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Comparative evaluation of encapsulation using β-cyclodextrin versus freeze-drying for better retention and stabilizing of black Périgord truffle (*Tuber melanosporum*) aroma

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Abstract: This study aimed to develop a novel technique to retain and stabilize compounds contributing to truffle aroma by encapsulation using β-cyclodextrin. Two experiments were conducted. In the first experiment, the key volatile profile and microbial population of products resulting from three different encapsulation methods, namely direct mixing method (M1), direct mixing followed by ethanol addition method (M2), and paste method (M3), were compared with untreated truffles (positive control) over a 90-day period. The M2-derived product was the least optimal for 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 the M3-derived product could retain its volatile profile better than the 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 the 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 the volatile stability of truffle products and allow for longer storage times. Microbes were found in all encapsulated truffle products and freeze-dried truffles on days 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, encapsulation, truffle aroma retention and stability, volatile compounds, β-cyclodextrin

Practical Application: A technique to capture and stabilize compounds responsible for truffle aroma by encapsulation using β-cyclodextrin was developed and compared with freeze-drying in this study. The overall finding suggests that while freeze-drying of truffle could sufficiently preserve volatiles, encapsulating truffle volatiles with β-cyclodextrin may improve its stability, extending its shelf life, which can be applied in the development of a natural truffle ingredient that can be applied in food product development.

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., 2014; Zambonelli et al., 2016). For this reason, artificial truffle flavor has been used extensively in the food industry (Cataldo et al., 2016; Lukin et al., 2018). There are consumer concerns over the consumption of artificially flavored food products, and thus there is value in providing a more complex and naturally derived product (Phong, Gibberd, et al., 2022; 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, Gibberd, et al., 2022). Long-term preservation of truffles is essential to extend the supply. However, improving the shelf life of fresh truffles for long-term storage while safeguarding their sensory qualities is challenging (Rivera et al., 2011). 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; W. Zhu & Cadwallader, 2019). Developing a mild processing method to retain and stabilize truffle aroma before fresh truffles deteriorate and produce off-flavor is therefore imperative (Campo et al., 2017; Culleré et al., 2013; Phong, Gibberd, et al., 2022). Furthermore, the current truffle grading standards are based on size and physical appearance rather than aroma quality (TheAustralianTruffleGrowersAssociation, 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, Gibberd, et al., 2022).

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). Although it may lead to some volatile changes and a reduction in the aroma intensity (Campo et al., 2017; Palacios et al., 2014), freeze-drying appears to be the most preferred preservation method for truffles compared to canning, hot air drying, and freezing at −80°C (Campo et al., 2017; Marco et al., 2016).

On the other hand, encapsulation has shown promise in retaining the aroma and increasing the shelf life of food products by inhibiting volatilization of aroma compounds and preventing off-flavor 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 flavoring ingredients (Zuidam & Heinrich, 2010). Among these materials, cyclodextrins (CDs) are commonly used as carriers and stabilizers for flavors in the food industry (Marques, 2010; Zuidam & Heinrich, 2010). Numerous investigations have demonstrated the potential of using cyclodextrin as a vehicle to encapsulate various types of flavors and volatile compounds for use as a food ingredient, such as oregano (*Origanum onites* L.) essential oil (Kotronia et al., 2017), sweet orange flavor (G. Zhu et al., 2014), and strawberry flavor (Balci-Torun & Ozdemir, 2021). However, research on capturing the aroma volatiles of truffles via encapsulation has yet to be reported in the literature. We hypothesized that encapsulation could be a promising technique to capture truffle aroma before fresh truffles deteriorate, thereby achieving longer shelf stability and allowing natural truffle aroma to be readily available for food application throughout the year. Furthermore, the production of a natural flavoring 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 the 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 truffles using β-CD as an encapsulating material to produce a natural flavoring 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.

2 | MATERIALS AND METHODS

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 mature truffles harvested from the Manjimup region of Western Australia in August 2020. No direct comparison was made between experiments 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., 2013). The cleaned truffles were packed in an insulated container held at 4 ± 2°C (Rivera et al., 2010) and immediately shipped to our laboratory (approximately 7 days for experiment 1 and 3 days for experiment 2).

2.2 | Starting material preparation: Crushed truffles for experiments 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 had 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). The selected truffles were crushed in a mortar and pestle and mixed well to reduce possible natural variation in the volatile profile and microbial community (interspecies), which may be due to the growing conditions and natural habitat (Strojnik et al., 2020; Torregiani et al., 2017). The homogenized 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-dried, and M3 were assessed in triplicate) for experiment 2. There was 100 g of mixture in each portion, and all of them were separately vacuum-packaged into individual 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 needed (experiments were conducted within the same day of starting material preparation).

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 2.2). Untreated truffles (starting material) served as the positive control. Three sets of negative controls (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 (Sections 2.5 and 2.6). Then, the control sets and encapsulated products were separately vacuum-packed and stored at 4 ± 2°C until needed, and the same analyses (Sections 2.5 and 2.6) were repeated after storage for 90 days. The formulation of the three encapsulation methods is summarized in Table 1.

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 and 0.37 mbar until constant weight. The dried complex was then ground into a fine powder using a

TABLE 1 Truffle-β-cyclodextrin (CD) complexes derived from M1, M2, and M3

Encapsulation method	Truffles (g)	β-CD (g)	Deionized 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

Note: C1*, C2*, C3*: Negative control for each method (without adding truffles).

mortar and pestle. The controls (C1) were prepared in the same manner, except no truffles were added.

2.3.2 | Direct mixing followed by ethanol addition method (M2)

Analytical reagent grade 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 homogenized 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 and 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.

2.3.3 | Paste method (M3)

To 100 g of β-CD, 100 ml of deionized water was added and mixed to form a paste before adding 100 g of crushed truffles. Next, the mixture was homogenized 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 and 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.

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. According to previous studies, freeze-drying is regarded as the preferred method for 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 (Sections 2.5 and 2.6). The balance of each product was separately vacuum-packed and stored at 4 ± 2°C until subsampling for volatile and microbial analyses (Sections 2.5 and 2.6) after 90 days of storage.

2.4.1 | Paste method (M3)

The same procedures as in Section 2.3.3 were performed to obtain the powdered complexes (M3) and their corresponding controls (C3).

2.4.2 | Freeze-drying

Crushed truffles were freeze-dried at 0.37 mbar and −30°C using a freeze drier (Christ Alpha 1–2 LD plus; Martin Christ Gefriertrocknungsanlagen GmbH) until the weight was constant (approximately 48 h).

2.5 | Volatiles analysis

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

2.5.1 | Headspace sampling by SPME

A flexible fused silica fiber 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 fiber was conditioned based on the manufacturer's instructions prior to use (Supelco) (Díaz et al., 2009). For the untreated truffles (positive control) analysis, approximately 1 g of crushed truffles (containing approximately 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 a moisture content similar to that of 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., 2017) 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 fiber) 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. Afterward, the fiber 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 analytes 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 that no carryover occurred during chromatography analysis (Splivallo et al., 2012).

2.5.2 | GC-MS analysis

The volatile compounds were analyzed by GC-MS according to the method described by Dıáz et al. (2003), with some modifications. An Agilent-6890 GC system coupled to a GC 5973 mass spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a 30 m (length) × 250 μm (diameter) × 0.25 μm (film thickness) i.d. An 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 electron ionization (EI) mode. Data acquisition was performed in scanning mode in the range of 40–250 m/z at 0.46 s/scan (3 μ-scans) after a 1.5 min 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 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 and the Kovats retention indices (RI) were calculated based on a series of n-alkanes under the same chromatographic conditions (Wernig et al., 2018).

The percentage composition of each identified compound was determined based on peak area normalization by integration. The relative number of individual compounds was expressed as a 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 coelution with other peaks with similar retention time, making the integration rather difficult.

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, 2013; Feng et al., 2019; Mustafa et al., 2020; Schmidberger & Schieberle, 2017; Strojnik et al., 2020). Therefore, 10 key volatile constituents (Table 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, 2013) and used as indicators to evaluate the volatile profile of each truffle product processed by different methods in the current study.

2.6 | Microbiological analysis

Each sample (1 g) was homogenized in 9 ml of sterile buffered peptone water for 2 min using a homogenizer (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

TABLE 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

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

on polymyxin egg yolk mannitol bromothymol blue agar (PEMBA) (Oxoid Ltd) after incubation for 24 h at 37 ± 1°C (Phong, Chang, et al., 2022).

To detect the presence of *Salmonella* spp., 1 ml of each sample from the first decimal dilution (10^{-1}) was inoculated into 9 ml of mannitol selenite cystine (MSC) and Rappaport-Vassiliadis (RV) enrichment broths (Oxoid Ltd). 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-deoxycholate 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 was 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, Chang, et al., 2022).

After incubation, microbial colonies that grew on TSA, *Pseudomonas*, and PEMBA medium were counted. Only counts of 30–300 colony forming units (CFU) were considered, and each count (expressed as log CFU/g) was calculated based on the mean of triplicate. A presence or absence was reported for *Salmonella* spp. and *Listeria* spp. Characterization 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).

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 was conducted on these samples after 90 days of storage.

2.7 | Data analysis

All experiments were performed in triplicate, and the data were reported as the mean ± standard error and statistically analyzed using IBM SPSS version 26 software. One-way analysis of variance 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. A paired samples *t*-test was used to compare two means that were from the same group at two different time points (days 0 and 90). Differences with $p < 0.05$ (95% confidence level) were considered statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | Key volatile compound analysis

As mentioned in Section 2.5.3, 10 key volatile compounds were selected as indicators to evaluate the encapsulation methods (Table 2) (Culleré et al., 2010, 2013). In the present study, nine key volatiles except 1-octen-3-one were detected in the untreated truffles (Tables 3 and 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 impact of the growing location, environmental factor, and maturation stage that led to natural variation in the volatile profile (Strojnik et al., 2020; Torregiani et al., 2017).

3.2 | Experiment 1—Comparison of three encapsulation methods

Three different encapsulation methods, namely M1, M2, and M3, as described in Section 2.3, were compared with

TABLE 3 Relative percentages of the key volatiles in the untreated truffles and truffle-*β*-cyclodextrin (CD) complexes derived from M1, M2, and M3 on days 0 and 90

Volatiles	Relative percentage (%)			
	Untreated truffles	M1-derived product	M2-derived product	M3-derived product
Day 0				
Methanethiol[a]	0.0051 ± 0.0002 ^{Aa}	0.0053 ± 0.0004 ^{Aa}	0.0023 ± 0.0005 ^{Aa}	0.0052 ± 0.0014 ^{Aa}
Dimethyl sulfide (DMS)[a]	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)[a]	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)[a]	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[a]	0.0032 ± 0.0002 ^{Bb}	0.0099 ± 0.0016 ^{Aa}	0.0032 ± 0.0001 ^{Ab}	0.0057 ± 0.0023 ^{Aab}
Dimethyl sulfide (DMS)[a]	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)[a]	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)[a]	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}

Notes: Data are reported as the mean of triplicate measurements ± standard error. 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 × 100%. Means within the same row with different lowercase letters differ significantly ($p < 0.05$). Means within the same column (compare days 0 and 90) with different uppercase letters differ significantly ($p < 0.05$).

Abbreviation: ND, not detected.

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

regard to their volatile profile and microbial quality. A storage study for a period of 90 days was conducted to evaluate whether the truffle aroma can be encapsulated and retained over time. This is the first step in determining the success of encapsulating the truffle aroma over time. This has not been reported before and needs to be performed before further investigation on the physicochemical characteristics of the encapsulated products. The results of volatile and microbial analyses are shown in Tables 3 and 5, respectively.

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 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 3). This suggests that water (M3) may be a better option than ethanol (M2) to

TABLE 4 Relative percentages of the key volatiles in the untreated truffles, freeze-dried truffles, and truffle-β-cyclodextrin (CD) complexes derived from M3 on days 0 and 90

Volatiles	Relative percentage (%)		
	Untreated truffles	Freeze-dried truffles	M3-derived product
Day 0			
Methanethiol[a]	0.0031 ± 0.0001 ^{Aa}	0.0038 ± 0.0013 ^{Aa}	0.0051 ± 0.0004 ^{Aa}
Dimethyl sulfide (DMS)[a]	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)[a]	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)[a]	0.0017 ± 0.0004 ^{b}	0.0053 ± 0.0007 ^{Aab}	0.0064 ± 0.0013 ^{Aa}
1-Octen-3-ol[a]	2.3682 ± 0.2382 ^{a}	1.9250 ± 0.3559 ^{Aa}	0.7014 ± 0.0587 ^{Ab}
Methional[a]	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[a]	0.0006 ± 0.0001 ^{Ba}	0.0038 ± 0.0006 ^{Aa}	0.0041 ± 0.0013 ^{Aa}
Dimethyl sulfide (DMS)[a]	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)[a]	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)[a]	ND	0.0104 ± 0.0013 ^{Aa}	0.0078 ± 0.0005 ^{Aa}
1-Octen-3-ol[a]	ND	1.7058 ± 0.2088 ^{Aa}	0.7146 ± 0.1301 ^{Ab}
Methional[a]	ND	0.2119 ± 0.0416 ^{Ab}	0.7200 ± 0.1051 ^{Aa}
p-Cresol	ND	ND	0.0284 ± 0.0065 ^{A}

Notes: Data are reported as the mean of triplicate measurements ± standard error. 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 × 100%. Means within the same row with different lowercase letters differ significantly ($p < 0.05$). Means within the same column (compare days 0 and 90) with different uppercase letters differ significantly ($p < 0.05$).

Abbreviation: ND, not detected.

[a]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.

be added during truffle aroma encapsulation. A possible explanation for this could be that water might have encouraged the hydrophobic volatile molecules to enter 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. For the undetectable *p*-cresol in the M2-derived product (Table 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.

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 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 stabilized 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 any of 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 the 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 3). We speculate that the addition of water in M3 would have facilitated the absorption of aroma volatiles

TABLE 5 Microbial loads (log colony forming unit [CFU]/g) in the untreated truffles, β- cyclodextrin (CD) only negative control (C1, C2, and C3), and truffle-β-CD complexes derived from M1, M2, and M3 on days 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}	TFTC	6.67 ± 0.22^{Ab}	ND	TFTC	TFTC	6.44 ± 0.09^{Ab}
Pseudomonas spp.	6.06 ± 0.06^{Aa}	ND	3.73 ± 0.15^{b}	ND	ND	ND	4.03 ± 0.13^{b}
Listeria spp.	ND	ND	ND	ND	ND	ND	ND
Salmonella spp.	ND	ND	ND	ND	ND	ND	ND
Bacillus spp.	ND	ND	TFTC	ND	TFTC	ND	ND
Day 90							
Total plate count	6.85 ± 0.04^{Ba}	ND	5.58 ± 0.29^{Ab}	ND	TFTC	TFTC	6.00 ± 0.12^{Ab}
Pseudomonas spp.	5.46 ± 0.11^{B}	ND	TFTC	ND	ND	ND	TFTC
Listeria spp.	ND	ND	ND	ND	ND	ND	ND
Salmonella spp.	ND	ND	ND	ND	ND	ND	ND
Bacillus spp.	ND	ND	TFTC	ND	TFTC	ND	ND

Notes: Data are 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 days 0 and 90) with different uppercase letters differ significantly ($p < 0.05$).
Abbreviations: ND, not detected; TFTC, too few to count.

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.

3.3 | Experiment 2—Comparison of encapsulation and freeze-drying

Due to the reason mentioned in Section 2.4, a comparison was made between the M3-derived product and the freeze-dried truffles regarding their volatile profile (Table 4) and microbial quality (Table 6) in experiment 2.

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. While 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 significantly higher than those 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 4). The overall result suggests that the freeze-dried truffles exhibited an overall better volatile profile than the M3-derived product on day 0.

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 was 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 4). A previous study by Savini et al. (2020) reported an increase in ethyl-3-methylbutanoate throughout 35 days of refrigerator storage under different packaging conditions, and this phenomenon was related to the decay of fresh black truffles. The decrease of 1-octen-3-ol after 90 days (Table 4) is most likely due to truffle degradation, which 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 4). The contradictory results suggest that not all volatiles show similar changing patterns 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

TABLE 6 Microbial loads (log colony forming unit [CFU]/g) in the untreated truffles, freeze-dried truffles, β-cyclodextrin (CD) only negative control (C3), and truffle-β-CD complexes (M3) on days 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}	TFTC	5.56 ± 0.20^{Ab}
Pseudomonas spp.	5.47 ± 0.11^{Aa}	3.55 ± 0.05^{b}	ND	TFTC
Listeria spp.	ND	ND	ND	ND
Salmonella spp.	ND	ND	ND	ND
Bacillus spp.	TFTC	TFTC	ND	TFTC
Day 90				
Total plate count	6.46 ± 0.20^{Aa}	5.70 ± 0.14^{Bb}	TFTC	5.04 ± 0.15^{Bb}
Pseudomonas spp.	3.91 ± 0.29^{B}	TFTC	ND	ND
Listeria spp.	ND	ND	ND	ND
Salmonella spp.	ND	ND	ND	ND
Bacillus spp.	TFTC	TFTC	ND	TFTC

Notes: Data are 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 days 0 and 90) with different uppercase letters differ significantly ($p < 0.05$).
Abbreviations: ND, not detected; TFTC, too few to count.

of the truffle-bacteria interactions under different storage conditions (Niimi et al., 2021; Savini et al., 2020). Niimi et al.'s (2021) study was based on white truffles from the Marche Region (central Italy), Hungary, and Croatia, and Savini et al.'s (2020) study was based on black truffles from the Abruzzo region (southern Italy), whereas we used black truffles harvested from western Australia in the present study. It can be expected that in addition to the natural truffle to truffle variability, different growing regions will host different bacteria that can lead to differences in interactions with the truffles, 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 4).

On the other hand, most of the volatiles in the freeze-dried truffles did not change except for ethyl-3-methylbutanoate, isoamyl alcohol, and *p*-cresol, which showed a significant reduction after 90 days of storage. 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 of approximately 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 4). Given that the volatile changes in the untreated truffles, freeze-dried truffles, 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 although freeze-drying could adequately retain and stabilize truffle aroma, the encapsulation of volatiles in β-CD could help to further extend the storage period of truffle aroma. Apart from freeze-drying, encapsulation could be another option for stabilizing 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 needed. This method provides kinetic data on the volatile stability of the encapsulated products (Mishkin et al., 1984; Mizrahi & Karel, 1977).

3.4 | Microbial analysis in experiments 1 and 2

The microbial results of all samples are summarized in Table 5 (experiment 1) and Table 6 (experiment 2). According to the manufacturer's specification (Wacker Chemie AG), a low level of microorganisms (maximum 3 log CFU/g) would be present in the β-CD. Hence, this is within our expectation when one or two bacterial colonies (TFTC) were detected in the controls in experiments 1 (C1 and C3 on day 0; C3 on day 90) and 2 (C3 on day 0 and 90) (Tables 5 and 6).

In experiment 1, approximately 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 (TFTC) and no *Pseudomonas* spp. were observed in the M2-derived product (Table 5). This indicates that the addition of ethanol in M2 could act as a disinfectant in the process. Although there was no pretreatment 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 counts in the products derived from M1 and M3 were significantly lower than those in 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 those in the untreated truffles, with 3.73 ± 0.15 log CFU/g and 4.03 ± 0.13 log CFU/g, respectively, on day 0 (Table 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) were 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 (TFTC) were significantly reduced compared to the untreated truffles (5.47 ± 0.11 log CFU/g) (Table 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 the survival and growth of some microorganisms. 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 6). The *Pseudomonas* counts in the products derived from M1 and M3 decreased from approximately 3–4 log CFU/g to TFTC, respectively, after 90 days of storage (Table 5). Similarly, the *Pseudomonas* counts in the freeze-dried truffles and the M3-derived product (from day 0 to 90) decreased from 3.55 ± 0.05 log CFU/g to TFTC and from TFTC to ND, respectively (Table 6).

Salmonella spp. and *Listeria* spp. were not detected in all samples in experiments 1 and 2 (Tables 5 and 6). Likewise, *Salmonella* spp. was not detected in *T. melanosporum* and *Triticum aestivum* (harvested in Spain) by Rivera et al. (2010) or in *T. aestivum* (harvested in Italy) by Reale et al. (2009). Reale et al. (2009) did not find *Listeria monocytogenes* in *T. aestivum* collected in Italy. Research conducted by Rivera et al. (2010) 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., 2010). 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), *Bacillus* spp. were detected in some products derived from M1 and M2 (days 0 and 90) but were undetected in the untreated truffles and the M3-derived product (days 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 enables *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 (Tables 5 and 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).

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 fresh truffles before processing them into dried products via encapsulation or freeze-drying.

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 that M3 is the preferred method to retain and stabilize 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 stabilize 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 days 0 and 90 suggests the need to incorporate a preprocessing step to reduce microbial counts, 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. To the best of our knowledge, this is the first study to report the possibility of encapsulating volatile truffle aroma compounds. This study confirmed that aroma volatiles can be encapsulated. Before the process can be commercialized, other encapsulation parameters need to be determined in future research to confirm the encapsulation of 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 characterizing the formation of an inclusion complex between the volatile molecules and β-CD by NMR/FTIR/DSC techniques.

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AUTHOR CONTRIBUTIONS

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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