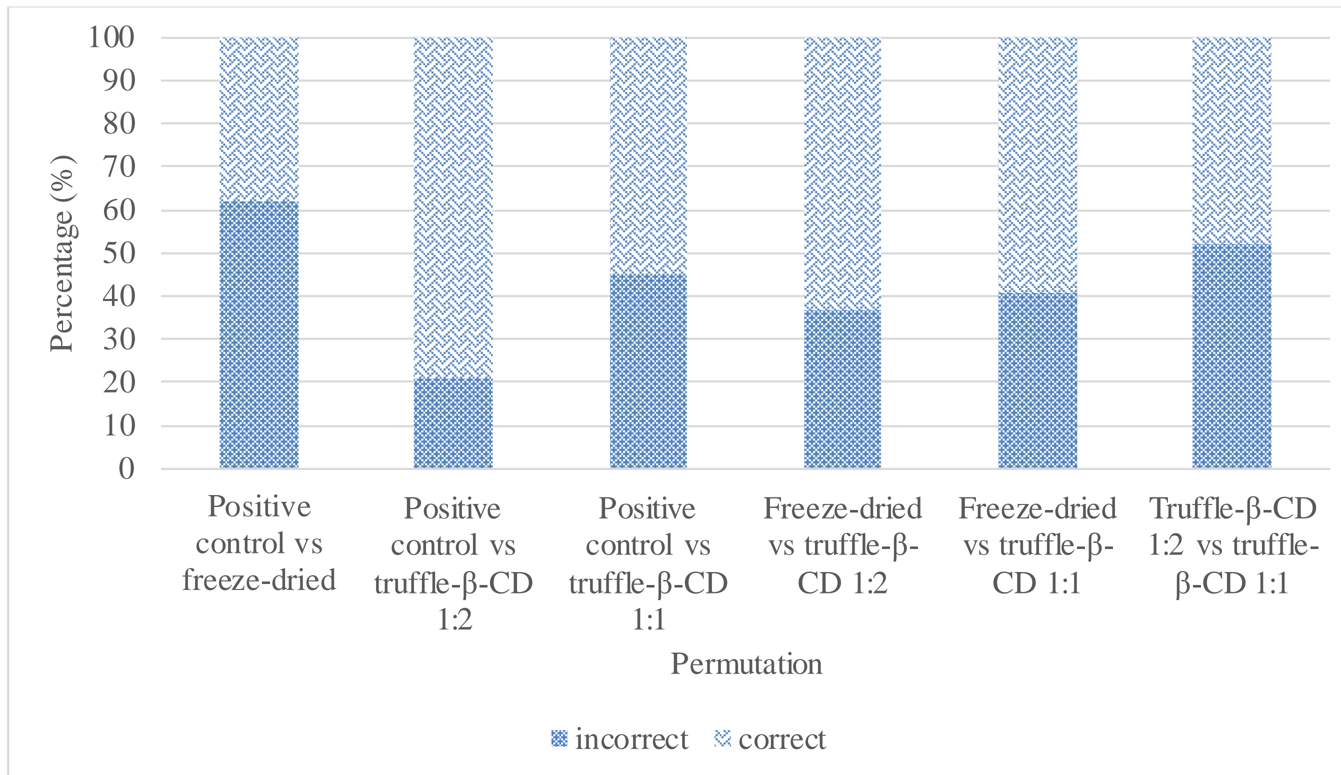

Figure 6.1: Score of participants in percentage (%) for each permutation in a triangle test

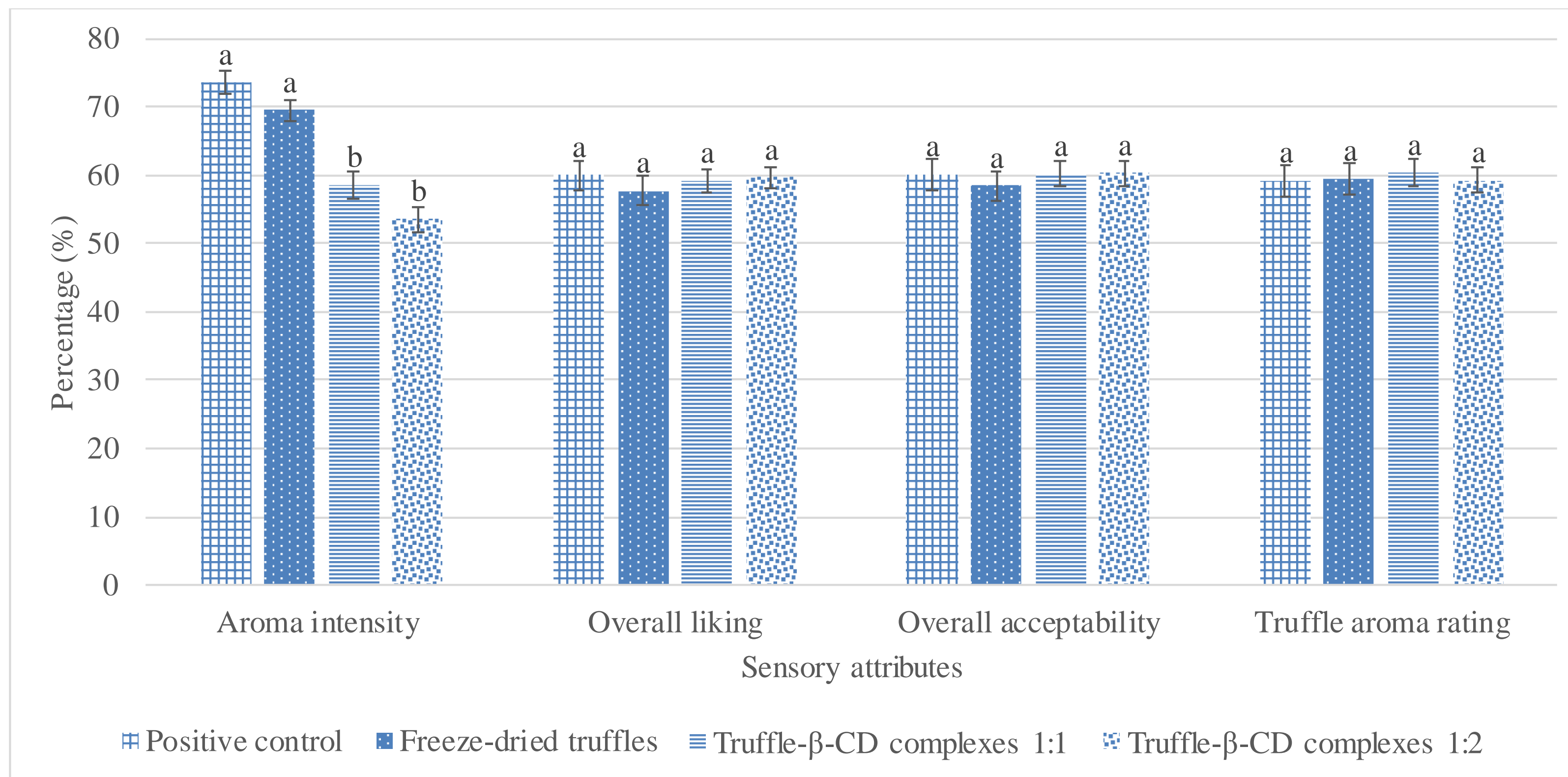

Figure 6.2: Score of sensory ranking test of the four truffle products in percentage (%) based on the general labelled magnitude scale corresponding to the consumers' degree of sensation (4 missing data in aroma intensity, overall acceptability and truffle aroma rating; 5 missing data in overall liking). Data are reported as the mean of scores ± standard error

Table 6.4: Pearson correlation coefficients (r) between key volatiles and consumer sensory testing scores of four truffle products

Key volatiles	Intensity	Overall liking	Overall acceptability
Dimethyl sulfide (DMS)	0.3943	0.8282	0.5780
Dimethyl disulfide (DMDS)	-0.9813	0.1220	0.4484
Isoamyl alcohol	0.9236	0.1711	-0.1742
Dimethyl trisulfide (DMTS)	-0.4398	-0.7629	-0.4804
1-Octen-3-ol	-0.5362	0.9070	0.9399
Methional	-0.9401	0.4790	0.7001
***p*-Cresol**	0.9784	-0.3620	-0.6198

6.4 Conclusion

This study evaluated the impacts of different processing techniques on the quality of truffles from both analytical and sensory aspects. Some volatile changes were observed when comparing the truffle products with respect to the positive control which were aligned with the consumer discrimination (triangle test) and the aroma intensity score (sensory ranking test). Nevertheless, differences in the key volatile profile and olfactory sense did not influence the overall sensory impression across all the four truffle products. Given that the overall sensory impression of the truffle products was not affected by some volatile changes and sensory differences, this provides an important indication about the potential value of processing truffles into a flavouring ingredient for food application via freeze-drying or encapsulation which would provide a positive impact to the truffle and food industry. Nonetheless, some volatile compounds may significantly influence the consumer acceptance of a truffle product based on the correlation analysis. It is important to ensure that 1-octen-3-ol and methional can be retained as much as possible while reducing the amount of *p*-cresol when developing a truffle product. Determining the absolute content of the key volatiles as well as evaluating the odour-active compounds using gas chromatography-olfactometry (GC-O) to study the aromatic characteristic of black truffle may be considered in future work. Future study to determine the efficiency of encapsulation is required before the process can be commercialised. This study further establishes the importance of conducting a sensory evaluation to complement the volatile analysis mainly based on the analytical instruments so as to provide a complete profile of a food product. Hence, incorporating the truffle products onto food as a flavouring ingredient to evaluate the consumer' purchase interest may be another interesting topic to explore in the near future.

6.5 Ethics approval

The sensory evaluation was approved by the Curtin University Human Research Ethics Committee (Approval number: HRE2019-0374).

CHAPTER 7: Effects of ozonated water and citric acid decontamination treatment on the microbial population and organoleptic qualities of fresh black truffle (*Tuber melanosporum*)

The content in this chapter has been submitted for publication as follows:

Phong, W. N., Payne, A. D., Dykes, G. A., & Coorey, R. Effects of ozonated water and citric acid decontamination treatments on the microbial population and organoleptic qualities of fresh black truffle (*Tuber melanosporum*). *Postharvest Biology and Technology*. (under review)

Abstract

Truffles usually contain a high microbial load and establishing a decontamination strategy without affecting the organoleptic features of fresh truffles before any postharvest processing is an imperative. In this study, the impacts of decontamination treatments including ozonated water (3 ppm and 6 ppm) and citric acid (5% and 10%) on the microbiological populations (total aerobic, total anaerobic, and *Pseudomonas* spp. counts) and organoleptic characteristics (firmness and colour) of fresh truffles were investigated. Furthermore, the effect of time of exposure (5, 10, 15, and 20 min) to each treatment was assessed. Treatment that demonstrated the most effective microbial reduction without compromising the colour and firmness of truffles was deemed preferred and its impact on the key volatile profile of truffles was assessed. Truffles treated with different concentrations and exposure times of ozonated water exhibited no changes in microbial counts, firmness, or colour compared to the control. A significant microbial reduction without compromising the colour and firmness of truffles was observed with citric acid treatments. In particular, the antimicrobial effect of citric acid against *Pseudomonas* spp. was improved significantly with increasing concentration and exposure time. Truffles treated with 10% citric acid treatment for 15 min promoted about 1.0 log CFU/g of reduction for the total aerobic and anaerobic microbes and the *Pseudomonas* spp. count was reduced from 6.18 ± 0.19 log CFU/g (control) to < 3.48 log CFU/g. The key volatile profile of the selected treatment was not different from the control. The overall finding shows that a single-step washing with citric acid could be a promising decontamination treatment for fresh truffles before any postharvest processing.

Keywords: Citric acid, decontamination treatment, fresh truffles, organoleptic quality, ozonated water, volatile compounds

7.1 Introduction

Truffles (*Tuber* spp.), including Black Périgord truffle (*Tuber melanosporum* Vittad. 1831), are underground mushrooms that are heavily colonised by a complex bacterial community with some of them having the potential to cause spoilage and affect aroma quality (Rivera et al., 2010a; Romanazzi et al., 2015). Rinsing fresh truffles with tap water after gentle brushing to remove soil is the commonly used postharvest cleaning procedure (Phong et al., 2022c; Rivera et al., 2010a) but based on the studies conducted by Rivera et al. (2010a) and Saltarelli et al. (2008), a high microbial load around 10^7 to 10^8 CFU/g were still detected in *T. melanosporum*. In the case of mushrooms, washing with water alone may increase microbial spoilage as it could lead to a higher water content which promotes bacterial growth (Brennan et al., 2000), therefore we speculate that the same would also happen to truffles. Considering that microbial spoilage is the main factor that affects the quality of fresh truffles (Campo et al., 2017), the need to supplement the simple cleaning procedure with an effective non-thermal decontamination treatment after harvest without affecting their organoleptic features is evident (Saltarelli et al., 2008; Sorrentino et al., 2018). In this regard, the use of a simple, safe, environmentally friendly, and low-cost decontaminating agent such as ozonated water or citric acid for fresh truffle decontamination is an attractive potential option (Behera et al., 2021; Zalewska et al., 2021).

Ozone (O_3) is a highly reactive form of oxygen (O_2) and is characterised by a broad spectrum of antimicrobial activities (Chen et al., 2020). Ozone oxidises the bacterial cell walls and cytoplasmic membrane, causing leakage of cellular components followed by cell lysis. Additive-free food products, along with an extended shelf life, can be achieved via ozone as it quickly auto-decomposes into molecular oxygen (Zalewska et al., 2021). As it leaves no chemical residue or taste, ozone has been widely used to disinfect drinking water (Bermúdez-Aguirre & Barbosa-Cánovas, 2013) and its application to decontaminate vegetables and fruits is also gaining considerable interest in the food industry (Sarron et al., 2021). However, there is a usage threshold in ozone concentration above which the exposure may induce surface oxidation that lead to colour change as observed in acai fruit (Bezerra et al., 2017) and in Brazil nuts (Freitas-Silva et al., 2013) and/or deterioration of flavour as reported in orange juice (Alves Filho et al., 2019). As

far as we know, the impacts of ozonated water on the microbial population and organoleptic quality of fresh truffles have not been reported.

Organic acids are natural antimicrobial compounds that are extensively employed in the food industry to increase the shelf life of fresh products but when using at a higher concentration, organic acids could inactivate microorganisms by causing acidification of the bacterial cytoplasm (Bermúdez-Aguirre & Barbosa-Cánovas, 2013; Carpenter & Broadbent, 2009; Mani-López et al., 2012; Virto et al., 2005). Among these organic acids, citric acid is considered one of the most broadly used and it can be found naturally in a variety of fruits like lemons, limes, oranges, grapefruits, and pineapples (Behera et al., 2021). Citric acid is commonly used in the food industry as a natural flavouring agent, preservative, and decontaminating agent (Behera et al., 2021; Mani-López et al., 2012) but there is a lack of information about its decontamination effect on fresh truffles such as *T. melanosporum*. Miao et al. (2014) proposed that dipping Chinese truffle *T. indicum* (harvested from Sichuan, China) in 3% citric acid and 3% apple polyphenol for 20 min at 45°C could extend its shelf life up to 45 days under modified atmosphere packaging cold storage (4°C). The antimicrobial effect of citric acid alone at different concentrations (0%, 1%, 2 %, and 3%), soaking time (0, 10, 20, and 30 min), and heating temperatures (35°C, 45°C, and 55°C) in *T. indicum* was investigated (Miao et al., 2014) as part of the study. The antimicrobial effect of citric acid was found to improve as the concentration, temperature, and exposure time increased. However, the high temperature (35 – 55°C) used in the citric acid treatment may be less suitable to treat fresh truffles as they contain some heat-labile volatiles but this aspect was not evaluated by Miao et al. (2014); instead, a sensory analysis was only performed on the quality of truffles (texture, aroma, flavour, internal colour, weight loss, general acceptability) treated with combined treatment (citric acid and apple polyphenol) under packaging and cold storage conditions (Miao et al., 2014). The decontamination effects of citric acid at room temperature on the microbial population and organoleptic qualities of popular truffle species, *T. melanosporum* has not yet been reported and was therefore evaluated in this study.

The senescence and spoilage occur immediately after fresh truffles are harvested and the degradative process is further exacerbated by the proliferation of microbes (Rivera et al., 2010a; Romanazzi et al., 2015) which adversely affects the visual appearance and the aroma quality of fresh truffles (Rivera et al., 2010b; Rivera et al., 2011b). Hence, there is a need to establish a practical decontamination step to supplement the simple cleaning

procedure (tap water rinsing) before any postharvest processing of fresh truffles (Saltarelli et al., 2008; Sorrentino et al., 2018). For this reason, this study aimed to evaluate the effects of ozonated water (3 ppm and 6 ppm) and citric acid (5% and 10%) in decontaminating *T. melanosporum*. The total aerobic, total anaerobic, and *Pseudomonas* spp. counts in the treated truffles were determined after exposure to each treatment for 5, 10, 15, and 20 min. The effects of ozonated water and citric acid on the firmness and colour of truffles were also assessed. Treatment that demonstrated the most effective microbial reduction without compromising the firmness and colour of truffles was further studied for its impact on the profile of key volatile compounds of truffles.

7.2 Materials and methods

7.2.1 Fresh truffles

Truffles were harvested from the Manjimup region of Western Australia, Australia in August 2021 at the standard commercial harvesting stage. The freshly collected truffles were brushed gently with a soft brush to remove soil, followed by rinsing with tap water and air-dried in a laminar flow cabinet. The packaged truffles were placed into an insulated container with ice packs held at 4 ± 2°C and immediately couriered to our laboratory (Phong et al., 2022b).

7.2.2 Truffle sample preparation

All sample preparations were conducted under aseptic conditions. Knife and forceps used for truffle cutting and handlings were flamed at regular intervals to ensure sterility. Upon receiving the truffles, a qualitative selection was made by carefully inspecting their visual appearance. Those free of defects with a similar appearance were selected, whereas those with indications of parasite infestations, spoilage or soft texture were rejected (Culleré et al., 2012).

For microbial analysis (Section 7.2.5), about 30 randomly selected fresh truffles were cut into small cubes of approximately 6 × 6 × 6 mm and mixed well in a sterile bag to ensure homogeneity before ozonated water treatment (Section 7.2.3). All subsamples were divided into three portions (four subsamples in each portion for four different exposure times): portion 1 were treated with sterile water alone (control), portion 2 were treated with 3 ppm ozonated water (lower treatment concentration), and portion 3 were treated with 6 ppm ozonated water (the higher treatment concentration) for 5, 10, 15, and 20 min, respectively. Each treatment was done in triplicate. The same sample preparation

procedures with the same replications were repeated for citric acid treatment (Section 7.2.4).

For firmness analysis (Section 7.2.6), 12 subsamples of comparable size (approximately $6 \times 6 \times 6$ mm) were divided from the same single truffle and three single truffles were required so that the analysis can be performed in triplicate. Another three single truffles were used for colour analysis (Section 7.2.7). Twelves subsamples of comparable size (approximately $12 \times 12 \times 3$ mm) were divided from the same single truffle before decontamination treatment and followed by the colour analysis. For both analyses, of the 12 subsamples from a single truffle, four subsamples were treated with sterile water alone (control); four subsamples were subjected to a lower treatment concentration (3 ppm ozonated water); another four subsamples were treated with a higher treatment concentration (6 ppm ozonated water) for 5, 10, 15, and 20 min, respectively. After treatment, the samples were air-dried in a laminar cabinet before firmness (Section 7.2.6) and colour (Section 7.2.7) analyses. The same sample preparation procedures with the same replications were repeated for citric acid treatment (Section 7.2.4).

For volatile compound analysis (Section 7.2.8 and 7.2.9) in experiment 3, two subsamples of comparable size (approximately $6 \times 6 \times 6$ mm) were cut from a single truffle; one subsample was treated with sterile water alone (control), another subsample was treated with the selected treatment (10% citric acid for 15 min). After treatment, the samples were air-dried in a laminar cabinet and crushed using a mortar and pestle before being immediately analysed by gas chromatography-mass spectrometry (GC-MS) (Section 7.2.8 and 7.2.9). Experiments were performed in triplicate using three single fresh truffles.

7.2.3 Experiment 1: Ozonated water decontamination treatment (3 ppm and 6 ppm)

Gaseous ozone was attained by an ozone generator (MP-1000 Multi-Purpose Ozone Generator, Pacific Water Technology Pty Ltd, Queensland, Australia) using oxygen as the feed gas. Ozonated water was generated by bubbling gaseous ozone with a bubble diffuser stone (Pacific Water Technology Pty Ltd, Queensland, Australia) into deionised water. The concentration of ozonated water was measured immediately before use with a commercially available ozone CHEMets visual kit (K-7404, CHEMetrics, Midland, VA, USA) (Garcia et al., 2003). After attaining the desired concentration of ozonated water (3

ppm and 6 ppm), 100 mL of ozonated water was poured into each beaker containing 10 g of truffle samples. The mixture was agitated at 200 rpm on a shaker (SkyLine Digital Orbital Shaker DOS-20L, ELMI Ltd., Newbury Park, CA, USA) at different exposure times (5, 10, 15, and 20 min). The treatment water was drained from the treated truffles before analyses (Section 7.2.5, 7.2.6, 7.2.7). The same procedures were repeated for control samples using sterile water alone as the treatment. Experiments were carried out in a fume hood at room temperature (approximately 20°C). Each treatment was performed in triplicate.

7.2.4 Experiment 2 and 3: Citric acid decontamination treatment (5% and 10%)

Two different concentrations of citric acid solutions (5% and 10%, w/v) were prepared by mixing granular citric acid monohydrate (Univar, Ajax Finechem Pty Limited, NSW, Australia) with sterile water. For each treatment and each exposure time (5, 10, 15, and 20 min), 100 mL of sterile water (control), 5% citric acid solution, and 10% citric acid solution was poured into a beaker containing 10 g of truffle samples and shaken at 200 rpm (SkyLine Digital Orbital Shaker DOS-20L, ELMI Ltd.), respectively. Next, the treatment solution was decanted and the treated truffles were rinsed with sterile water (Miao et al., 2014) before analyses (Section 7.2.5, 7.2.6, 7.2.7). Volatile analysis (Section 7.2.8 and 7.2.9) was performed on the selected treatment (10% citric acid for 15 min). Experiments were conducted at room temperature (approximately 20°C). Each treatment was performed in triplicate.

7.2.5 Microbiological analysis

About 10 g of samples treated with ozonated water were homogenised (PRO250, PRO Scientific Inc., Oxford, USA) for 2 min into 90 mL of sterile buffered peptone water (Oxoid Ltd, Victoria, Australia) containing 0.05% of analytical grade sodium thiosulfate (Univar, Ajax Finechem Pty Limited, NSW, Australia) capable of neutralising excessive ozone (Yuk et al., 2007). Serial dilutions were performed in sterile phosphate-buffered saline. Subsequently, 0.1 mL of aliquots from each dilution were spread plated onto respective culture medium and incubated accordingly. The same analytical procedures were repeated for samples treated with citric acid except that the sterile buffered peptone water used did not contain any additive to neutralise citric acid; therefore, an additional rinsing step with sterile water right after the treatment was performed to wash off any residual citric acid from the truffle samples (Miao et al., 2014) (Section 7.2.4).

Total aerobic counts were determined after spread plating onto tryptone soya agar (TSA) (Oxoid Ltd, Victoria, Australia) and incubated at 37 ± 1°C for 48 h. To culture anaerobic microbes, tryptone soya agar (TSA) (Oxoid Ltd, Victoria, Australia) plates were placed into anaerobic jars containing AnaeroGen sachets (Oxoid Ltd, Victoria, Australia) and resazurin anaerobic indicator strip (Oxoid Ltd, Victoria, Australia) before the jars were sealed and incubated at 37 ± 1°C for 48 h. *Pseudomonas* spp. were enumerated on *Pseudomonas* isolation agar (Becton Dickinson Pty Ltd, NSW, Australia) after incubation at 37 ± 1°C for 48 h (Phong et al., 2022b).

After incubation, microbial colonies on agar medium were counted by only considering counts of 30 to 300 colony forming units (CFU). The limit of detection was set at one colony from the first dilution (10^{-1}) and is denoted by < 2.00 log CFU/g whereas plates with less than 30 colonies are considered below the limit of quantification and are denoted by < 3.48 log CFU/g. Each microbial count (expressed as log CFU/g) was calculated based on the mean of three replicates. Ten bacterial colonies from each growth medium were isolated and identified by oxidase and catalase reactions (Bullock & Aslanzadeh, 2013; Harvey et al., 2007; Phong et al., 2022b) before Gram-staining (Fluka, Australia). Once stained, the morphological characteristics of each isolate were observed using a fluorescence microscope (Olympus BX51, Japan) (Phong et al., 2022b).

7.2.6 Firmness analysis

Following Jiang et al. (2012) method with some modifications, firmness measurements were performed on truffles from each treatment using a Perten texture analyser (TVT 6700, Perten instruments, Hägersten, Sweden) with a 2 mm diameter cylindrical probe. Each truffle sample was penetrated 3 mm in depth at three random locations. The speed of the probe was maintained at 1 mm s^{-1} during pre-test and penetration. Firmness is defined as the maximum force of the first compression and was measured at the maximum peak force in Newton (N) from the force vs time curve.

7.2.7 Colour analysis

Colour changes of truffles were measured by a colorflex EZ (45°/0° design) spectrocolorimeter (Hunter Lab, Hunter Associates Laboratory Inc., Reston, VA, 20190, USA). The instrument was calibrated with a white tile followed by a black glass according to the manufacturer’s instructions before taking any measurement. Colour measurement was performed at three random locations on each truffle sample. The colour of each

sample was expressed in three dimensions as L* denotes lightness (high values) or darkness (low values) varying from 0 to 100, +a* redness, -a* greenness, +b* yellowness, and -b* blueness.

7.2.8 Headspace solid-phase microextraction (SPME)

SPME was automated using GERSTEL MultiPurpose Sampler (Díaz et al., 2009). A flexible fused silica fibre coated with a 50/30 μm layer of divinylbenzene/ Carboxen/ Polydimethylsiloxane (DVB/CAR/PDMS) (57298-U, Supelco, Bellefonte, Pennsylvania, USA) was chosen for volatile sampling. The fibre was conditioned as per the manufacturer's instructions (Supelco) before its use (Phong et al., 2022a; Phong et al., 2022d).

About 1 g of crushed truffles was weighed into a 20 mL headspace glass vial. 1,2-Dichlorobenzene (Sigma Aldrich, Sydney, NSW, Australia) was added to all samples as an internal standard as it was not present in truffles and separated well from other compounds in the chromatogram (Liu et al., 2017b). The vials were tightened with a magnetic screw cap (Thermo Fisher Scientific, Australia) and equilibrated for 5 min at 53 °C. The fibre was then introduced into the headspace of each vial for 13.6 min for volatile sampling. Volatiles that bound to the fibre were desorbed in a splitless injector for 2 min at 200 °C (Phong et al., 2022a; Phong et al., 2022d).

7.2.9 Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was performed as per previous studies (Phong et al., 2022a; Phong et al., 2022d). An Agilent-6890 GC system coupled to a GC 5973 mass spectrometer (Agilent, Santa Clara, California, USA) was used to carry out the analysis. The GC oven temperature programme was increased from 35 °C (held 5 min) to 240 °C at 5 °C min^{-1} (held 3 min). Mass spectra were recorded in electron impact (EI) ionisation mode, scanning the 40-250 m/z range at 0.46 sec/scan (3 μ-scans) after 1.5 min of solvent delay. Helium gas (at 6.74 psi, 1.0 mL/min flow rate) was used as a carrier gas. The capillary GC column used was a 30 m (length) x 250 μm (diameter) x 0.25 μm (film thickness) i.d. HP-INNOWax GC column (Agilent) fused silica capillary column (Model Number: Agilent 19091N-133).

The chromatographic data obtained from MSD ChemStation F.01.03.2357 (1989-2015 Agilent Technologies, Inc.) was analysed using the National Institute of Standards

Technology mass spectral database program (NIST, Version 2.3, 2017, USA). Volatile compound identification was based on the comparison between the mass spectra for each compound with those in the NIST 2017 Mass Spectral library (a minimum mass spectral match quality of 700 was used as the criterion) (Watson & Sparkman, 2007) as well as with published mass spectra, GC retention time and Kovats retention indices (calculated based on n-alkanes) (Wernig et al., 2018).

The peak area of each compound was determined based on area normalisation by integration. Each identified compound was expressed in a relative percentage (%) by dividing the proportion of its peak area to the total peak areas of all the compounds of interest (Díaz et al., 2003). The integration of some compounds such as methanethiol, dimethyl sulfide (DMS), dimethyl disulfide (DMDS), and dimethyl trisulfide (DMTS) was difficult as they did not produce any fully resolved peaks in the chromatogram due to their low concentration and/or co-elution with other peaks with a similar retention time. Therefore, a single ion chromatogram was extracted to determine their relative percentage.

7.2.10 Data analysis

All experiments were carried out in triplicate and data were expressed as mean ± standard error. Statistical analyses were performed using IBM SPSS statistics 26 software. The means of two different groups were compared by an independent samples t-test with a 5% significance level. The means of more than two independent groups were compared by a one-way analysis of variance (ANOVA) followed by a post-hoc Tukey's test (if a significant difference was identified) with a 5% significance level.

7.3 Results and discussion

This study was divided into three experiments. The impacts on the microbial, firmness, and colour of fresh truffles after being treated with 3 ppm and 6 ppm of ozonated water (Experiment 1) and 5% and 10% citric acid (Experiment 2) for 5, 10, 15, and 20 min, respectively were studied. Sterile water alone was used as the control treatment for experiments 1 and 2. As there was no significant microbial reduction in truffles treated with ozonated water (Experiment 1), no further investigation on the key volatile profile was performed for this treatment. The citric acid treatment achieved a significant microbial reduction and did not impact the firmness and colour of the truffles (Experiment 2). The most effective microbial reduction was achieved after exposing truffles to 10%

citric acid for 15 and 20 min treatment. The 15 min was selected to determine its impact on the key volatile profile of truffles (Experiment 3).

7.3.1 Experiment 1: Ozonated water treatment

7.3.1.1 Microbiological analysis

The effects of ozonated water treatment (3 ppm and 6 ppm) against the total aerobic count, total anaerobic count, and *Pseudomonas* spp. count of the treated truffles compared to the control are presented in Table 7.1. Both concentrations did not show any effect in reducing the microbial counts compared to that of the control (Table 7.1). Increasing the exposure time up to 20 min showed no impact on the microbial reduction either (Table 7.1), most likely due to the instability of ozone in an aqueous medium which spontaneously decomposes to oxygen (Sarron et al., 2021). As ozone diminishes quickly upon contact with organic matter, this could be the reason for its poor antimicrobial efficacy (Yucel Sengun & Kendirci, 2018) when in contact with a high organic matter content in truffles (Rivera et al., 2011b). Moreover, the peridium structure of truffle ascocarps which is irregular, rough, and wrinkled (Rivera et al., 2011b) may provide an ideal niche for microbial attachment and hinder the access of ozonated water to microorganisms which would have further reduced the antimicrobial efficacy of ozonated water. Alexopoulos et al. (2013) reported a better antimicrobial efficacy of ozonated water in bell pepper that had a smooth and regular surface compared to lettuce with irregular and rugged surface.

Contrary to the poor antimicrobial efficacy of ozonated water as observed in the present study, previous studies reported the positive effect of ozone in reducing the microbial load in various fresh produces. For example, ozonated water treatment (9 ppm for 10 min) reduced the total aerobic mesophilic bacterial load by 0.9-2.4 log CFU/g and *E. coli* by 1.3-2.1 log CFU/g in tomato, cucumber, carrot, and lettuce as compared to their untreated corresponding controls (Pounraj et al., 2021). In spinach that was inoculated with approximately 6–7 log CFU/g of *E. coli* and *Listeria monocytogenes*, these microbes were reduced by 1.22 log CFU/g and 1.40 log CFU/g, respectively after being treated with 5 ppm ozonated water for 3 min (Rahman et al., 2010). Ozonated water treatment at 5 ppm for 1 min decreased *Yersinia enterocolitica* and *L. monocytogenes* from potato surfaces by 1.6 log CFU/g and 0.8 log CFU/g, respectively (Selma et al., 2006). As the antimicrobial efficacy of ozone may be influenced by various factors such as microbial population, species, and strain, pH, temperature, ozone dosage, contact time, temperature,

produce type (root, leaf or fruit) and surface (smooth, intact, rugged, rough, porous, wrinkled or irregular), and the delivery method (such as spraying, soaking, agitation, or bubbling) (Dawley et al., 2021; Glowacz & Rees, 2016; Sarron et al., 2021), these may explain the contradictory findings between the present study and the previous studies.

7.3.1.2 Firmness and colour analyses

An ideal decontamination treatment should be mild but effective and produce no physical and sensory damage to the treated truffles (Rivera et al., 2011b). Firmness was evaluated in this study as it is one of the important organoleptic properties for fresh truffles. In general, truffles with a firm texture are highly desirable because consumers associate this attribute with freshness (Rivera et al., 2011b), whereas truffles with soft texture are associated with senescence and susceptible to injuries during handling. Based on the results shown in Table 7.2, there was no significant change in the firmness of truffles between the control and the treated truffles at any exposure time and concentration. Colour is another critical organoleptic attribute for fresh truffles as it impacts directly on consumer visual perception and any changes in appearance may be reflected as a symptom of deterioration (Rivera et al., 2011b). The characteristic colour of truffles was maintained after treatment, even when treated with a higher concentration and longer exposure time (Table 7.3). Our findings were consistent with the results of previous investigations on carrots, mushrooms, and lettuce. No changes to the firmness and the colour were observed after exposing the carrots to ozonated water up to 10 ppm and 60 min (de Souza et al., 2018). Exposing shiitake mushroom to 3.21 mg/m^3 ozonated water for 30 min did not cause any changes to the colour (Liu et al., 2020). White button mushrooms showed no alteration in their colour and firmness after being treated with gaseous ozone up to 2 ppm and 60 min (Zalewska et al., 2021). Lettuce treated with ozonated water at 2 ppm for 15 min did not show any colour differences compared to the pure water treatment (Yucel Sengun & Kendirci, 2018).

Despite leaving no adverse effect on the firmness and colour of the treated truffles across different exposure times (Table 7.2 and 7.3), ozonated water treatment was excluded from further consideration due to its poor antimicrobial efficacy under the tested conditions in the present study (Table 7.1). A higher concentration of ozonated water may be required to achieve a significant decontamination effect in truffles.

Table 7.1: The effects of water treatment (control) and ozonated water treatment (3 ppm and 6 ppm) at varying exposure times (5, 10, 15, and 20 min) on the total aerobic count, total anaerobic count and *Pseudomonas* spp. count (log CFU/g) in fresh truffles.

		Ozonated water treatment	
Exposure time (min)	**Water treatment (control)**	**3 ppm**	**6 ppm**
Total aerobic count (log CFU/g)			
5	6.71 ± 0.12^{Aa}	6.38 ± 0.29^{Aa}	6.34 ± 0.11^{Aa}
10	6.40 ± 0.22^{Aa}	6.37 ± 0.28^{Aa}	6.35 ± 0.32^{Aa}
15	6.15 ± 0.07^{Aa}	6.16 ± 0.15^{Aa}	5.99 ± 0.10^{Aa}
20	6.52 ± 0.27^{Aa}	6.30 ± 0.45^{Aa}	5.78 ± 0.36^{Aa}
Total anaerobic count (log CFU/g)			
5	6.35 ± 0.19^{Aa}	6.22 ± 0.31^{Aa}	5.88 ± 0.06^{Aa}
10	6.05 ± 0.08^{Aa}	5.96 ± 0.26^{Aa}	5.82 ± 0.19^{Aa}
15	5.88 ± 0.07^{Aa}	5.87 ± 0.29^{Aa}	5.79 ± 0.21^{Aa}
20	6.13 ± 0.22^{Aa}	5.93 ± 0.32^{Aa}	5.53 ± 0.12^{Aa}
***Pseudomonas* spp. count (log CFU/g)**			
5	6.23 ± 0.18^{Aa}	6.29 ± 0.30^{Aa}	5.72 ± 0.13^{Aa}
10	6.17 ± 0.18^{Aa}	5.87 ± 0.34^{Aa}	5.90 ± 0.15^{Aa}
15	5.85 ± 0.19^{Aa}	5.90 ± 0.31^{Aa}	5.79 ± 0.06^{Aa}
20	6.11 ± 0.34^{Aa}	5.99 ± 0.42^{Aa}	5.71 ± 0.29^{Aa}

Data are expressed as mean ± standard error of three replicate samples.

Means within the same column with different uppercase letters differ significantly ($p < 0.05$) across different time points.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$) between different treatments.

Table 7.2: The effects of water treatment (control) and ozonated water treatment (3 ppm and 6 ppm) at varying exposure times (5, 10, 15, and 20 min) on the firmness (N) of fresh truffles.

Exposure time (min)	Water treatment (control)	Ozonated water treatment	
		3ppm	6ppm
5	3.91 ± 0.12 Aa	3.74 ± 0.05 Aa	3.78 ± 0.08 Aa
10	3.91 ± 0.22 Aa	3.76 ± 0.21 Aa	3.71 ± 0.04 Aa
15	3.85 ± 0.09 Aa	3.81 ± 0.12 Aa	3.69 ± 0.07 Aa
20	3.92 ± 0.09 Aa	3.71 ± 0.09 Aa	3.80 ± 0.08 Aa

Data are expressed as mean ± standard error in Newton (N) unit of three replicate samples.

Means within the same column with different uppercase letters differ significantly ($p < 0.05$) across different time points.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$) between different treatments.

Table 7.3: The effects of water treatment (control) and ozonated water treatment (3 ppm and 6 ppm) at varying exposure times (5, 10, 15, and 20 min) on the colour (L*, a*, b*) of fresh truffles.

Exposure time (min)	Colour coordinates	Water treatment (control)	Ozonated water treatment	
			3ppm	6ppm
5	L*	23.12 ± 0.15 Aa	24.19 ± 0.76 Aa	24.61 ± 0.96 Aa
	a*	3.85 ± 0.40 Aa	3.95 ± 0.39 Aa	4.29 ± 0.32 Aa
	b*	5.60 ± 0.30 Aa	6.76 ± 0.24 Aa	6.87 ± 0.43 Aa
10	L*	22.97 ± 0.35 Aa	24.43 ± 0.84 Aa	24.33 ± 1.12 Aa
	a*	3.95 ± 0.20 Aa	4.13 ± 0.31 Aa	4.07 ± 0.11 Aa
	b*	6.55 ± 0.39 Aa	6.98 ± 0.08 Aa	6.74 ± 0.42 Aa
15	L*	24.58 ± 0.62 Aa	23.86 ± 0.50 Aa	25.04 ± 0.85 Aa
	a*	3.80 ± 0.11 Aa	3.73 ± 0.37 Aa	4.13 ± 0.12 Aa
	b*	6.59 ± 0.52 Aa	6.28 ± 0.49 Aa	6.54 ± 0.67 Aa
20	L*	23.69 ± 0.46 Aa	23.60 ± 0.97 Aa	24.75 ± 1.01 Aa
	a*	3.62 ± 0.37 Aa	4.23 ± 0.33 Aa	4.06 ± 0.21 Aa
	b*	6.24 ± 0.21 Aa	6.50 ± 0.51 Aa	7.14 ± 0.53 Aa

L* (dark to light), a* (green to red), b* (blue to yellow)

Data are expressed as mean ± standard error of three replicate samples.

Means within the same column with different uppercase letters differ significantly ($p < 0.05$) across different time points.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$) between different treatments.

7.3.2 Experiment 2: Citric acid treatment

7.3.2.1 Microbiological analysis

The efficacy of citric acid treatment in reducing the total aerobic microbial count, total anaerobic microbial count, and *Pseudomonas* spp. count in the treated truffles compared to the control is shown in Table 7.4. The aerobic and anaerobic microbial populations were significantly reduced when the truffles were treated with citric acid compared to the control but increasing the concentration of citric acid from 5% to 10% and exposure time up to 20 min did not enhance its antimicrobial efficacy against these microbes. In contrast, the bactericidal activity of citric acid against *Pseudomonas* spp. *was* significantly enhanced when increasing the concentration of citric acid from 5% to 10% and exposure time. For example, the *Pseudomonas* spp. count was reduced by about 0.9 log CFU/g when the truffles were treated for 10 min with 10% citric acid compared to 5% citric acid. A dramatic reduction was observed when the truffles were exposed to citric acid for 15 min, in which the *Pseudomonas* spp. counts were reduced from 3.94 ± 0.23 log CFU/g (5% citric acid) to < 3.48 log CFU/g (10% citric acid). However, an increase in the exposure time to 20 min for 10% citric acid treatment did not result in any further *Pseudomonas* spp. reduction and the count remained < 3.48 log CFU/g (Table 7.4), suggesting that the exposure of truffles to 15 min of 10% citric acid could achieve the maximum efficacy on the inactivation of *Pseudomonas* spp. in this study. Being the major postharvest spoilage microorganism in truffles, the reduction of *Pseudomonas* spp. count is particularly important to slow down the deterioration of truffles after harvest (Phong et al., 2022b; Rivera et al., 2010a). *Pseudomonas* spp. may cause degradation and softening by breaking down the intracellular matrix, reducing the central vacuole, and affecting the cell turgidity as reported in mushrooms (Lagnika et al., 2014). Taken together, the findings suggest that 15 min of 10% citric acid treatment may allow an overall effective reduction of microbial counts in truffles.

Pseudomonas spp. are aerobic and able to grow on the total aerobic plate as well. Hence, we expected both the total aerobic microbes and *Pseudomonas* spp. would exhibit a similar reduction pattern after citric acid treatment. However, it is interesting to note that although a significant reduction was observed in the total aerobic count, the reduction was not as great as that observed in the *Pseudomonas* spp. count after treatment. Based on the finding, we speculate that *Pseudomonas* spp. have not been able to grow well on the total aerobic plate, therefore displaying variability in the reduction pattern between the total aerobic count and the *Pseudomonas* spp. count after treatment. A possible reason

to justify our speculation could be that the culture medium used for the total aerobic count (tryptone soy agar) is for general and nonselective purposes, so it may not be favourable for *Pseudomonas spp*. growth. Given that the components of each culture media used were different, the type and number of microbes that grow in the nonselective (tryptone soy agar) and selective (*Pseudomonas* isolation agar) media may vary. Another possible reason could be that certain bacterial species may produce antimicrobial toxins which facilitate interference competition with other bacterial species (Hibbing et al., 2010), this explains that the growth of *Pseudomonas* spp. may be inhibited by other bacterial species in the tryptone soy agar. The access to nutrients for *Pseudomonas* spp. may also be limited as a result of microbial competition for nutritional resources among different microbial species (Hibbing et al., 2010). All these reasons may explain the above experimental observation and justify our speculation.

Knowledge on the antimicrobial action of citric acid remains limited. But it is believed that citric acid exhibits antibacterial activity through multiple mechanisms: at low pH, citric acid in its uncharged and undissociated form penetrates the microbial cytoplasmic membrane and dissociates into anions and protons once inside the microbial cytoplasm, resulting in the cytoplasmic acidification that eventually leads to cell damage (Burel et al., 2021; Mani-López et al., 2012). Based on this theory, we expected an increase in the treatment time would allow sufficient time for citric acid to complete the antimicrobial action and exert an antimicrobial effect. Our expectation was supported by other researchers who observed that the antimicrobial efficacy of citric acid was enhanced with increasing concentration, time, and temperature (Miao et al., 2014). A maximum reduction of 5.64 and 3.69 log units in the mesophilic aerobic and mesophilic anaerobic counts, respectively, was achieved after treating *T. indicum* with 3% citric acid at 55°C for 30 min (Miao et al., 2014). In the current study, however, increasing the citric acid exposure time with truffles from 5 min to 20 min showed a significant reduction against the *Pseudomonas* spp. count but not the total aerobic and anaerobic microbial counts (Table 7.4). For example, the *Pseudomonas* spp. count was drastically reduced from 3.90 ± 0.45 log CFU/g (after 5 min of exposure to 10% citric acid) to < 3.48 log CFU/g (after 15 min of exposure to 10% citric acid). The variability in the antimicrobial efficacy of citric acid against *Pseudomonas*, aerobic, and anaerobic microbes when increasing the exposure time may be due to the inherent differences in the cell structure and outer layers of microbes that would have influenced the penetration of citric acid into the cell membrane which is necessary for the antimicrobial action to take place. In general,

bacterial spores have been described as being the most resistant to disinfectants, followed by mycobacteria, then Gram-negative bacteria, and lastly Gram-positive bacteria (Mai-Prochnow et al., 2016; Russell, 1999). Given that the microbiome of truffles may vary significantly from species to species or from a single truffle to another which is closely associated with different ecological niches and maturity stages (Rivera et al., 2011a), the presence of some highly resistant microbes like the spore-forming *Bacillus* species in the truffles (Vahdatzadeh et al., 2015) would probably explain the insignificant effects of the citric acid against the total aerobic and anaerobic microbial counts despite the prolonged exposure time (Table 7.4). The antimicrobial effect of citric acid on truffles is consistent with previous studies using other fresh produces. Brennan et al. (2000) observed that soaking mushrooms (*Agaricus bisporus*) in 4% citric acid for 10 min reduced the *Pseudomonas* spp. counts by more than 2 log units. Similarly, *Pseudomonas* spp. counts in *A. bisporus* were reduced from 6.04-6.73 log CFU/g (water alone treatment) to 4.63-5.00 log CFU/g after treating with 1% citric acid for 5 min (Simón & González-Fandos, 2010). A reduction of *E. coli* , *Salmonella* typhimurium, and *L. monocytogenes* by more than 2 log units was observed when lettuce and unwaxed fresh apples were treated with 2% citric acid for 10 min compared to the treatment with water alone (Park et al., 2011). Likewise, the exposure of spinach inoculated with approximately 6-7 log CFU/g of *E. coli* and *L. monocytogenes*, respectively to 1% citric acid treatment for 3 min showed a reduction in the *E. coli* and *L. monocytogenes* counts by 1.5 log CFU/g and 1.70 log CFU/g, respectively (Rahman et al., 2010).

7.3.2.2 Firmness and colour analyses

For the firmness and colour (Table 7.5 and 7.6), it was observed that the organoleptic attributes of fresh truffles did not significantly differ from the controls after being treated with 5% and 10% of citric acid, respectively. Increasing the exposure time up to 20 min did not significantly alter the firmness and colour of the treated truffles either. Similarly, Park et al. (2011) did not find significant differences in the colour of lettuce and unwaxed fresh apples after 2% citric acid treatment for 10 min. In contrast, soaking *A. bisporus* in 4% citric acid for 10 min induced a slight yellowness to their surface (Brennan et al., 2000). Simón and González-Fandos (2010) also noticed that immersing *A. bisporus* in 1% citric acid for 5 min caused colour deterioration and induced a darkening effect at the slice edges due to damage in the hyphae cell wall that allowed contact between polyphenols and polyphenol oxidase. Likewise, Lagnika et al. (2014) observed that *A. bisporus* soaked in 1% citric acid for 10 min showed a slight yellowness

on the surface but their firmness was maintained. The discrepancy in the observation may be due to the type of produce. It appears that any colour changes may generally be more noticeable in fresh produces that are lighter in colour like the white mushroom (*A. bisporus*). Park et al. (2011) suggested that water rinsing after organic acid treatment may minimise the colour changes in fresh produces. The results of the current study indicate that citric acid treatment may be utilised to decontaminate fresh truffles before any postharvest processing and/or extend truffle shelf life, without impacting its organoleptic properties.

Table 7.4: The effects of water treatment (control) and citric acid treatment (5% and 10%) at varying exposure times (5, 10, 15, and 20 min) on the total aerobic count, total anaerobic count and *Pseudomonas* spp. count (log CFU/g) in fresh truffles.

Exposure time (min)	Water treatment (control)	Citric acid treatment 5%	Citric acid treatment 10%
	Total aerobic count (log CFU/g)		
5	6.69 ± 0.18 ^{Aa}	6.28 ± 0.14 ^{Aab}	6.03 ± 0.05 ^{Ab}
10	6.71 ± 0.12 ^{Aa}	5.83 ± 0.25 ^{Ab}	5.93 ± 0.07 ^{Ab}
15	6.62 ± 0.09 ^{Aa}	5.92 ± 0.20 ^{Aab}	5.65 ± 0.30 ^{Ab}
20	6.54 ± 0.03 ^{Aa}	5.92 ± 0.04 ^{Ab}	5.67 ± 0.12 ^{Ab}
	Total anaerobic count (log CFU/g)		
5	6.69 ± 0.12 ^{Aa}	6.18 ± 0.15 ^{Ab}	6.01 ± 0.04 ^{Ab}
10	6.73 ± 0.08 ^{Aa}	5.93 ± 0.27 ^{Ab}	5.95 ± 0.12 ^{Ab}
15	6.50 ± 0.05 ^{Aa}	5.67 ± 0.24 ^{Aab}	5.55 ± 0.27 ^{Ab}
20	6.59 ± 0.05 ^{Aa}	5.76 ± 0.11 ^{Ab}	5.62 ± 0.18 ^{Ab}
	***Pseudomonas* spp. count (log CFU/g)**		
5	6.37 ± 0.24 ^{Aa}	4.42 ± 0.10 ^{Ab}	3.90 ± 0.45 ^{Ab}
10	6.23 ± 0.20 ^{Aa}	4.49 ± 0.04 ^{Ab}	3.64 ± 0.09 ^{Ac}
15	6.18 ± 0.19 ^{Aa}	3.94 ± 0.23 ^{ABb}	< 3.48
20	6.02 ± 0.16 ^{Aa}	3.56 ± 0.25 ^{Bb}	< 3.48

Data are expressed as mean ± standard error of three replicate samples.

Means within the same column with different uppercase letters differ significantly ($p < 0.05$) across different time points

Means within the same row with different lowercase letters differ significantly ($p < 0.05$) between different treatments.

Table 7.5: The effects of water treatment (control) and citric acid treatment (5% and 10%) at varying exposure times (5, 10, 15, and 20 min) on the firmness (N) of fresh truffles.

		Citric acid treatment	
Exposure time (min)	**Water treatment (control)**	**5%**	**10%**
5	5.84 ± 0.27 ^{Aa}	5.82 ± 0.18 ^{Aa}	5.99 ± 0.37 ^{Aa}
10	5.78 ± 0.29 ^{Aa}	5.74 ± 0.07 ^{Aa}	5.86 ± 0.18 ^{Aa}
15	5.89 ± 0.10 ^{Aa}	5.78 ± 0.08 ^{Aa}	6.15 ± 0.52 ^{Aa}
20	5.76 ± 0.71 ^{Aa}	5.80 ± 0.14 ^{Aa}	5.90 ± 0.10 ^{Aa}

Data are expressed as mean ± standard error in Newton (N) unit of three replicate samples.

Means within the same column with different uppercase letters differ significantly ($p < 0.05$) across different time points.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$) between different treatments.

Table 7.6: The effects of water treatment (control) and citric acid treatment (5% and 10%) at varying exposure times (5, 10, 15, and 20 min) on the colour (L*, a*, b*) of fresh truffles.

Exposure time (min)	Colour coordinates	Water treatment (control)	Citric acid treatment	
			5%	10%
5	L*	21.63 ± 1.04 Aa	21.84 ± 0.13 Aa	22.55 ± 1.03 Aa
	a*	2.77 ± 0.26 Aa	2.21 ± 0.17 Aa	2.80 ± 0.23 Aa
	b*	5.88 ± 0.05 Aa	5.56 ± 0.25 Aa	5.36 ± 0.39 Aa
10	L*	22.27 ± 1.25 Aa	20.36 ± 0.92 Aa	21.26 ± 0.79 Aa
	a*	2.51 ± 0.11 Aa	1.90 ± 0.07 Ab	2.10 ± 0.12 Aab
	b*	6.31 ± 0.51 Aa	4.84 ± 0.37 Aa	4.84 ± 0.30 Aa
15	L*	21.86 ± 1.47 Aa	21.46 ± 0.57 Aa	21.52 ± 1.26 Aa
	a*	2.51 ± 0.07 Aa	2.17 ± 0.20 Aa	2.13 ± 0.19 Aa
	b*	5.87 ± 0.71 Aa	4.99 ± 0.25 Aa	4.68 ± 0.23 Aa
20	L*	22.12 ± 1.88 Aa	22.45 ± 1.16 Aa	21.56 ± 0.61 Aa
	a*	2.25 ± 0.21 Aa	2.49 ± 0.11 Aa	2.28 ± 0.06 Aa
	b*	6.52 ± 1.09 Aa	5.04 ± 0.43 Aa	5.08 ± 0.23 Aa

L* (dark to light), a* (green to red), b* (blue to yellow)

Data are expressed as mean ± standard error of three replicate samples.

Means within the same column with different uppercase letters differ significantly ($p < 0.05$) across different time points.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$) between different treatments.

7.3.3 Experiment 3: Key volatile profile of the preferred treatment

Aroma is one of the main determinants of the quality of fresh truffles apart from the firmness and colour (Phong et al., 2022c). A further investigation on the volatile profile of truffles treated with 10% citric acid for 15 min was conducted in experiment 3 as the selected treatment showed the highest microbial reduction, especially in the *Pseudomonas* spp. count (< 3.48 log CFU/g), while retaining the firmness and colour of the treated truffles in Experiment 2 (Section 7.3.2).

A total of 75 volatile compounds were selected as the compounds of interest. Given that not all volatiles contributes to the final aroma impression of truffles (Culleré et al., 2010; Culleré et al., 2013a; Schmidberger & Schieberle, 2017), ten key volatiles which are associated to those that humans perceive as the aroma in *T. melanosporum* (Culleré et al., 2010; Culleré et al., 2013a) were selected as the indicators for volatile profile evaluation in the present study.

The key volatile profile of truffles treated with water alone (control) and 10% citric acid for 15 min is shown in Table 7.7. Overall, the key volatile profile of the treated truffles did not significantly differ from the control. This suggests that citric acid treatment at 10% for 15 minutes could effectively reduce microbes without causing any distinctive changes to the volatile profile of truffles to achieve a better decontamination level for fresh truffles before any postharvest processing and thereby it may be possible to extend its shelf life.

Table 7.7: Relative percentages (%) of the key volatile profile of treated truffles after 15 min of exposure to 10% citric acid treatment.

Key volatiles	Aromatic descriptor	Relative percentage (%)	
		Water treatment	10% Citric acid treatment for 15 min
Methanethiol [$]	Cooked cabbage, vegetable	ND	ND
Dimethyl sulfide (DMS) [$]	Truffle, sulphur	0.1795 ± 0.0296 [a]	0.1127 ± 0.0304 [a]
Ethyl-3-methylbutanoate	Fruit, anise	1.1043 ± 0.0224 [a]	1.2127 ± 0.1013 [a]
Dimethyl disulfide (DMDS) [$]	Truffle, sulphur	0.0296 ± 0.0113 [a]	0.0202 ± 0.0013 [a]
Isoamyl alcohol	Cheese	26.5841 ± 5.447 [a]	22.5039 ± 1.7276 [a]
1-Octen-3-one	Mushroom	ND	ND
Dimethyl trisulfide (DMTS) [$]	Pungent	0.0142 ± 0.0002 [a]	0.0315 ± 0.009 [a]
1-Octen-3-ol	Mushroom, earth, mould	0.6536 ± 0.0766 [a]	0.4934 ± 0.1396 [a]
Methional	Boiled potatoes	0.257 ± 0.0379 [a]	0.1772 ± 0.0278 [a]
***p*-Cresol**	Phenolic/ leather	0.0202 ± 0.0013 [a]	0.0234 ± 0.0074 [a]

Data reported as the mean of triplicate measurements ± standard error.

ND: not detected

Values were expressed as relative percentages (%) with the total peak area of all analytes of interest being 100%.

Relative percentage (%) of each identified volatile: peak area of an individual component / total peak area of all components X 100%.

Footnote [$]: The selective mass chosen (m/z) for identifying methanethiol, DMS, DMDS, and DMTS was 47, 62, 94, and 126, respectively.

Means within the same row with different lowercase letters differ significantly ($p < 0.05$).

7.4 Conclusion

This study demonstrated the potential of a single-step washing with citric acid as a promising supplementation to simple cleaning procedure using water alone that have been commonly used in the truffle industry to achieve a better decontamination of the produce. Apart from its decontamination efficacy, other advantages such as low-cost, simplicity, non-thermal, free from chemical residue, and non-hazardous may facilitate the implementation of citric acid treatment in the truffle industry. The finding should be of interest to the truffle industry in controlling the microbial population of truffles at the postharvest stage. Future research needs to determine the possibility of this treatment to extend shelf life of fresh truffles from its current 14 days.

CHAPTER 8: General discussion and conclusion

The ultimate goal of this thesis was to turn the smaller, offcut or excessive unsold lower-priced truffles into a natural flavouring ingredient that would be available throughout the year for food application. This is important to prevent the highly perishable truffles from deterioration and losing aroma rapidly after harvest (Campo et al., 2017; Rivera et al., 2011b; Vahdatzadeh et al., 2019). A series of studies were performed to explore the potential application of extraction and encapsulation to achieve this notion.

8.1 General discussion

8.1.1 Microbial storage study of the whole, sliced, and freeze-dried truffles

Chapter 3 provides an understanding of the microbial changes of the whole, sliced, and freeze-dried truffles under vacuum packaging and cold storage over 30 days of storage period. The key finding of the study was that the total aerobic microbes, *Pseudomonas* spp., and yeast were presented at a much lower count in the freeze-dried truffles than the whole and sliced truffles. In particular, *Pseudomonas* spp. and yeasts which are known truffle spoilage agents were undetectable (< 2.00 log CFU/g) in the freeze-dried truffles at the end of storage. This implies that the removal of moisture content from truffles via freeze-drying did not favour the growth of these microbes. All truffle samples showed that mould, *Listeria* spp., and *Salmonella* spp. were below the detection limit (< 2.00 log CFU/g) except *Bacillus* spp. which were detected at a very low number (< 3.48 log CFU/g) in all sample types throughout the storage period. Nonetheless, the presence of *Bacillus* spp. in the current study was too low to cause any foodborne illness but more studies with a larger sample size are required to confirm this finding. Apart from this, we speculate that the risk of contracting any foodborne illness is very unlikely due to the presence of antimicrobial compounds in truffles that may react against the pathogens (Beara et al., 2014; Neggaz et al., 2018; Palacios et al., 2011; Schillaci et al., 2017; Tejedor-Calvo et al., 2021a) allowing the safe consumption of truffles. As there is no previous literature to support our speculation, more research to understand the role of antimicrobial compounds that are present in truffles may be helpful to confirm this. Overall, the survival of some microbes throughout storage highlights the importance of developing an effective decontamination treatment to control the microbial population in truffles before packaging and storage. The findings of this chapter are however limited due to a lack of information on the aroma quality of the whole, sliced, and freeze-dried truffles throughout storage. The volatile changes of truffles under vacuum-packaging and

refrigerator storage condition has been investigated previously but the focus of the study was only on fresh truffles with a storage period of up to 14 days (Choo et al., 2021). Since aroma is considered the main quality attribute that influences consumer acceptance and appreciation of truffles (Hall et al., 2017), it is therefore recommended that the impact of extended storage conditions up to 30 days on the aroma quality of the whole, sliced, and freeze-dried truffles should be investigated in future work. Investigating the synergy effect of combining an antimicrobial treatment with a preservation method to achieve a better microbial quality of truffles without compromising their aroma quality and extending their shelf life can also be an interesting topic for future work.

8.1.2 The production of a natural truffle flavouring ingredient using truffles as the raw materials

8.1.2.1 Extraction

The study in Chapter 4 advances our understanding of the impacts of extraction on the mass recovery, microbial quality, and key volatile profile of truffle extract derived from different extraction methods. The study concentrated on the ten key volatiles that are required to produce the black truffle aroma, which are methanethiol, DMS, ethyl-3-methylbutanoate, DMDS, isoamyl alcohol, 1-octen-3-one, DMTS, 1-octen-3-ol, methional, and *p*-cresol. These key volatiles were used as indicators to assess and compare the volatile profile of each extract obtained by different methods in this study. The recovery of different volatiles using different solvent types (ethanol, acetone, hexane, and liquefied butane) were first evaluated. Based on the results, ethanol was deemed the preferred solvent for Soxhlet extraction in the next phase of the study. A comparison between Soxhlet extraction and SC-CO_2 extraction in the subsequent study showed that the mass recovery of Soxhlet extract was significantly higher (6.62 ± 0.33 %) than the SC-CO_2 extract (2.49 ± 0.38 %). Despite a significant difference in the mass recovery, both extraction methods produced extracts that contained six (methanethiol, DMDS, isoamyl alcohol, DMTS, 1-octen-3-ol, and *p*-cresol) of the ten key volatiles. Our results were in agreement with Tejedor-Calvo et al. (2021b) who claimed that not all key volatile compounds identified in the truffles such as methional and ethyl-2-methylbutanoate were successfully recovered by SC-CO_2 extraction. Nonetheless, our finding was encouraging as the recovery of the six key volatiles in both extracts indicates that the natural extracts were more aromatically complex than the synthetic truffle aroma which typically contain a mixture of only two to five molecules (Wernig et al., 2018). It is believed that the natural truffle extract, although did not contain all of the key volatiles required to produce the

typical black truffle aroma, would still be able to provide a sufficiently similar aroma to satisfy truffle flavour craving when fresh truffles are unobtainable during the off-season and accepted by consumers. Evaluating the overall aroma impression and consumer acceptance of the extracts is therefore required in a future study to determine the preferred and acceptable extract from a consumer perspective (Andrewes et al., 2021; Fan et al., 2016).

Another encouraging result in the present study was that all the microbes tested in the extracts were below the detection limit (< 2.00 log CFU/g), showcasing the advantage of adopting a truffle aroma extraction process. The outcome suggests that the manufacture of a microbiologically safe truffle flavouring ingredient for food application is possible with extraction. Overall, this study provides insights into the factors that need to be considered when developing an extraction process for volatile extraction from truffles which can serve as a basis for future research. Unfortunately, there are still some limitations ahead that need to be overcome before extraction can be a viable option for industrial application. The limitations of the extraction include the removal of extractant from volatiles and not all ten key volatiles were successfully extracted under the current operating conditions in the present study. Hence, future studies to optimise the operating conditions should be carried out in order to obtain a truffle extract that closely resembles the actual truffle aroma with a maximal mass recovery. Integrating the current extraction methods with other technologies may also be needed to improve the current approach.

8.1.2.2 Encapsulation

Chapter 5 presents the promising application of encapsulation using β-CD to capture truffle volatiles for food application, which to the best of our knowledge has not been reported before in scientific literature yet. Fresh truffles were used as the source of guest molecules for the encapsulation study. In this study, three different encapsulation methods (M1, M2, and M3) were evaluated over 90 days of storage and the most preferred encapsulation method, namely the paste method (M3) was selected for comparison with freeze-drying (which has been the preferred preservation method among all the existing preservation techniques). It was observed that encapsulation could preserve the truffle volatiles for a longer period of time over freeze-drying, allowing the extended supply of truffle aroma for food product application. However, some microbes in all the encapsulated truffle products and freeze-dried truffles survived over 90 days of storage. This could be the key shortcoming in applying encapsulation to truffles. Hence, it is

suggested that incorporating a decontamination treatment for fresh truffles before postharvest processing should be a focus of future studies (please see Chapter 7). The knowledge obtained from this study opens an exciting avenue of research for applying encapsulation of truffle aroma as an attractive option to preserve the key volatiles of truffles. The findings of this study are however limited due to the lack of sensory data to assess the consumer acceptance of the encapsulation products compared to the freeze-dried truffles, which was therefore performed in the next study (please see Chapter 6). Consumer perception is essential for market success (Andrewes et al., 2021; Fan et al., 2016), thus conducting a sensory evaluation that is directly associated with consumer acceptance is highly recommended in a future study (please see Chapter 6) as the human sensory response can serve as a complementary to the instrumental analysis, aid in providing a comprehensive interpretation of the tested products (Lawless, 1991).

As stated previously in Chapter 5, a consumer sensory evaluation is required to evaluate the consumer acceptance of the truffle products obtained by encapsulation and freeze-drying. In Chapter 6, the impacts of freeze-drying and encapsulation on the key volatiles, consumer discrimination, and overall sensory impression of truffles was evaluated. After processing, there were some changes in the key volatile profile between products. Significant sensory differences between products in terms of consumer discrimination (triangle test) and aroma intensity (sensory ranking test) were also observed. Despite some volatile and sensory differences between truffle products, there were no significant differences in the consumers' preference and acceptance towards any of them. The overall finding showcases the potential value of processing truffles into a natural flavouring ingredient via freeze-drying or encapsulation. Based on the results of Chapter 5, all the detected key volatiles remained unchanged in the encapsulated product whereas three key volatiles showed a significant reduction in the freeze-dried truffles at the end of the storage study, due to which encapsulation may be the technique to pursue for commercial application. Since the finding was encouraging, a further study may be needed to evaluate the consumer's purchase interest after incorporating the processed truffle products onto food as a flavouring ingredient. This study also demonstrated the significance of conducting a consumer sensory evaluation which would be helpful in providing a complete profile of the tested products (Andrewes et al., 2021; Fan et al., 2016; Lawless, 1991).

8.1.3 Decontamination of truffles before postharvest processing

In Chapter 7, fresh truffles were treated with ozonated water (3 ppm and 6 ppm) and citric acid (5% and 10%) for 5, 10, 15, and 20 min, respectively. The same procedures were repeated using sterile water alone (control treatment) to mimic the common industry practice. Subsequently, the effects of each treatment on microbial populations (total aerobic microbes, total anaerobic microbes, and *Pseudomonas* spp.), firmness, and colour of truffles were assessed. Of all the treatments, 10% citric acid for 15 min was deemed the preferred decontamination method based on the microbial, firmness, and colour analyses. The subsequent volatile analysis showed that the key volatile profile of truffles treated with this method did not significantly differ from the untreated truffles. This study showcases the potential application of a single-step washing with citric acid to achieve a better decontamination level for fresh truffles compared to the simple water cleaning procedure that has commonly been used in the industry prior to any postharvest processing. Although this study was performed at a laboratory scale, some potential advantages of this method such as simplicity, non-toxic, and low cost (Behera et al., 2021; Mani-López et al., 2012) suggest that its application on a commercial scale would be feasible and easily adopted. Since the outcome was encouraging, further study to optimise the treatment parameters is required before its application on a commercial scale. There is a need to conduct a consumer sensory evaluation to determine if there are any organoleptic and sensory differences from a consumer perspective between the untreated and treated truffles in a future investigation. In addition, only one truffle species was investigated in the present study. Therefore, it would be interesting to study the effects of this treatment on other truffle species so as to understand how effective this method can be at decontaminating other truffle species. In view of the microbial population of fresh truffles can be controlled at the postharvest stage using a single-step washing with citric acid, determining the possibility of this treatment to extend the shelf life of fresh truffles can be another interesting topic to explore in the future.

8.2 General conclusion

Considering the findings presented in the entire thesis showcase the feasibility and potential advantages of processing the highly perishable truffles into a natural truffle flavouring ingredient that would benefit both the truffle and food industries. Truffle spoilage and undesirable aroma changes could be avoided or mitigated by turning truffles into aroma extract or encapsulated material which can be used as a high-value flavouring

ingredient that is available all year round. Processing of truffles via extraction and encapsulation would be an ideal approach to add value to the smaller, offcut or surplus truffles as a result of the rapid growth in truffle cultivation, maximising the truffle growers' profit. The readily available natural truffle flavouring ingredient would lead to the development of new truffle-flavoured food products, benefiting both truffle and food sectors with new business opportunities. The production of a value-added natural flavouring ingredient from truffles holds commercial potential as it could help strengthen the position of Australia as one of the leading truffle producers and exporters in the world.

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Appendix A – Truffle volatiles

Table S1: List of volatile compounds identified in *T. melanosporum*

No.	Retention time (min)	Compound Name
1	1.754	Methanethiol
2	1.813	Acetaldehyde
3	1.985	Dimethyl sulfide
4	2.211	Propanal
5	2.353	2-Methyl propanal
6	2.383	Acetone
7	2.982	Butanal
8	3.045	Methacrolein
9	3.144	Ethyl acetate
10	3.320	2-Butanone
11	3.507	2-Methylbutanal
12	3.625	3-Methylbutanal
13	3.993	Ethanol
14	5.550	Decane
15	5.908	Methyl-2-methylbutanoate
16	6.242	Methyl-3-methylbutanoate
17	6.488	2-Butanol
18	6.851	2-Butenal
19	6.915	1-Propanol
20	7.308	Ethyl-2-methylbutanoate
21	7.833	Ethyl-3-methylbutanoate
22	7.882	Dimethyl disulfide

23	8.236	Hexanal
24	8.560	2-Methyl-2-butenal
25	9.119	2-Methyl-1-propanol
26	9.458	2-Methyl-1-butanol acetate
27	9.910	Propyl 2-methylbutanoate
28	10.975	1-Butanol
29	11.064	2-Methylpropyl-2-methylbutanoate
30	11.285	D-limonene
31	11.452	Heptanal
32	11.535	Methyl hexanoate
33	11.594	2-Methylpropyl-3-methylbutyrate
34	11.732	2-Methylbutyl-2-methylpropanoate
35	12.792	Isoamyl alcohol
36	12.807	2-Pentylfuran
37	13.033	Ethyl hexanoate
38	13.656	3-Octanone
39	14.437	2-Methylbutyl-2-methylbutanoate
40	14.658	2-Octanone
41	14.766	Octanal
42	14.972	2-Methylbutyl-3-methylbutanoate
43	15.149	1-Octen-3-one
44	15.778	2-Heptenal
45	15.945	2,3-Octanedione
46	16.053	2-Ethyl-2-hexenal
47	16.377	Anisole/ Methoxybenzene
48	16.720	2-Isopropyl-5-methylhex-2-enal

49	16.804	1-Hexanol
50	17.305	Dimethyl trisulfide
51	17.737	Nonanal
52	17.904	3-Octanol
53	18.709	2-Octenal
54	19.161	1-Methyoxy-3-methylbenzene
55	19.446	1-Octen-3-ol
56	19.529	Methional / Propanal, 3-(methylthio)-
57	21.213	Benzaldehyde
58	21.277	1-Ethyl-4-methoxybenzene / 4-ethylanisol
59	22.082	1-Octanol
60	23.015	2-Undecanone
61	23.295	5-Methyl-2-propylphenol
62	24.066	Alpha-methyl benzeneacetaldehyde
63	24.174	Benzeneacetaldehyde
64	24.282	Acetophenone
65	25.858	3-(Methylthio)-1-propanol / methionol
66	26.089	1,2-Dimethoxybenzene
67	26.561	1,3-Dimethoxybenzene
68	27.611	2,5-Dimethoxytoluene
69	27.867	3,4-Dimethoxytoluene
70	30.037	Phenylethanol / phenylethyl alcohol
71	30.420	Alpha-Ethylidene-phenylacetaldehyde
72	32.040	Phenol
73	33.243	5-Methyl-2-phenyl-2-hexenal
74	33.661	*p*-Cresol

75	33.725	1,2,3-Trimethoxy-5-methylbenzene

Appendix B – Sensory panel screening questionnaire

Truffle Aroma Sensory Evaluation Curtin University

SENSORY PANEL SCREENING QUESTIONNAIRE

Personal Information

Name:________________________________

Contact number / e-mail address (for confirmation of evaluation time only):

____

Gender : Male ____ Female ____ Other: _______________________

Availability

1. Availability for sensory evaluation: please indicate your preferred day and time

	Preferred time (between 10 am and 4pm)
Monday	
Tuesday	
Wednesday	
Thursday	
Friday	

2. Please indicate any days on which you would **not** be available on a regular basis if you are selected to participate in sensory evaluation.

Monday	
Tuesday	
Wednesday	
Thursday	
Friday	

3. Is there any period that you would be unavailable for an extended period of time between July and August 2021?

____

Health Details

		Yes	No
1.	Are you over the age range of 18?		
2.	Are you allergic to truffle?		
3.	Are you allergic to any other food item?		
4.	Do you have frequent head colds?		
5.	Do you have nasal disease?		
6.	Are you currently pregnant? (female only)		
7.	Are you currently breast feeding? (female only)		
8.	Are you a smoker? If yes, how many cigarettes per day would you smoke? ____________		
9.	Do you take any medication that will affect your senses, especially your sense of smell or taste?		

Food Habits

1. Have you ever smelt truffles in the past one year? Yes / No

____

2. Have you purchased or consumed truffles or truffle products in the past one year?

____

3. Please give three words that you would associate with the aroma of mushrooms or truffles.

____

For questions or more information, you may contact any of the following:

Miss Win Nee Phong (Student researcher)
Contact Number : +61 474449699
Email address : winnee.phong@postgrad.curtin.edu.au

Associate Professor Ranil Coorey (Chief Investigator)
Contact Number : +61 8 9266 1043
Email address : R.Coorey@curtin.edu.au

Ethics officer or Manager of Research Integrity
Contact Number : +61 8 9266 9223 or +61 8 9266 7093
Email address : ORD-ethicshs@curtin.edu.au

Curtin University Human Research Ethics Committee (HREC) has approved this study (HREC number HRE2019-0374). Should you wish to discuss the study with someone not directly involved, in particular, any matters concerning the conduct of the study or your rights as a participant, or you wish to make a confidential complaint, you may contact the Ethics Officer on (08) 9266 9223 or the Manager, Research Integrity on (08) 9266 7093 or email hrec@curtin.edu.au.

Thanks for your participation!

Appendix C – Participant information statement

Truffle Sensory Evaluation

PARTICIPANT INFORMATION STATEMENT

HREC Project Number:	HRE2019-0374
Project Title:	Extraction of the volatile compounds from Black Périgord truffle (*Tuber melanosporum*) for innovative value added products
Chief Investigator:	Associate Professor Ranil Coorey
Student researcher:	Miss Win Nee Phong
Collaborator:	Associate Professor Billy Sung
Version Number:	2.0
Version Date:	18/05/2021

What is the project about?

Two types of sensory testing (triangle test and consumer test) will be conducted in this study. Fresh black Périgord truffles are supplied by the Truffle Producers Western Australia Inc. (TPWA). The triangle test aims to determine whether a sensory difference exists between the two samples. The overall liking and acceptability of the truffle samples will be determined by the consumer test. These two tests will be provided to the test subjects separately. The panel will use the sensory evaluation facilities at Curtin University, Bentley.

Who is doing the research?

The project is being conducted by Associate Professor Ranil Coorey, Miss Win Nee Phong with the help of Associate Professor Billy Sung (collaborator). The results of this research project will be used by Win Nee Phong to obtain her Doctor of Philosophy at Curtin University. The project is funded by Truffle Producers Western Australia Inc. (TPWA).

Why am I being asked to take part and what will I have to do?

Prior to the commencement of the sensory evaluation, you are required to complete an anonymous pre-screening questionnaire. To be eligible to take part in this sensory evaluation, you must be over 18 years old without any known food allergies or intolerances, neither currently pregnant nor currently breastfeeding.

For the triangle test, you will be asked to evaluate 6 sets of three truffle aroma samples. In each set, you will be receiving a set of three different randomly coded samples. You will be asked to sniff the samples (please DO NOT consume the sample). Your task is to pick the sample which is different from the other two, or the odd one out in each set and record your answer during the sensory evaluation.

With regard to the consumer acceptance test, you will be asked to sniff the samples and assess the intensity of the aroma in general, overall liking, and overall acceptability of the samples by completing a sensory questionnaire wherein you are required to give a score to each sample (4 in total) based on the linear hedonic scale.

This study will be conducted at the Sensory Room located on Level 1 of Building 400 (School of Public Health building) in Curtin University. This room has six individual booths with a small window. You may choose any booth that you want to do the evaluation in. The samples, along with the questionnaire, will be given to you through the window. After completing the evaluation, you will return the questionnaire through the same window.

You will only need to complete the questionnaire once, and the questionnaire will be collected from you when you have completed the sensory evaluation. The sensory evaluation will take approximately 60 minutes to complete. Taking part in this study will not incur any costs to you, and you will be paid for participating in the sensory study.

Are there any benefits from being in the study?

There may be no direct benefit to you from being in this study. However, your inputs will allow us to assess the overall acceptability of the truffle samples from a consumer perspective. The results of this project may contribute to the stable and sustainable growth of the Australian truffle industry.

Are there any risks, side-effects, discomforts or inconveniences from being in the study?

The samples you will test contain Black Périgord truffles (frozen and freeze-dried) as well as food-grade edible encapsulating agent namely β-cyclodextrin (please DO NOT consume the samples). Please inform the researcher if you are allergic to the Black Périgord truffle and β-cyclodextrin so that you may be exempted as a participant in this study. Also, if you are allergic to any other food, kindly inform the researcher.

The sensory evaluation will be performed in a sensory room within Building 400 in Curtin University. Participants will be required to travel to the location in Curtin University at a scheduled date and time to take part in the sensory evaluation.

Who will have access to my information?

Some of the information collected from you will be identifiable while some will not be identifiable. Identifiable information is any information that can identify you. This includes information you provide on the consent form and on the screening questionnaire. Non-identifiable information, on the other hand, is anonymous information. This includes information you provide on the sensory questionnaire during the actual sensory evaluation. No one, not even the research team will be able to identify you based on this information. The collated information may be used in scientific peer-reviewed publications. All the information collected during this study including the identifiable and non-identifiable information will only be used in the research project and will be treated as confidential.

All electronic information will be stored within the secure network at Curtin University, protected with a password and properly backed-up. Whereas, all hard copies of the information will be stored securely in locked storage at the Curtin University. It is Curtin University's policy to securely keep all the collected information for seven years and then it will be destroyed. The research team will have access to the information we collect from the sensory evaluation. The Curtin Ethics Office may access the information for audit purposes.

The results of this sensory evaluation may be published in peer-reviewed journals and you will not be identifiable in any results published.

Will the results of the study be disclosed to me?

You will not be informed of any results from this study. However, the collated results of this study may be published in peer reviewed journal or presented at conference. Nonetheless, you will not be identified in any results that will be published or presented.

Do I have to take part in this study?

Taking part in this study is totally voluntary and is entirely up to you. You are not forced to take part in this study if you do not want to. If you decide to take part but change your mind later on, you can withdraw from this study at any time, without any consequences to your employment or studies. You

are not required to provide us with the reasons for your withdrawal. Kindly inform us if you wish to withdraw from the study as we would like to ensure that you are aware of anything that needs to be done so that you can withdraw safely. Not taking part in this study or withdrawing from the study at any time will not affect your relationship with the University, staffs, colleagues or students in any manner.

If you choose to withdraw from the study, we will continue to use any information that has been collected. As your information will be collected in an anonymous way, we would not be able to destroy them.

What is next and who can I contact about the research?

Any further information, please contact:

Miss Win Nee Phong

Contact Number: +61 474449699

Email Address: winnee.phong@postgrad.curtin.edu.au

Or

Associate Professor Ranil Coorey (Research Supervisor)

Contact Number: +61 8 9266 1043

Email Address: R.Coorey@curtin.edu.au

If you decide to take part in this study, you will be asked to sign a consent form. Signing the consent form indicates that you understand what you have read, and that you agree to be in the study without any known food allergies or intolerances, neither currently pregnant nor currently breastfeeding. Please take your time and ask any questions that you may have before deciding what to do. If you wish, you will be given a copy of this information sheet and the consent form to keep.

Curtin University Human Research Ethics Committee (HREC) has approved this study (HREC number HRE2019-0374). Should you wish to discuss the study with someone not directly involved, in particular, any matters concerning the conduct of the study or your rights as a participant, or you wish to make a confidential complaint, you may contact the Ethics Officer on (08) 9266 9223 or the Manager, Research Integrity on (08) 9266 7093 or email hrec@curtin.edu.au.

Appendix D – Consent form

Truffle Sensory Evaluation

CONSENT FORM

HREC Project Number:	HRE2019-0374
Project Title:	Extraction of the volatile compounds from Black Périgord truffle (*Tuber melanosporum*) for innovative value added products
Chief Investigator:	Associate Professor Ranil Coorey
Student researcher:	Miss Win Nee Phong
Collaborator:	Associate Professor Billy Sung
Version Number:	2.0
Version Date:	18/05/2021

- I have read the information statement and I understand its contents.
- I believe I understand the purpose, extent and possible risks of my involvement in this project.
- I am over 18 years old.
- I do not have any food allergies or intolerances.
- I am not pregnant or breast feeding.
- I understand that participants will not be identified in any manner nor the organisation itself by name, address or any such method.
- I understand the confidentiality of the information I provide will be safeguarded and will not be distributed to any other agency.
- I understand that participation is voluntary and that I can withdraw at any time with no prejudice or negative impacts and without penalty.
- I have had the opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that this project has been approved by Curtin University Human Research Ethics Committee and will be carried out in line with the National Statement on Ethical Conduct in Human Research (2007) – updated March 2014.
- I voluntarily consent to participate in this study.
- I understand I will receive a copy of this Information Statement and Consent Form.
- I hereby acknowledge the above and give my voluntary consent for participation in this project.

Participant Name	
Participant Signature	
Date	

Declaration by researcher: I have supplied an Information Letter and Consent Form to the participant who has signed above, and believe that they understand the purpose, extent and possible risks of their involvement in this project.

Researcher Name	
Researcher Signature	
Date	

Note: All parties signing the Consent Form must date their own signature.

Curtin University Human Research Ethics Committee (HREC) has approved this study (HREC number HRE2019-0374). Should you wish to discuss the study with someone not directly involved, in particular any matters concerning the conduct of the study or your rights as a participant, or you wish to make a confidential complaint, you may contact the Ethics Officer on (08) 9266 9223 or the Manager, Research Integrity on (08) 9266 7093 or email hrec@curtin.edu.au.

Appendix E – Sensory evaluation questionnaire

SENSORY EVALUATION QUESTIONNAIRE FOR TRUFFLE SAMPLES

Assessor No: _______________

Date: __.07.2021 **Time:** 10-11am / 11-12pm / 12-1pm / 2-3pm / 3-4pm

Instructions: Triangle test

1. In total, six (6) sets of samples (set 1 to set 6) will be presented to you, separately.
2. For each set, you will be presented with three (3) randomly coded samples. Two samples are alike; one is different. You need to pick the one sample that differs from the other two after sniffing (answer the questions).
3. Please DO NOT eat the samples.
4. You should sniff the samples from left to right.
5. First, please open the first sample bottle, sniff the sample, and tighten up the lid after sniffing to reduce the loss of aroma over time. Please DO NOT keep the bottle open.
6. Next, please sniff a glass bottle filled with **sliced lemon** for about 5 seconds and breathe normally for approximately 10-15 seconds before sniffing the next sample. Repeat the same between samples.
7. Answer the question after sniffing the three (3) samples.
8. After you have answered the first question, please slide the samples through the window.
9. The second set of samples will be presented to you.
10. Repeat step 4 to step 8 until you have answered all the six (6) questions.
11. After completing the evaluation, please slide the questionnaires through the window along with the samples.

Question 1 (Set 1)

Assess samples from left to right. Two samples are alike; one is different. Write the number of the sample that differs from the others in the space below. (Please tighten up the lid after sniffing each sample and sniff **sliced lemon** between samples to calibrate your olfactory sense)

The one sample that differs from the other two is: ________________ (sample code in three digits)

If you are not sure, record your best guess; you may note under **remarks** that you were **guessing**.
Remarks (if any): ____

Question 2 (Set 2)

Assess samples from left to right. Two samples are alike; one is different. Write the number of the sample that differs from the others in the space below. (Please tighten up the lid after sniffing each sample and sniff **sliced lemon** between samples to calibrate your olfactory sense)

The one sample that differs from the other two is: ________________ (sample code in three digits)

If you are not sure, record your best guess; you may note under **remarks** that you were **guessing**.
Remarks (if any): ____

Question 3 (Set 3)

Assess samples from left to right. Two samples are alike; one is different. Write the number of the sample that differs from the others in the space below. (Please tighten up the lid after sniffing each sample and sniff **sliced lemon** between samples to calibrate your olfactory sense)

The one sample that differs from the other two is: _______________ (sample code in three digits)

If you are not sure, record your best guess; you may note under **remarks** that you were **guessing**.
Remarks (if any): ____

Question 4 (Set 4)

Assess samples from left to right. Two samples are alike; one is different. Write the number of the sample that differs from the others in the space below. (Please tighten up the lid after sniffing each sample and sniff **sliced lemon** between samples to calibrate your olfactory sense)

The one sample that differs from the other two is: _______________ (sample code in three digits)

If you are not sure, record your best guess; you may note under **remarks** that you were **guessing**.
Remarks (if any): ____

Question 5 (Set 5)

Assess samples from left to right. Two samples are alike; one is different. Write the number of the sample that differs from the others in the space below. (Please tighten up the lid after sniffing each sample and sniff **sliced lemon** between samples to calibrate your olfactory sense)

The one sample that differs from the other two is: _______________ (sample code in three digits)

If you are not sure, record your best guess; you may note under **remarks** that you were **guessing**.
Remarks (if any): ____

Question 6 (Set 6)

Assess samples from left to right. Two samples are alike; one is different. Write the number of the sample that differs from the others in the space below. (Please tighten up the lid after sniffing each sample and sniff **sliced lemon** between samples to calibrate your olfactory sense)

The one sample that differs from the other two is: _______________ (sample code in three digits)

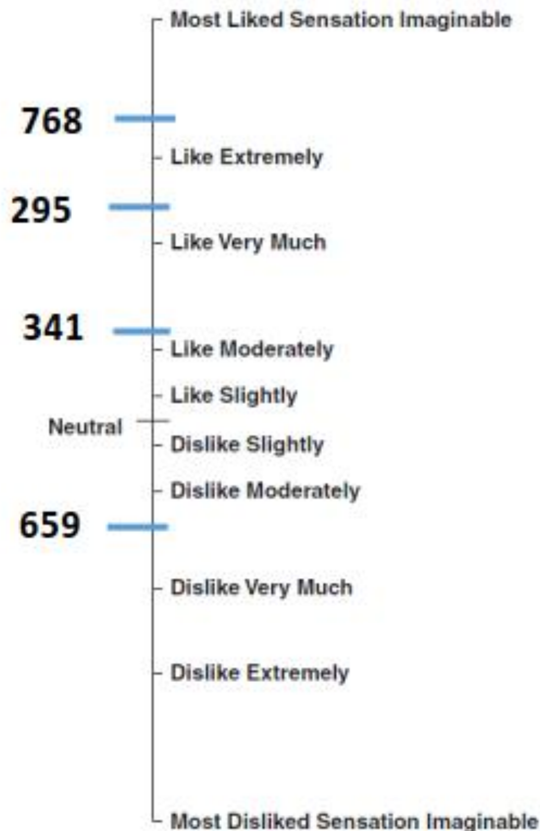
If you are not sure, record your best guess; you may note under **remarks** that you were **guessing**.
Remarks (if any): ____

Assessor No: ______________

Date: __.07.2021 **Time:** 10-11am / 11-12pm / 12-1pm / 2-3pm / 3-4pm

Instructions: Consumer acceptance test

1. In total, five (5) sets of samples will be presented to you, separately.
2. For each set, you will be presented with four (4) randomly coded samples.
3. Please DO NOT eat the samples.
4. You should sniff the samples from left to right.
5. First, please open the first sample bottle, sniff the sample, and tighten up the lid after sniffing to reduce the loss of aroma over time. Please DO NOT keep the bottle open.
6. Next, please sniff a glass bottle filled with **sliced lemon** for about 5 seconds and breathe normally for approximately 10-15 seconds before sniffing the next sample. Repeat the same between samples.
7. Answer the question after sniffing the four (4) samples.
8. After you have answered the first question, please slide the samples through the window.
9. The second set of samples will be presented to you.
10. Repeat step 4 to step 8 until you have answered all the five (5) questions.
11. **Mark a horizontal line** on the scale which corresponds to your degree of sensation with mentioning the **sample code (in three digits)**, **example** as follows:



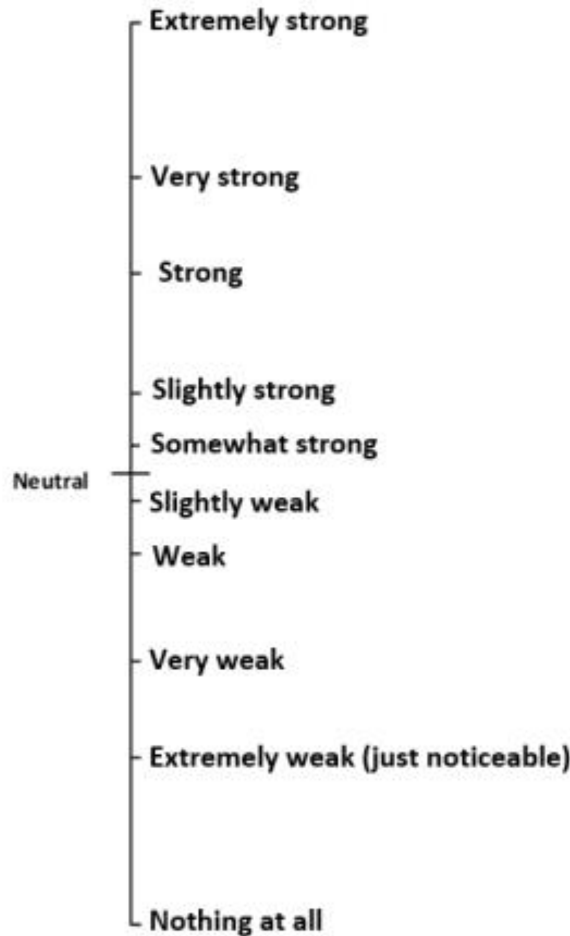
CRICOS Provider Code 00301J (WA)

Question 7

How would you rate the **intensity** of the aroma in general?

(Please tighten up the lid after sniffing each sample and sniff **sliced lemon** between samples to calibrate your olfactory sense)

*Please **make a mark "anywhere" on the line** corresponding to your degree of sensation with mentioning the **sample code** (in three digits) for each sample, as per example on page 3.

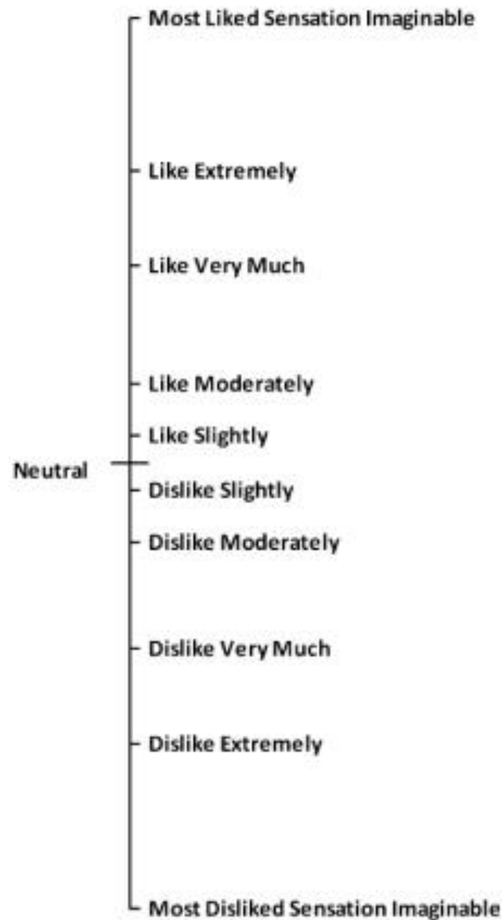


Remarks (if any): ____

Question 8

How would you rate the **overall liking** of the samples?
(Please tighten up the lid after sniffing each sample and sniff **sliced lemon** between samples to calibrate your olfactory sense)

*Please **make a mark "anywhere" on the line** corresponding to your degree of sensation with mentioning the **sample code** (in three digits) for each sample, as per example on page 3.



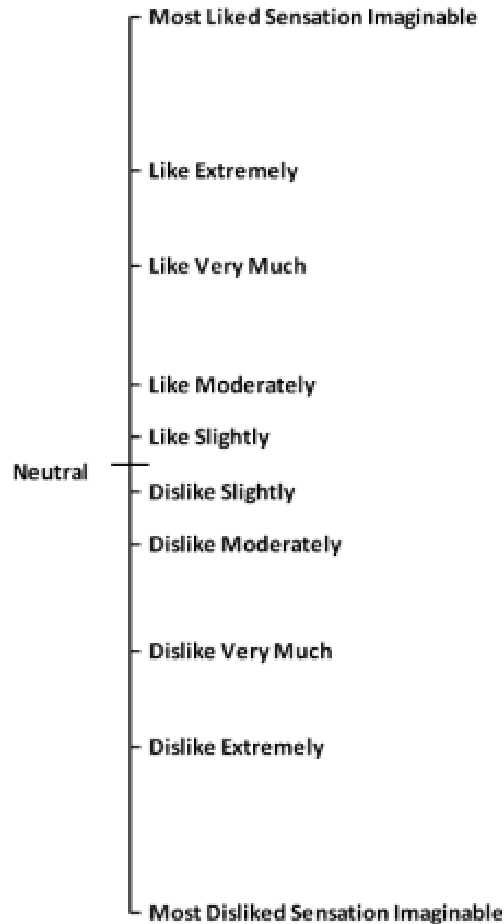
Remarks (if any): ____

CRICOS Provider Code 00301J (WA)

Question 9

How would you rate the **overall acceptability** of the samples?
(Please tighten up the lid after sniffing each sample and sniff **sliced lemon** between samples to calibrate your olfactory sense)

*Please **make a mark "anywhere" on the line** corresponding to your degree of sensation with mentioning the **sample code** (in three digits) for each sample, as per example on page 3.



Remarks (if any): ____

CRICOS Provider Code 00301J (WA)

Question 10

Out of the four (4) samples, which one do you **prefer the most**?

(Please tighten up the lid after sniffing each sample and sniff **sliced lemon** between samples to calibrate your olfactory sense)

Sample code in three digits ____

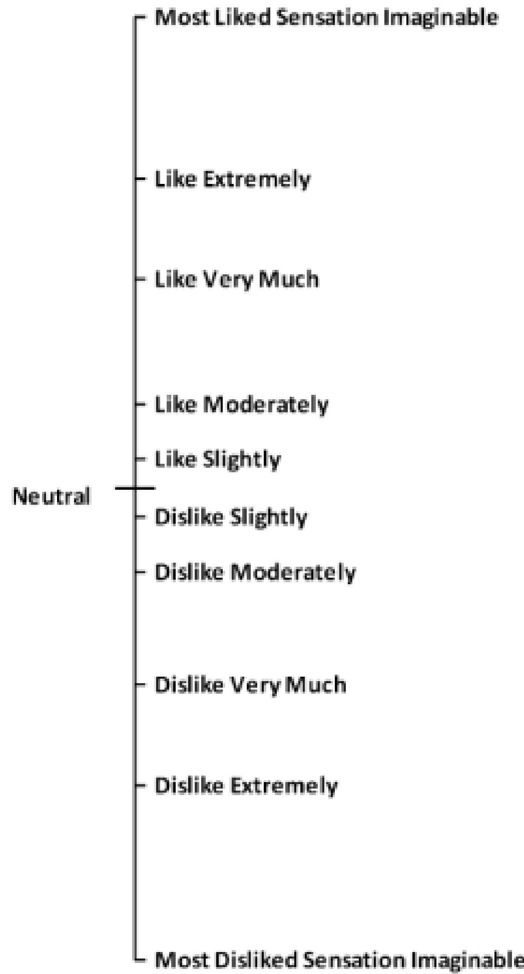
Remarks (if any): ____

CRICOS Provider Code 00301J (WA)

Question 11

How would you rate the **truffle aroma**?
(Please tighten up the lid after sniffing each sample and sniff **sliced lemon** between samples to calibrate your olfactory sense)

*Please **make a mark "anywhere" on the line** corresponding to your degree of sensation with mentioning the **sample code** (in three digits) for each sample, as per example on page 3.



Remarks (if any): ____

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