Biotechnological production and characterization of natural flavors

Cumulative Dissertation

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I. Abstract

Biotechnological production of flavors is economically relevant and a key to enable a tasty and diverse food supply. Fungi are suitable biocatalysts for the production of flavor compounds and often more efficient than the classical flavor extraction from plants and foods. Fungi from the department of Basidiomycota have a powerful enzymatic toolbox to generate flavor compounds.

Based on a broad screening of Basidiomycota, media optimization was performed to find suitable conditions for an efficient biotransformation of black currant pomace. During fermentation, the scents were investigated on a sensory and instrumental level via gas chromatography-mass spectrometry (GC-MS). *W. cocos* was grown on a solid-state medium composed of 30 g kg^{-1} pomace, 6.24 g kg^{-1} sodium aspartate-monohydrate and 30 g kg^{-1} agar-agar for 10 days. The flavor was characterized by means of a self-validated aroma dilution analysis (ADA), quantified, and proven with recombination experiments. Solid-state fermentation of pomace and aspartate led to a pleasant odor reminiscent of wild strawberries. Methyl anthranilate (2,206 µg kg^{-1}, flavor dilution factor 2^{10}), (*R*)-linalool (1,879 µg kg^{-1}, 2^{11}), 2-amino benzaldehyde (771 µg kg^{-1}, 2^{5}), and geraniol (138 µg kg^{-1}, 2^{5}) were determined as aroma-relevant for the wild strawberry like flavor in recombination experiments. The substrate-fungus combination made use of the nutrients provided by the black currant pomace leading to an appealing natural flavoring for food.

In a second approach, 80 g L^{-1} pomace and 60 g L^{-1} sucrose (pH 4.4) were fermented in a submerged culture until the medium reached a pH-value of 3.5. Supernatants of the fermented product were evaluated for a potential use as a beverage. Submerged fermentation of black currant pomace and sucrose led to a pleasant flowery and honey-like odor and a fruity, slightly sour taste. The produced fruit acids and flavor compounds were characterized and quantified. The scaled-up fermentation product was processed and carbonized in cooperation with the Geisenheim University. A panel evaluated the carbonated fermented beverage on a hedonic scale, ranging from 0 to 10, with 8.0 ± 1.4, whereas the value for the non-inoculated medium was 2.5 ± 2.5. A rise of the oxalic acid concentration was detected, which led to the characteristic fruity taste in combination with citric acid (935 mg L^{-1} citric acid and 192 mg L^{-1} oxalic acid). Linalool (23 µg L^{-1}), geraniol (42 µg L^{-1}), phenylacetic acid (950 µg L^{-1}), eugenol (23 µg L^{-1}) and methyl phenylacetate (94 µg L^{-1}) were detected with odor activity values above one, which imparted fruity and honey-like odors. Fermentation of black currant pomace led to a tasty beverage, which suits the current beverage trends. Upcycling of pomace with its physiologically valuable nutrients as a fermented beverage could contribute to a healthy human nutrition.

In a third study, 28 potentially aroma active non-canonical terpenes were investigated in cooperation with the Dechema Research Institute. For twelve especially potent compounds, the odor thresholds (OT) were determined in comparison to their non-methylated equivalents. In addition to the classical approach according to Ullrich and Grosch (1987), a novel method including the individual OT determination of the internal standards was established. The odors of several non-canonical terpenes were described for the first time. Twelve compounds were identified as highly intense flavors, *e.g.*, the flowery smelling 2-methyllinalool with an OT of 1.8 ng L^{-1} air, the earthy smelling 2-methyl-α-fenchol (3.6 ng L^{-1} air), and the flowery scent 2-methylgeraniol (5.4 ng L^{-1} air). The newly developed method for the OT determination appeared as an improvement compared to the method used so far. Methylated derivatives of linalool, citronellol, and geraniol were highly attractive due to their flowery and citrus-like scents, which are highly interesting for the cosmetic industry.

II. Zusammenfassung

Die biotechnologische Herstellung von Aromen spielt sowohl wirtschaftlich, als auch für die Sicherstellung einer wohlschmeckenden und abwechslungsreichen Ernährung eine entscheidende Rolle. Durch die Verwendung von Speisepilzen ist die Herstellung im Vergleich zur klassischen Extraktion aus Lebensmitteln effizient und nachhaltig. Pilze der Abteilung der Basidiomycota bieten durch ihre enzymatischen Werkzeugkoffer vielfältige Möglichkeiten für die Aromenproduktion.

Basierend auf einem umfangreichen Screening von Basidiomycota wurden die Kulturbedingungen für die Biotransformation von Johannisbeertrester optimiert. Die Kulturen wurden während der Fermentation sensorisch und mittels Gaschromatographie-Massenspektrometrie (GC-MS) untersucht. Im ersten Ansatz wurden 30 g kg^{-1} Johannisbeertrester mit 6,24 g kg^{-1} Natriumaspartat-Monohydrat und 30 g kg^{-1} Agar-Agar emers für 10 Tage fermentiert. Der Geruch wurde durch eine eigens validierte Aroma-Verdünnungsanalyse (AVA) charakterisiert, die Aromen quantifiziert und durch Rekombinationsexperimente bestätigt. Die Emers-Fermentation führte zu einem intensiven, an Walderdbeeren erinnerndes Aroma. Als Schlüsselaromastoffe wurden Methylanthranilat (2.206 µg kg^{-1}, Aromaverdünnungsfaktor 2^{10}), (*R*)-Linalool (1.879 µg kg^{-1}, 2^{11}), 2-Aminobenzaldehyd (771 µg kg^{-1}, 2^{5}) und Geraniol (138 µg kg^{-1}, 2^{5}) identifiziert und durch Rekombinationsexperimente bestätigt. In der Substrat-Pilz-Kombination wurden die Nährstoffe des Johannisbeertresters genutzt, um ein Aroma zu erzeugen, das für die natürliche Aromatisierung von Lebensmitteln eingesetzt werden kann.

In einem zweiten Ansatz wurden 80 g L^{-1} Johannisbeertrester und 60 g L^{-1} Saccharose (pH 4,4) submers fermentiert bis ein pH-Wert von 3,5 erreicht wurde. Der Kulturüberstand wurde auf dessen Eignung als Getränk untersucht und gebildete Fruchtsäuren und Aromen wurden identifiziert und quantifiziert. Die Submers-Fermentation zeigte einen blumigen, an Honig erinnernden Geruch und einen fruchtigen, säuerlichen Geschmack. Nach dem Up-Scaling wurde das Getränk in Kooperation mit der Hochschule Geisenheim filtriert und karbonisiert. Von einem Panel wurde das karbonisierte Getränk auf einer Skala von 0 bis 10 mit einer Gesamtakzeptanz von 8,0 ± 1,4 bewertet, während das nicht prozessierte Medium lediglich eine Gesamtakzeptanz von 2,5 ± 2,5 erzielte. Bei der Fermentation wurde ein Anstieg der Oxalsäure-Konzentration nachgewiesen, die zusammen mit Zitronensäure charaktergebend für den fruchtigen Geschmack war (935 mg L^{-1} Zitronensäure und 192 mg L^{-1} Oxalsäure). Außerdem wurden die Aromen Linalool (23 µg L^{-1}), Geraniol (42 µg L^{-1}), Phenylessigsäure (950 µg L^{-1}), Eugenol (23 µg L^{-1}) und Phenylessigsäuremethylester (94 µg L^{-1}) mit Aromawerten größer eins gebildet. Diese haben fruchtige, an Honig erinnernde Gerüche. Durch die Fermentation des Johannisbeertresters mit *W. cocos* konnte ein wohlschmeckendes Getränk hergestellt werden, das den aktuellen Trends des Getränkemarkts entspricht. Durch das Upcycling besteht die Möglichkeit, die physiologisch wertvollen Nährstoffe in Johannisbeertrester direkt für die menschliche Ernährung zu nutzen.

In Kooperation mit dem Dechema Forschungsinstitut wurden 28 neuartige nicht-kanonische Terpene auf ihre Eignung als Aromastoffe untersucht. Zwölf besonders interessante aromaaktive Vertreter wurden für die Bestimmung von Geruchsschwellen (GS) im Vergleich mit den nicht-methylierten Äquivalenten ausgewählt. Die klassische Methode zur GS-Bestimmung nach Ullrich und Grosch (1987) wurde mit einer eigens entwickelten Technik, bei der die GS des internen Standards individuell von den Probanden bestimmt wurden, verglichen. Die Gerüche zahlreicher nicht-kanonischer Terpene wurden erstmals beschrieben. 2-Methyllinalool mit einem blumigen Geruch und einer GS von 1,8 ng L^{-1} Luft, das erdig riechende 2-Methyl-α-fenchol (3,6 ng L^{-1}), 2-Methylgeraniol (5,4 ng L^{-1}) mit blumigen

Geruchseindrücken und neun weitere Substanzen zeigten niedrige GS. Die neu entwickelte Methode zur Ermittlung der GS stellt eine Verbesserung des bisher verwendeten Verfahrens dar. Die methylierten Derivate von Linalool, Geraniol und Citronellol zeichneten sich durch ihr angenehmes blumiges, Citrus-artiges Aroma aus, das im Speziellen interessant für den Einsatz in der Kosmetikindustrie sein könnte.

III. List of publications

Peer-reviewed publications

I. Wild strawberry-like flavor produced by the fungus *Wolfiporia cocos* - Identification of character impact compounds by Aroma Dilution Analysis after Dynamic Headspace Extraction
S. Sommer, M.A. Fraatz, J. Büttner, A.A. Salem, M. Rühl, and H. Zorn
J. Agr. Food Chem. 2021, 69 (47), pp. 14222–14230
https://doi.org/10.1021/acs.jafc.1c05770, [1]

II. Upcycling of black currant pomace for the production of a fermented beverage with *Wolfiporia cocos*

S. Sommer, J.L. Hofmann, M.A. Fraatz, H. Zorn

Submitted to *J. Food Sci. Technol.*

III. Odor characteristics of novel non-canonical terpenes

S. Sommer, L.M. Lang, L. Drummond, M. Buchhaupt, M.A. Fraatz, H. Zorn

Molecules 2022, 27 (12), p. 3827

https://doi.org/10.3390/molecules27123827, [2]

Publications for science communication

I. Neue Methode zur Getränkeherstellung: Medizinalpilz *Wolfiporia cocos* als vielversprechender Biokatalysator zur Aromabildung

S. Sommer, M. Rühl

Der Champignon, Bund Deutscher Champignon- und Kulturpilzanbauer e.V. 14.05.2021

Patents

I. Biotechnologische Herstellung von Aromastoffen aus Johannisbeertrester
H. Zorn, S. Sommer, N. Sella, C. Schlering, M. Rühl, M.A. Fraatz, J. Büttner
Europäische Patentanmeldung EP 3 739 055 A1, Anmeldenummer: 1917437.7
15th May 2019

II. The use of non-canonical terpenes or terpenoids as aroma chemicals
S. Sommer, M.A. Fraatz, H. Zorn
Europäische Patentanmeldung Anmeldenummer 22174377.6
19th May 2022

Conference contributions

- **Flash-poster presentation**

I. Flavor composition of wild strawberry-like odor produced by *Wolfiporia cocos* on black currant pomace
S. Sommer, J. Büttner, N. Sella, P.C. Sturm, L.M. Lang, M.A. Fraatz, M. Rühl, H. Zorn
16th Weurman Flavour Research Symposium 2021

- **Presentations**

I. Flavor analysis of fungal biotransformation products
S. Sommer, M.A. Fraatz, H. Zorn
13th Annual GGL Conference 2020

- **Poster**

I. Production of natural wild strawberry-like aroma by biotransformation of black currant pomace with basidiomycetes
S. Sommer, J. Büttner, C. Schlering, M.A. Fraatz, M. Rühl, H. Zorn
13th Wartburg Symposium on Flavor Chemistry and Biology 2019

II. Fermentation of *Wolfiporia cocos* on black currant pomace as a promising way for natural wild strawberry-like flavor synthesis
S. Sommer, J. Büttner, C. Schlering, M.A. Fraatz, M. Rühl, H. Zorn
12th Annual GGL Conference 2019

IV. List of abbreviations

ADA	Aroma Dilution Analysis
AEDA	Aroma Extract Dilution Analysis
DHS	Dynamic Headspace
FID	Flame Ionization Detector
GC-O	Gas Chromatography – Olfactometry
GC-MS	Gas Chromatography – Mass Spectrometry
HMG	3-Hydroxy-3-methylglutaryl
IS	Internal Standard
MS	Mass Spectrometry
OAV	Odor Activity Value
ODP	Olfactory Detection Port
PP	Pyrophosphate
RT	Recognition Threshold
SAFE	Solvent Assisted Flavor Evaporation
SBSE	Stir Bar Sorptive Extraction
UN	United Nations

3 Chapter 1

1.1. Sustainable food production

In a world of declining resources and a rising population, efficient and sustainable food production gets more and more important. The world population has increased from 2.5 billion in 1950 to 7.8 billion in 2022 and is estimated to rise to 8.5 billion in 2030.[3] Worldwide, nearly 690 million people suffered hunger in 2019.[4] According to the recent trend, this number could even rise up to 840 million in 2030.[4] Especially nutrients such as proteins, fiber, vitamins, and secondary metabolites are lacking.[4] The United Nations (UN) published "Sustainable Development Goals" to tackle the biggest problems the present population is facing. Especially the fight against poverty and hunger, the demand for good health, responsible production and consumption, as well as efficient and sustainable crop growing on minimal spaces are necessary to improve the well-being of the world's population.[5,6] More efficient and fairer distribution of high-quality food is one tool to fight hunger.[4,6] Conservation of food is a key to less supply shortages. Examples for value-adding processes are well described; fermentation of cabbage saved thousands from scurvy and was the first example to conserve vitamins.[7] Canning is an example of thermic and physical conservation of a broad variety of food and enables save food supply over years.[8] Upcycling of side streams to produce a flavoring agent offers great potential. For example, Liebig developed a meat extract out of meat, bones, and cooking water and revolutionized the cooking of soups and sauces.[9] Finding new solutions to improve the efficiency of food production is challenging but necessary to allow a sustainable and healthy food supply.[10]

1.2. Currants

Currants are highly tasty small fruits with over 150 species differing in color or taste.[11] Red, white currants (both *Ribes rubrum* L., *R. sativum*, or *R. vulgare* Jancz.), and black currants (*Ribes nigrum*) are highly popular in Europe.[11] European countries produce currants almost exclusively, with Poland being the biggest exporter while Germany imports the highest amounts (**Figure 1**).

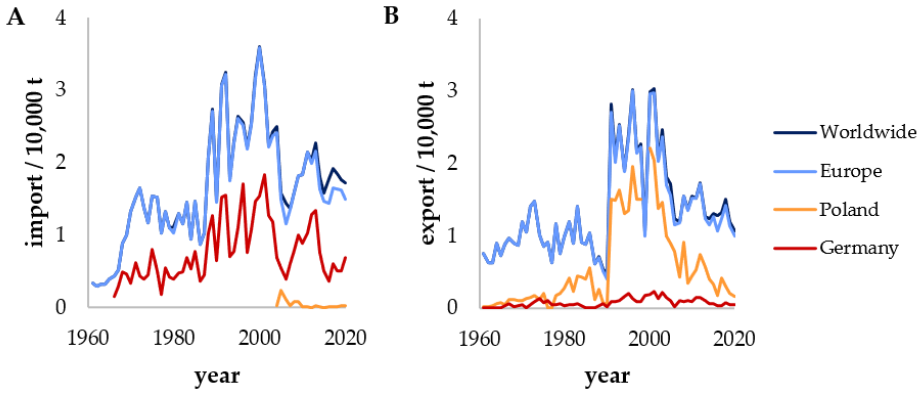


Figure 1: Development of traded currants, with **A** import and **B** export, worldwide (dark-blue), in Europe (light blue), Poland (orange) and Germany (red) from 1961 to 2020. The data were obtained from the FAOSTAT.[12]

Overall, the traded amounts increased over the past 60 years. People eat them as fresh fruit and as ingredients in jams, desserts, liquor, baked goods, and dairy products.[11,13] The berries are highly sensitive and need to be harvested by hand if they are traded as fresh fruits. This is very time-consuming and labor-intensive, but leads to a highly delicate product.[11]

Economically, black currants are most interesting. Poland is the biggest producer of black currents with a yearly total production of 80,000 t. This excels the production of Germany (5,200 t), Latvia (3,900 t) and France (3,500 t) by far.[14] Black currents have a very thick skin, big kernels, an intense odor and a characteristic sour taste. Most consumers do not like the pure taste of the fruits and prefer only low doses of black currant.[15] The famous French liquor *Crème de cassis* as well a wide variety of spritzers and candies include its juice, which is obtained by berry pressing.[13] After harvesting, the berries are crushed, and treated with enzymes (maceration) (**Figure 2**).[16,17] Due to their high pectin concentration (1.7 g kg^{-1}) the output would otherwise be low with emerging gelation.[17,18] The use of commercially available enzyme mixtures including pectin lyase, pectin esterase, pectinase, cellulase, and hemi-cellulase overcomes this problem.[16,17] After maceration, mashed berries are pressed.[16,17] The pressing procedure is optimized to improve yields and phenol extraction, and to warrant an intense odor and taste.[16] The so-called mother juice is normally not consumed pure but as a nectar. Commercially available black currant nectar has a juice concentration of 25 to 30% and is produced by adding water and sugar.[19–21]

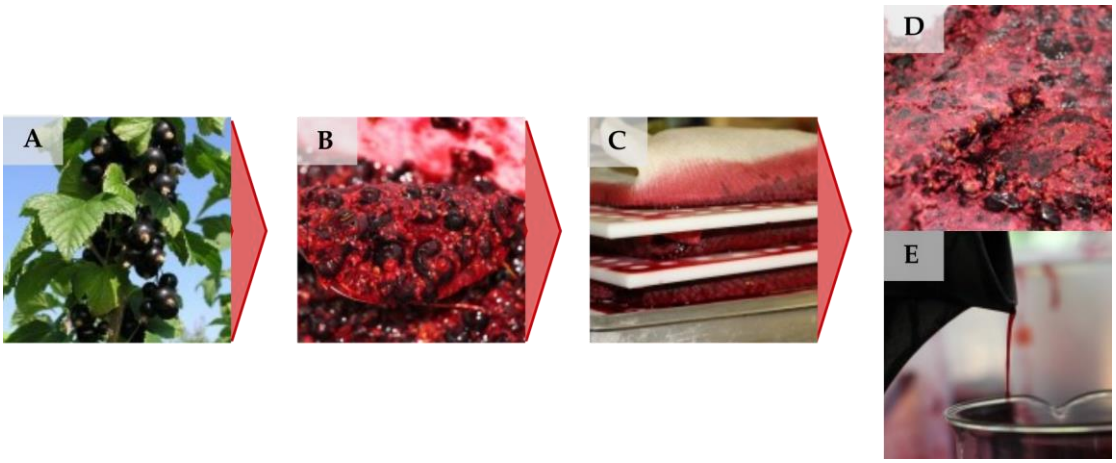


Figure 2: Black currant processing starting with the berries on the bush (**A**), berries after maceration (**B**), pressing (**C**), pomace (**D**) and produced mother juice (**E**). Pictures were provided by Tina Kissinger and Christine Schlering (Geisenheim University).

This procedure leads to high amounts of solid leftovers, the so-called pomace, making up to approximately 20 to 30% of the fruit.[22] Comparing the ingredients of the whole berry, juice and pomace, elucidates the presence of high-value nutrients in this side-stream (**Table 1**). Pomace is rich in protein, fat, and fiber and contains high amounts of anthocyanins and vitamin C. The fatty acid profile is particularly valuable. The kernels contain high amounts of unsaturated fatty acids, approximately 79% polyunsaturated, 13 % monounsaturated, and 8% saturated fatty acids.[23,24] Furthermore, the skin is rich in anthocyanins (1150 mg $(100\ g)^{-1}$).[25] The comparison of juice and pomace shows that a high portion of the health-beneficial compounds remains in the pomace and does thus not contribute to human nutrition. Maceration is one tool to optimize the extraction of secondary metabolites and leads to varying concentrations in the mother juice. Nevertheless, not all high quality nutrients can be extracted with the juice.[16,17]

Table 1: Comparison of the nutrients of fresh black currant berries, black currant mother juice, and black currant pomace, with * = calculated of Glc, Fru, and Sac content, # = related to 100 mL, + = calculated with a water content of 60 g (100 g)$^{-1}$.

		Whole berry		Mother juice		Pomace	
Water	g (100 g)$^{-1}$	74–83	11,26,27	86–89	26,27	52–63	27,28
Protein	g (100 g)$^{-1}$	1.4	11	≤ 0.5#	29,30	7.5	28
Fat	g (100 g)$^{-1}$	0.4	11	≤ 0.5#	29,30	9.7	28
Carbohydrate	g (100 g)$^{-1}$	6.3*–15.4	11,18,27	7.2#–13.5*	27,29,30	1.0–2.4*	27,28
Glucose	g (100 g)$^{-1}$	2.4–5.6	18,27	6.0	27	1.2	27
Fructose	g (100 g)$^{-1}$	3.2–4.1	18,27	4.2	27	1.0	27
Sucrose	g (100 g)$^{-1}$	0.7–0.9	18,27	3.3	27	0.2	27
Fiber	g (100 g)$^{-1}$	6.8	18	< 0.5#	29	28.7	28
Citric acid	g (100 g)$^{-1}$	2.4–3.0	18,26,27,31	2.6–3.7#	32	0.5	27
Anthocyanins	mg (100 g)$^{-1}$	410	27	66	27	1500	27
Flavonol glycosides	mg (100 g)$^{-1}$	10	27	4	27	29	27
Flavonol aglycons	mg (100 g)$^{-1}$	3.0	27	1.3	27	8.3	27
Hydroxycinnamic acids	mg (100 g)$^{-1}$	6.0	27	2.6	27	16.7	27
Vitamin C	mg (100 g)$^{-1}$	177	18	80# -250#	29,32	210+	33

Extracts of black currant were reported to have strong antioxidant, anti-inflammatory, antimicrobial, and other health-beneficial effects.[34,35] These are attributed to the phenolic compounds.[35] To enable a direct comparison of anthocyanins, flavonols, and hydroxycinnamic acids between juice, fruit, and pomace, the values from a single publication were used, wherein the concentrations were determined by HPLC-DAD after extraction with acidic methanol and ethyl acetate, respectively (**Table 1**).[27] Anthocyanins and flavonoids belong to the group of polyphenols, which are associated with health benefits.[36] Anthocyanins **1** of black currant have an intense red to purple color, depending on the pH (**Figure 3**).[37] Delphinidin (R_1 = OH, R_2 = OH) and cyanidin (R_1 = OH, R_2 = H) are the most prominent anthocyanins in black currant, which are present as aglycons (R_3 = OH) or as glycosides with R_3 bearing glucose or rutinose moieties.[38–40] Characteristic flavonols **2** in black currant are myricetin (R_4 = OH, R_5 = OH, R_6 = OH, R_7 = OH or bond to sugars) and quercetin (R_4 = OH, R_5 = H, R_6 = OH, R_7 = OH or bond to sugars) typically bond to sugars.[39,41,42] Examples for derivatives of hydroxycinnamic acids **3** in black currant are caffeic acid (R_8 = H, R_9 = H) and ferulic acid (R_8 = OCH_3, R_9 = H).

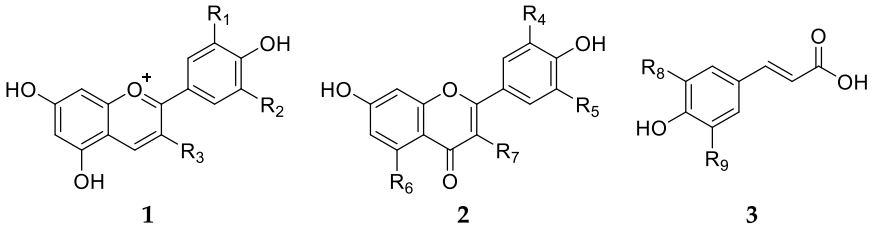


Figure 3: Basic structures of anthocyanins **1**, flavonols **2**, and hydroxycinnamic acids **3**.

Flavonols and hydroxycinnamic acids are potent antioxidants having a bitter taste, which is associated with the pleasant taste of currants.[39,43] The concentrations of phenolic compounds in the pomace are higher in comparison to those juice and berries (**Table 1**).

Until now, most companies do not see pomace as a valuable substrate and dispose it.[44] Therefore, a huge source of healthy nutrients is not supplied to human nutrition.[38,43] Therefore, different approaches for

the upcycling of pomace are conceivable.[45] Due to the low pH (2.6–2.8), usage for production of biogas, composting or animal feed is barely possible.[16] Pomace has a high water content and nutrient density. Therefore, it needs to be quickly processed, mostly dried or frozen.[22,28] One method to reuse pomace is extracting polyphenols and using them as a supplement or coloring agent.[38,46] Apart from that, it is used as an ingredient for cereal-based products and shortbread cookies.[33,45] Alonso González *et al.* (2019) showed that black currant pomace can be a decent substrate for *Saccharomyces cerevisiae* to produce a distilled alcoholic beverage.[47] Nevertheless, the present upcycling methods are economically not profitable.[44]

Effective treatment of food and its side products enables us to have a lower ecological footprint, save resources and money.[44,48] Therefore, it is necessary to search for high quality methods to use as many nutrients from the side streams as possible to redirect them to human nutrition. Fungal fermentation is an option to also use low value nutrients or to bio-transform valuable nutrients and bring them back to human nutrition.[49]

1.3. Basidiomycota

Basidiomycota is one of the two departments of Dikarya. Most medicinal and edible mushrooms, but also pathogenic strains, belong to the department of Basidiomycota.[50,51] Shiitake (*Lentinula edodes*), champignon (*Agaricus bisporus*), wood-ear (*Auricularia auricula*), and oyster mushroom (*Pleurotus ostreatus*) represent some of the famous examples of Basidiomycota.[51,52] The production of cultivated mushrooms increased more than 30-fold to 34 billion kg from 1978 to 2013.[52] Besides their pleasant aroma and taste, mushrooms are popular because of their texture, low calorie density, high amounts of dietary fiber, protein, and secondary metabolites.[53,54] Furthermore, fungi are very important for a healthy environment and biotechnological approaches. Fungi are the only organisms capable of degrading lignin, cellulose, and hemi-cellulose.[55,49] Successful approaches for upcycling of side streams with fungi are manifold. They can decay wood, produce valuable fruiting bodies on straw, express enzymes for biotechnological use, biosynthesize flavor compounds, and produce protein-rich mycelium in submerged cultures.[56–63]

1.4. Wolfiporia cocos

A fungus with great potential to upcycle side streams is *Wolfiporia cocos*. Other names for this brown rot fungus are *Fu Ling*, *Fuling*, *Hoelen*, China rot, *Pachyma*, *Wolfiporia extensa*, or *Poria cocos*.[64–68] It belongs to the department of Basidiomycota, the class of Agaricomycetes, the order of Polyporales, and the family of Polyporaceae.[69] *W. cocos* does normally not form fruiting bodies, but large sclerotia, which resemble a coconut (**Figure 4**).[69]

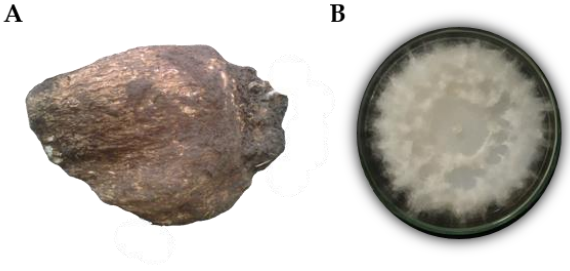


Figure 4: Sclerotium (**A**) and mycelium (**B**) on malt extract medium of *W. cocos* (picture **A** from Trappem, English Wikipedia, picture **B** from Svenja Sommer).[70]

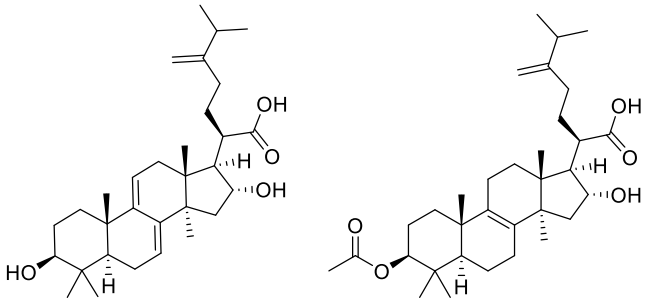
W. cocos lives on pine wood. Due to the high demand, indoor cultivation in culture bottles was also established.[71] After rotting, brown-rot fungi, such as *W. cocos*, leave behind brown organic material consisting mainly of lignin.[72] Wood decaying fungi have a toolbox of enzymes to degrade cellulose and hemi-cellulose.[72] Cellulose is hydrolyzed by several hydrolases, such as cellobiohydrolases, *β*-1,4-endoglucanases, and *β*-1,4-glucosidases.[72] Furthermore, oxidoreductases like lytic polysaccharide monooxygenases and cellobiose dehydrogenases oxidize the substrate thus forming acids, lactones, free electrons and hydroxyl radicals. The degradation products are mainly glucose monomers, which serve as carbon source for the fungus.[72–74] For degradation of heterogeneous hemicelluloses, different enzymes are necessary. For xylose-rich structures, hydrolysis with *β*-1,4-endoxylanases, *β*-1,4-xylosidases, and xylobiohydrolases are crucial. For mannose-rich hemicelluloses, debranching enzymes like *α*-, *β*-1,4-galactosidases, *β*-1,4-endomannanases, and galactomannan acetyl esterases are required. Additionally, *β*-1,4-endoglucanases, cellobiohydrolases, and *β*-1,4-endoglucanases also hydrolyze cellulose backbones of hemicelluloses. In the genome of *W. cocos*, several of these enzymes have been tentatively identified.[75,76]

Whereas white-rot fungi are also able to degrade lignin biochemically, brown-rot fungi cleave lignin via a non-enzymatic Fenton reaction. This reaction involves the oxidation of Fe^{2+} by H_2O_2 leading to the formation of Fe^{3+} and hydroxyl radicals as well as hydroxyl ions. These hydroxyl radicals and other low molecular mass radicals attack wood struts, which introduces degradation products fitting into the substrate portfolio of fungal enzymes, thus enabling an enzymatic degradation of (hemi)cellulose.[72] Formation of oxalic acid was postulated to support the Fenton reaction, as the fungus is able to stabilize and solve Fe^{3+} from plant material. Studies suggested that oxalic acid is a transporter for iron between hyphae and plant cell wall. The hydroxyl radicals oxidize cellulose and hemicellulose structures non-selectively. One-electron-transfer reactions lead to degradation and depolymerization and enable the attack of the selective enzymes.[72] Hydroxyl radicals attack lignin by several modes of action, leading to oxidation, demethylation, depolymerization, and hydroxylation events of *e.g.* aromatic rings. This results in extensively modified lignin structures.[72,77]

W. cocos is a rich source of pharmaceutical compounds. The white, inner part of the sclerotium has been used as a supplement and as a crude drug in Chinese, Japanese and Korean medicine for thousands of years.[64,68,78] It is often mixed with other ingredients like herbs and plants, prepared as tea or consumed in capsules. For example, *Yi-jin* is a combination with Ginseng, cactus, and another rhizomes to lower blood glucose.[65]. In Europe, *W. cocos* is barely used and considered as a novel food.[79] Nevertheless, the fungus is on the mushroom list of the federal office for consumer protection and food safety Germany as a food, supplement, and tea.[80] It is not a registered drug in Germany or Europe, but pharmacies sell it as a supplement.[81–83] In the USA, the fungus is known as a supplement without described side effects.[84] For 18 g d^{-1} of the mycelium, no chronic or acute toxicity has been detected.[64] Overall, the data are insufficient to evaluate the efficiency and the safety of *W. cocos* as a drug or as a supplement. Nevertheless, no severe side effects have been described.[64,84,85]

W. cocos contains approximately 70 (100 g)$^{-1}$ dry matter in the sclerotium. It is rich in triterpenes and polysaccharides and poor in other nutrients.[68] Several *β*-D-glycans with high glucose portions of 90–95% have been identified as healthy food ingredients.[86,87] The most prominent representative is pachyman with (1→3)-*β*-D-glucose strains, which are (1→6) branched.[68] According to several studies, *W. cocos* reduced blood glucose levels, has hypoglycemic effects and is anti-diabetic.[85,88] It was also tested to treat COVID-19.[89,90] Anti-inflammatory, anti-cancer, and anti-leukemia effects were also shown.[86]

For instance, dehydrotumulosic acid **4** and pachymic acid **5** show anti-diabetic and anti-cancer activity.[88,91,92] Overall, 35 different triterpenes have so far been described in *W. cocos* (**Figure 5**).[68]



4 **5**

Figure 5: Dehydrotumulosic acid **4** and pachymic acid **5** structures according to Fu *et al.* (2018).[92]

Several studies described bioconversion reactions catalyzed by *W. cocos*. The fungus is able to reduce the beany off-flavor of okara, a side product of soy milk production and to improve the scent of vine tea (*Ampelopsis grossedentata*).[93,94] While the sclerotium has a neutral odor and taste, submerged fermentations showed intense flavors. Depending on the culture conditions, *W. cocos* has been described to be a suitable biocatalyst for flavor production.[95]

1.5. Fermented beverages

The popularity of sugar-reduced fermented beverages and black currant spritzers is constantly growing due to their association with a healthy lifestyle.[96] Traditionally, beverages were fermented to enhance shelf life and improve taste, texture, and nutrients.[97] Well-known examples for fermented refreshing drinks are Kombucha, water kefir, *Bionade*, and Kvass.[96,97] Kvass is a drink from Russia and a fermentation product of bread, rye, and lactic acid bacteria. Water kefir is fermented sugar water using different *Lactobacillus* and yeast species. Traditionally from Mexico, water kefir is popular worldwide now.[96] *Bionade* is a German beverage, which is produced by fermentation of malt. A mixture of yeasts, *e.g.*, *Saccharomyces cerevisiae* and *Pichia* species, and bacteria like *Acetobacter*, *Lactobacillus*, and *Gluconacetobacter* ferments the drink and produces an alcohol-free beverage.[96] Similar microorganisms ferment brewed tea to Kombucha.[97] Kombucha is often fermented at home with a so-called SCOBY, a symbiotic consortium of bacteria and yeast.[96,98] Commercially, beverages have so far not been fermented with higher fungi.

Nevertheless, scientists made several approaches to use fungi from the department of Basidiomycota to bio-convert tea or other drinks. *Ganoderma lucidum, Naematelia aurantialba*, and *Lycoperdon pyriforme* were used to remove off-flavors from soymilk.[99–101] Furthermore, different fungi fermented wort to produce a non-alcoholic drink. Shiitake (*Lentinula edodes*) was used to produce a tasty beverage, which resembled of plum and was fruity, sweet, fresh and slightly sour.[102,103] Zhang *et al.* (2014) screened 31 strains for their suitability to produce a pleasant aroma in combination with wort. Whereas some candidates did not change the flavor or deteriorated the odor, some fermented products showed highly pleasant odor impressions. The product from *Polyporus umbellatus* reminded of kiwi and raspberry and the product from *Panellus seretinous* had a fresh, honey-like, and sweetish smell.[104,105] *Trametes versicolor* is an interesting fungus for the fermentation of wort. The odor changed to sweetish, fruity and flowery. Notably, the use of *T. versicolor* showed neither cytotoxic nor mutagenic activity.[103] Green tea was

fermented with the edible mushroom *Flammulina velutipes*. It produced a highly pleasant chocolate and nutty scent.[106]

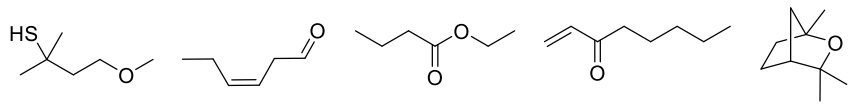
Recent studies investigated *W. cocos* as a biocatalyst to produce fermented beverages. The flavor of vine tea was enhanced without destroying polyphenols and flavonoids, which are responsible for the health-beneficial effect of traditional Chinese tea. Whereas the unfermented tea is not tasty, the fermented product was perceived as fruity.[94] Rigling *et al.* (2021) also fermented brewed green tea infusion with *W. cocos* . They produced a highly interesting flavor, which was associated with jasmine, flowers and citrus.[107]

1.6. Flavors

Flavor changes are major reasons for value-adding fermentation. Producing flavors with fungi has several benefits. Firstly, they are seen as natural and have a high consumer acceptance.[108] Furthermore, flavors aromatize food, cosmetics, cleaning agents, beverages, and several other products thus enhancing the overall assessment of consumables and foods. Adding pure flavors or flavor extracts to food is very common. Raspberry or vanilla flavors are very popular and mostly attributed to biotechnologically or synthetically produced flavors.[109] The raw materials are too rare to allow everyone the taste of real vanilla or raspberries. Due to the significant improvement of food by adding aroma compounds, a sustainable solution for the production of such aroma compounds is mandatory. Thus, healthy and tasty nutrition can be available for everyone.[108]

1.6.1. Black currant flavors

An intense and highly remarkable flavor is associated with black currants. 4-Methoxy-2-methyl-2-butanethiol **6** is the character impact compound, which has a very low odor threshold (0.001 µg L^{-1} water). In low concentrations, its scent is highly characteristic for black currant, but in high concentrations it reminds of cat urine.[110] Further odor active compounds are (3*Z*)-hexenal **7** with a grassy odor, the pineapple-like smelling ethyl butanoate **8**, champignon-like 1-octen-3-one **9**, and 1,8-cineol **10** with a coniferous scent (**Figure 6**). Furthermore, aldehydes with a green and cucumber-like flavor are present *e.g.*, hexanal and (2*E*)-nonenal. The musty smelling pyrazines 2-isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxypyrazine and 2-*sec*-butyl-3-methoxypyrazine also contribute to the characteristic black currant odor.[111]



6 **7** **8** **9** **10**

Figure 6: Structures of odor active compounds in black currants (OAV > 20), 4-methoxy-2-methyl-2-butanthiol **6**, (3*Z*)-hexenal **7**, ethyl butanoate **8**, 1-octen-3-one **9**, and 1,8-cineol **10**.[111]

1.6.2. Fungal flavors

The flavor spectrum of fungi is manifold and a rich source for research. Fraatz *et al.* (2011) divided fungi into three flavor groups. The first group mainly produces C_8-compounds like 1-octen-3-one, 1-octen-3-ol, or 2-octanone, which have an intense fungal flavor. Examples for this group are champignons (*Agaricus bisporus*) and gray shag (*Coprinopsis cinerea*).[112–114] The second group is characterized by sulfur-containing compounds, *e.g.*, garlic parachute (*Marasmius alliaceus*) and shitake (*Lentinula edodes*).

Characteristic flavors of the sulfur group are dimethyl trisulfide and 3,5-dimethyl-1,2,4-trithiolane with sulfurous, savory, and garlic-like odors.[112,115,116] The third group of fungi forms terpenes, which often release citrus-like, flowery, or coniferous scents.[112] The huge variety of the fungal flavors also includes anise-like smelling fungi, *e.g.*, oyster mushroom (*Pleurotus sapidus*) or sweet odors as described for *Hebeloma sacchariolens*.[57,117]

The flavor of fungi does not only depend on the species, but also on the culture substrate. Especially submerged fermentation is a highly promising method to produce flavors. It is fast, easy to breed, and relatively easy to scale up.[118] An especially interesting species for biotransformation is *Pleurotus sapidus*, which shows an immense biosynthetic toolbox. While this fungus forms an intense anise-like odor when grown on citrus side-streams, it is also able to produce high-value protein when cultured on apple pomace as a carbon and nitrogen source.[56,57] Submerged cultures grown on molasses bio-synthesized a broad variety of wine lactones and dill ethers.[60] For growth and flavor production, supply of nutrients is necessary and the composition of the medium needs to be optimized for the respective purpose.[119]

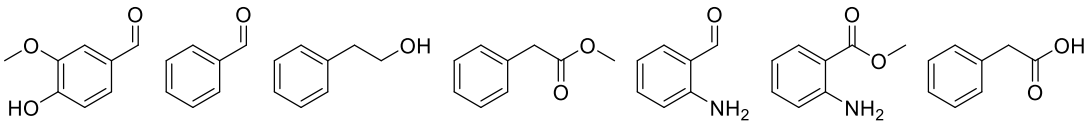
Cultures of *W. cocos* produced a mixture of different flavors depending on the culture substrate while the sclerotium does not exhibit a special odor or taste (**Table 2**).[66,120] Overall, different flavor groups have been reported. Besides the typical fungal odor 1-octen-3-ol, alcohols, aldehydes, terpenes and aromatic compounds are described in the literature.

Table 2. Selected aroma-active compounds produced by *W. cocos* on different culture media, with MM = minimal medium, * = medium composed of glucose, asparagine, and yeast extract; flavor description according to TGSC Information System.[121]

	green tea [107]	**wine press cake** [122]	**wort** [122]	**carrot peel** [123,124]	**MM** [125]	**GAY*** [126]	**okara** [93]	**vine tea** [94]	**Odor description**
β-Cyclocitral	X								tropical, saffron
Geraniol	X	X		X	X	X	X		flowery, citrus
Hexanal							X		fresh, green
Hexanol							X		Alcoholic
β-Ionone				X					raspberry, flowery
Linalool	X		X	X	X	X	X		(*R*) flowery, (*S*) citrus
Limonene						X			(*R*) lemon, (*S*) resinous
Methyl anthranilate	X				X				jasmine, wild strawberry
2-Methylbutanoic acid		X							pungent, cheesy
Methyl 2-methylvalerate								X	apple, fruity
Methyl phenylacetate	X					X	X		Honey
1-Nonanol							X		fresh, fatty
1-Octen-3-ol		X			X		X		Mushroom
2-Phenylethanol	X	X	X	X	X		X		Rose
Phenylacetic acid			X						honey, beeswax
Phenyl methanol			X			X	X	X	floral, rose
2-Undecanone							X		waxy, flowery
2-Undecanol	X								fresh, waxy

1.6.3. Aromatic flavor compounds

Many fungi have been reported to produce a variety of aromatic flavors (**Figure 7**). Vanillin **11,** a product of lignin degradation, is the best-known flavor compound and has an intense vanilla-like odor.[109,127] A co-fermentation of *Aspergillus niger* and *Pycnoporus cinnabarinus* can produce vanillin from ferulic acid.[128] Benzaldehyde **12** is a famous odorant, which has a marzipan and bitter almond-like flavor. Biosynthesis of **12** was investigated for *Bjerkandera adusta* starting from phenylalanine and is *de novo* synthesized by *Ischnoderma benzoium*.[109,129,130] 2-Phenylethanol **13** is a product from phenylalanine and lignin biotransformation and can also be synthesized *de novo*.[109,131] Production of the rose-like smelling 2-phenylethanol **13** by *W. cocos* was increased by supplementation of glucose and phenylalanine.[107] The *de novo* synthesis of methyl phenylacetate **14** was described for several fungi, *e.g.*, *Sarcodontia setosa*. It has an intense odor reminiscent of honey and beeswax.[131] The rather uncommon flavor 2-aminobenzaldehyde **15** has a jasmine-like odor and was shown to be produced from the precursor anthranilic acid by *Hebeloma sacchariolens*.[132] Methyl anthranilate **16** is known for its intense odor reminiscent of jasmine and wild strawberries. A putative pathway for **16** suggests a direct methylation of the carboxyl group from anthranilic acid. A correlation of anthranilic acid supplementation and methyl anthranilate formation was shown by feeding experiments with *Pycnoporus cinnabarinus* and *W. cocos*.[107,133]



11 **12** **13** **14** **15** **16** **17**

Figure 7: Structures of vanillin **11**, benzaldehyde **12**, 2-phenyl ethanol **13**, methyl phenylacetate **14**, 2-aminobenzaldehyde **15**, methyl anthranilate **16**, and phenylacetic acid **17**.

Black currant pomace is rich in proteins and hydroxycinnamic acids, like ferulic, *p*-coumaric, and caffeic acid, which are known precursors for the formation of aromatic flavor compounds.[27,109] Anthranilic acid is an intermediate of the shikimate pathway, which transforms pyruvate to aromatic amino acids in fungi.[134] Furthermore, several aromatic compounds have been described to be necessary for the Fenton reaction as a reduction agent for Fe^{3+} in *W. cocos*.[76] One example is the odor-active phenylacetic acid **17**. Additionally, benzoic, amino benzoic, 4-hydroxyphenylacetic, and 4-hydroxy cinnamic acid have been described in *W. cocos*.[76] *W. cocos* produces several enzymes such as cytochrome P450 monooxygenases, tyrosinases or hydroxylases putatively involved in the biotransformation of several aromatic precursors.[135] Nevertheless, further analysis would be necessary to proof biosynthetic pathways of aromatic compounds in *W. cocos.*

1.6.4. Terpenes

Terpenes are a huge and diverse group of flavor compounds. They occur in nearly every organism. All terpenes formally consist of isoprene (2-methyl-1,3-butadien) units, but have a huge structural diversity.[136] Precisely, terpenes have a hydrocarbon based scaffold whereas functionalized terpenes are collectively called terpenoids. Terpenoids occur in great structural diversity, such as linear, monocyclic, or polycyclic compounds with optional keto-, acid-, aldehyde- hydroxyl-, and carboxylic groups.[137] The so-called mevalonate pathway to biosynthesize terpenes was postulated by Lynen and Bloch, which were awarded with a Nobel Prize in 1964.[138] The proposed biosynthesis starts with isopentenyl

pyrophosphate (IPP) **24** and dimethylallyl pyrophosphate (DMAPP) **25** (**Figure 8**). While hemiterpenes (C_5) are directly derived from IPP and/or DMAPP, terpenes with longer carbon chain lengths (C_{10}, C_{15}, C_{20}, C_{30}, ...) are derived from the precursors geranyl-PP (C_{10}) **26** or farnesyl-PP (C_{15}) **27**, which are formed via consecutive condensation reactions of DMAPP and IPP.[139]

The group of hemiterpenes is rather small and includes examples like prenol **33**, isoprenol **34** and 3-methylbutanoic acid **35**. Especially 3-methyl butanoic acid has an intense cheesy scent.[121] Monoterpenes (C_{10}) include a wide range of ubiquitously occurring flavor compounds, *e.g.*, linalool **29**, geraniol **30**, limonene **31**, and terpinen-4-ol **32**. Linalool has two enantiomers: licareol (*R*) with a lavender, flowery, and woody odor and coriandrol (*S*) with a sweet and citrus-like scent. Geraniol smells flowery with a citrus-like odor. (*R*)-Limonene has a fresh and citrus-like flavor, whereas the herbal odor of (*S*)-limonene reminds of coniferous forest. Terpinen-4-ol is described as earthy, musty and peppery.[121] 1,8-Cineol **10**, which is present in black currants, is an example of a bicyclic monoterpene (**Figure 8**).[111]

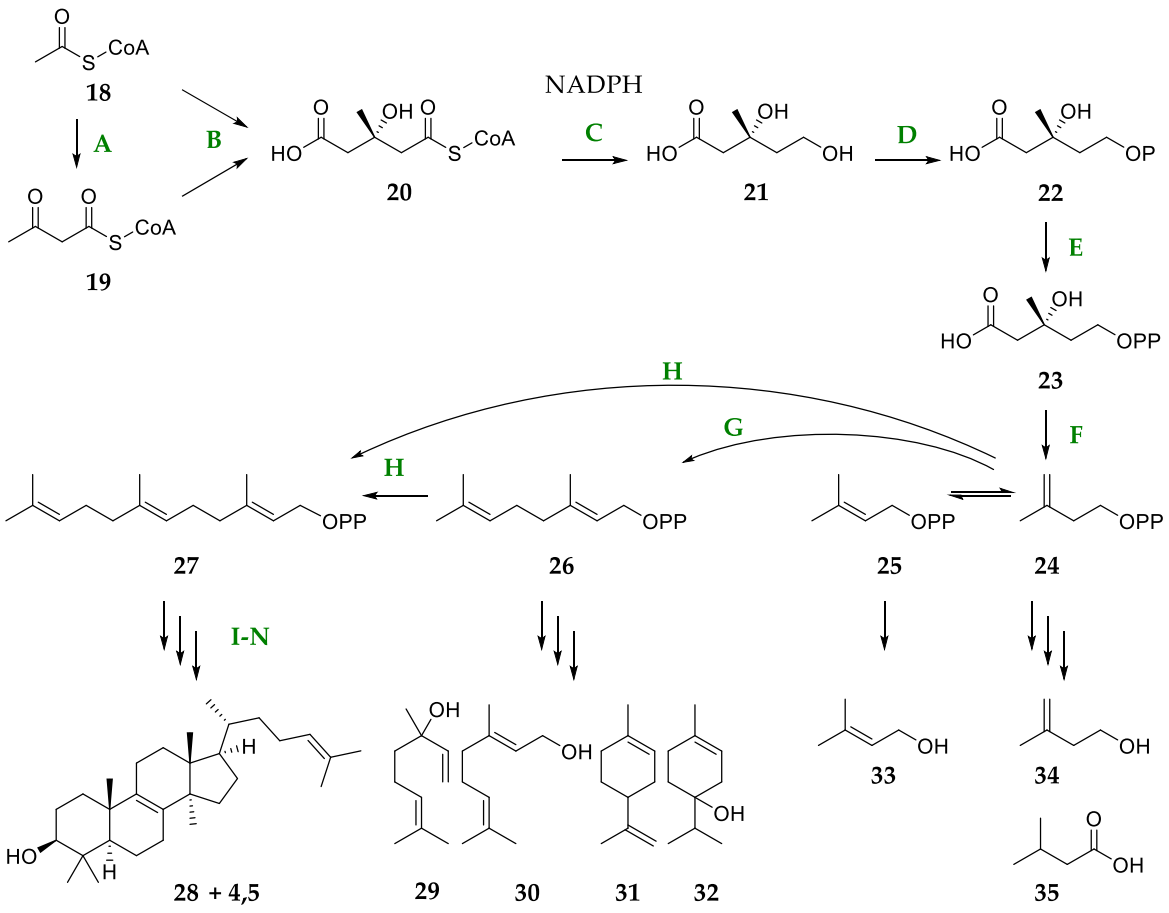


Figure 8: Biosynthetic reactions of the mevalonate pathway to form triterpenes (lanosterol **28**, **4**, **5**), monoterpenes (linalool **29**, geraniol **30**, limonene **31**, terpinen-4-ol **32)**, and hemiterpenes (prenol **33**, isoprenol **34**, 3-methylbutanoic acid **35**). Intermediates are acetyl-CoA **18**, aceto-acetyl-CoA **19**, hydroxymethyl glutaryl (HMG)-CoA **20**, mevalonate **21**, mevalonate phosphate **22**, mevalonate pyrophosphate (PP) **23**, isopentenyl-PP **24**, dimethyl allyl-PP **25**, geranyl-PP **26**, and farnesyl-PP **27.** Enzymes in *W. cocos* are **A** acetyl-CoA acetyltransferase, **B** HMG-CoA synthase, **C** HMG-CoA reductase, **D** mevalonate kinase, **E** phosphomevalonate kinase, **F** mevalonate-PP decarboxylase, **G** farnesyl-PP synthase, **H** farnesyl-PP synthase, **I** farnesyl-PP farnesyltransferase, **J** squalene monooxygenase, **K** lanosterol synthase, **L** cytochrome P450 monooxygenase, **M** sterol C-24 methyltransferase, and **N** O-acetyltransferase. Reaction scheme is modified from Shu *et al.* (2013) and Zhu *et al.* (2021).[140,141]

Several selective enzymes have been described to bio-convert farnesyl pyrophosphate **26** to a broad structural diversity.[139,142] Triterpenes (C_{30}) are not volatile but can have a sweet taste like glycyrrhizin,

act as a precursor for provitamins, *e.g.*, lanosterol **28** for ergosterol, or antidiabetic agents like dehydrotumulosic acid **4** and pachymic acid **5**.[140,143,144]

The biosynthesis of triterpenes in *W. cocos* was investigated in detail, as the described health benefits are associated mostly with pachymic acid and dehydrotumulosic acid. Several intermediates and enzymes have been characterized, which are also relevant for flavor synthesis.[140,141,145–148] For *W. cocos*, a positive correlation of linalool and geraniol formation with the addition of glucose and phenylalanine was observed.[107]

1.6.5. Non-canonical terpenes

Non-canonical terpenes have at least one additional methyl group and high structural similarities with terpenes. They are products of terpene degradation or produced by action of methyl transferases. The most common non-canonical terpene is 2-methyl isoborneol **36** (**Figure 9**). With its moldy odor and a low odor threshold, it is known as a contaminant in drinking water, derived from *Actinomyces* and *Streptomyces* species.[149] Several other non-canonical terpenes have been detected in trace amounts in soil, *e.g.* 1-methyl camphene **37** or 2-methylene bornane **38**.[150] 2-Methylgeraniol **39**, 2-methyllinalool **40**, and 2-methyllimonene **41** were detected as products of the bacterium *Nannocystis exedens*.[151]

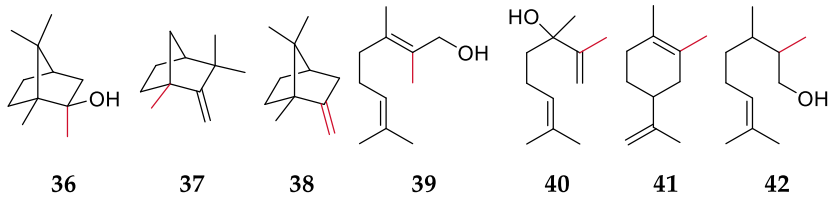


Figure 9: Structures of methylated terpenes with red-labeled bonds to the additional methyl group: 2-methyl isoborneol **36**, 1-methylcamphene **37**, 2-methylenbornane **38**, 2-methylgeraniol **39**, 2-methyllinalool **40**, 2-methyllimonene **41**, and 2-methylcitronellol **42**.

Since the compounds could only be detected in trace amounts, the respective genes were cloned into *Escherichia coli* or *Saccharomyces cerevisiae* to selectively produce C_{11} terpenes.[152,153] Genes encoding 2-methylisoborneol synthase from *S. griseus* and *S. coelicolor* and 2-methylene bornane synthase from *M. olivasterospora* and *P. fluorescens* were cloned into *E. coli*. Additionally, the genes for the mevalonate pathway, IPP-isomerase and the geranyl-PP isomerase were included.[152] This approach enabled the formation of several methylated monoterpenes and sesquiterpenes , *e.g.* of 2-methyllinalool **40**, 2-methyllimonene **41**, and 2-methylcitronellol **42**.[152] Furthermore, methyl transferases from *S. monomycini* were heterologously expressed in *E. coli* and produced in combination with the native farnesyl-PP synthase an additional substrate spectrum including (*E*/*Z*)-4-methylisoprenol **43**, (*E*/*Z*)-4-methylprenol **44**, 4-methylgeraniol **45**, and 8-methylgeraniol **46** (**Figure 10**).

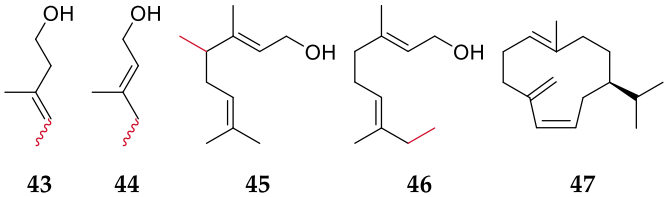


Figure 10: Structures of methylated terpenes with red-labeled bonds to the additional methyl group: (*E*/*Z*)-4-methylisoprenol **43**, (*E*/*Z*)-4-methylprenol **44**, 4-methylgeraniol **45**, 8-methylgeraniol **46**, and iso-germacrene **47**.

The C_{16}-compound iso-germacrene **47** has a citrus-like flavor.[154] Nevertheless, most of the known methylated terpenes have not been investigated as potential flavor compounds. Even if the flavor impressions are hard to predict, the structural similarities with highly potent aroma compounds from the group of monoterpenes and monoterpenoids are a promising starting point.

1.7. Flavor characterization

The characterization of flavors is challenging. Factors like age, hunger, genetics, and health status influence the perceived qualities and intensities of odors.[155] The description depends on memories, which are associated with the scent. Whereas an odor can be highly pleasant to one person, it can be uncomfortable to others.[156] The ability to describe scents can be trained and an experienced panel is necessary to investigate aroma impressions.[156] Aroma extract dilution analysis (AEDA) is the most common tool to determine the important odor-active compounds.[157]

1.7.1. Identification of odor active compounds

During AEDA, an organic extract of the sample material is prepared.[157] After extraction, the solution can be purified by means of solvent assisted flavor evaporation (SAFE) to remove non-volatile compounds.[158] The purified organic extract is then dried with anhydrous Na_2SO_4 and concentrated. Afterwards, the obtained extract is stepwise-diluted 1:2, and all of the dilutions are subjected to GC-O analysis.[159] This device has a splitter plate, which divides the gas flow equally to the instrumental detector and an olfactory detection port (ODP). Through the ODP, a panelist smells the gas flow. The device enables the contemporaneous identification of the compound by retention index, mass spectrum and odor.[160,161] In AEDA, all dilutions are measured and participants note the perceived odors. The compounds smelled in the highest dilutions are presumed to be most relevant.[157]

Aroma dilution analysis (ADA) was developed recently as an alternative to AEDA. It uses modern solvent-free techniques such as Solid Phase Microextraction (SPME), Stir Bar Sorptive Extraction (SBSE), or Dynamic Headspace (DHS) extraction.[105,162–165] For SPME, a coated fiber is used to extract volatile compounds from the headspace of the sample. In SBSE, a coated magnetic glass bar is used in and/or above liquids to extract flavor compounds.[166] By dynamic headspace extraction, flavors are trapped on a sorbent-filled liner with a nitrogen stream, which is blown though the sample.[167] Bound compounds are released in the injector of the GC by thermal desorption.[168,169] Solvent free techniques have several advantages, as they are more selective for flavor compounds, represent the perceived odor better, and are more sensitive for highly volatile compounds.[105,169] For ADA, the samples are stepwise-diluted 1:2 by adjusting the split vents in the injector system of the GC-O device. As described for AEDA, the substances which are perceived in the highest dilutions are most relevant for the overall odor.[164]

A method to distinguish between more and less relevant flavor compounds is the odor activity value (OAV). To determine the OAV, the determined concentration (c) is divided by the respective odor threshold (OT) (**Formula 1**). With a value of OAV higher than one, the human nose can perceive the compound while a value of smaller than one means that the odor may not be perceived.[170]

$$OAV = \frac{c}{OT} \quad (1)$$

After identification of the most relevant flavor compounds, it is necessary to proof whether all of the compounds have an influence on the overall flavor. Therefore, the identified compounds are combined in their respective concentrations in an aroma model. Because other ingredients like sugars, salt, and

other nutrients may influence the release of the odor-active compounds and the equilibrium of the flavors, the model system should reflect the composition of the original sample as closely as possible. If no significant difference of the sample and the recombinate is perceived, all compounds, which are responsible for the overall flavor, are defined as identified.[170]

1.7.2. Odor threshold

The odor threshold is an important parameter for the potency of a flavor compound and can be determined in air, water, or other media. Thresholds can barely be predicted and must be determined individually for each compound and stereoisomer.[171] Examples for compounds with extremely low recognition thresholds are bis-(2-methyl-3-furyl)-disulfide (0.76 ng L^{-1} water) and (*E*,*Z*)-2,6-nonadienal (9.1 ng L^{-1} water).[172] The composition and polarity of the medium are highly relevant for the distribution between the medium and the air. For instance, the odor threshold of δ-decalactone is 66 µg L^{-1} in pure water, 546 µg L^{-1} in a solution of 10% fat at pH 7, 294 µg L^{-1} in a medium with 10% fat at pH 5.5, and 1550 µg L^{-1} in pure oil.[173]

To determine the odor thresholds in water, the analyzed compounds are solved in water and the solutions are sequentially diluted, usually 1:3.[172,174] In a panel, each diluted solution is presented with two references which do not contain the aroma compounds. The subjects name the deviant sample and mark the dilution steps, at which the characteristic odor is still perceived. The recognition threshold is defined as the concentration, at which the characteristic odor can be described, whereas the detection threshold is defined as the concentration, where the odor is perceived in comparison to a reference.[172] The threshold in water depends on the individual, distribution between air and water, and interaction with the receptors in the nose.[172,175]

The odor threshold in air is independent from the medium and mostly determined by means of GC-O.[176] Other methods have been described, but are rarely used. For example, thresholds can be determined after preparing a gas-mix with the compound and are released with a dynamic gas blender.[175] Ullrich and Grosch (1987) diluted the aroma compound and an internal standard in an organic solvent. According to AEDA, GC-O analysis is performed and the dilution steps, where the odors are perceived in the lowest concentrations are defined as the D-value. To determine the odor threshold in air $OT_{air,X}$ the D-value of the internal standard (IS) D_{IS} and of the analyte D_X and additional parameters, such as the odor threshold of IS in air $OT_{air,IS}$, the concentrations of the IS c_{IS} and of the analyte c_X, are used for calculations (**Formula 2**).[176]

$$OT_{air,X} = \frac{OT_{air,IS} c_X D_{IS}}{c_{IS} D_X} \quad (2)$$

This method assumes that the OT_{air} of the analyte is proportional to the concentration of the analyte divided by the D-value of the analyte. To compensate for variations of the device, the use of an IS is necessary. Even if Ullrich and Grosch (1987) suggested to use a compound as an internal standard which is similar to the analyte, (2*E*)-decenal is the most common internal standard.[172,174,177] Its odor threshold OT_{air} was determined once as 2.7 µg L^{-1} using the air to water partition coefficient K_W and the odor threshold in water OT_W (**Formula 3**).[178]

$$OT_{air} = OT_w \cdot K_W \quad (3)$$

On the one hand, threshold determination of each compound needs to be performed with at least three participants because of their individual odor thresholds. On the other hand, one fixed odor threshold

of the internal standard is used for every participant. This leads to faults. Even if there are approaches to develop an electronic nose as a sensor for the detection, it is still not able to replace the determination with different human participants.[171] Finding new solutions to determine odor thresholds in air is challenging, but necessary for a precise determination of odor thresholds.

1.8. Objective

The aim of this dissertation was to find sustainable solutions to upcycle black currant pomace and to characterize biotechnologically produced flavors. The remaining nutrients of black currant pomace should be used as substrates for fungal growth, flavor biosynthesis, and to be redirected to human nutrition. Therefore, a screening for suitable media-fungus combinations should be performed.

On the one hand, the dissertation should investigate the optimal culture conditions to produce an intense flavor, which could be relevant as an industrial flavoring agent. Especially fruity and savory scents are highly requested. The produced flavor should be characterized in comparison to reference media, which did not contain pomace, to determine the influence of the substrate. To identify flavors, suitable techniques for identification and quantification of flavor compounds should be applied. Furthermore, additional fermentative methods to upcycle pomace should be investigated. Therefore, a suitable substrate-fungus combination should be evaluated and culture conditions should be optimized. The product should be characterized regarding its aroma profile. Besides fungal flavors, this dissertation aimed to investigate other novel compounds. For instance, methylated terpenes which were detected in genetically modified *E. coli* strains by the Dechema research institute should be investigated regarding their sensory properties, especially regarding their odor impressions and odor thresholds.

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4 Chapter 2

Wild strawberry-like flavor produced by the fungus *Wolfiporia cocos*

Identification of character impact compounds by aroma dilution analysis after Dynamic Headspace Extraction

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Wild Strawberry-like Flavor Produced by the Fungus *Wolfiporia cocos*—Identification of Character Impact Compounds by Aroma Dilution Analysis after Dynamic Headspace Extraction

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ABSTRACT: Brown-rot fungi are particularly suitable for the sustainable and cost-efficient biotechnological production of natural flavors. In this study, *Wolfiporia cocos* was employed for the fermentation of European black currant pomace supplemented with aspartate in surface cultures to produce a flavor reminiscent of wild strawberries. Aroma dilution analysis (ADA) by means of dynamic headspace extraction was developed as a suitable technique for solid samples. The character impact compounds were quantified by stable isotope dilution analysis and standard addition and validated by recombination experiments. (*R*)-Linalool (1879 $\mu g\ kg^{-1}$, ADA 2^{11}), methyl anthranilate (2206 $\mu g\ kg^{-1}$, 2^{10}), 2-aminobenzaldehyde (771 $\mu g\ kg^{-1}$, 2^{5}), and geraniol (138 $\mu g\ kg^{-1}$, 2^{5}) were identified as key aroma compounds. Recombination experiments demonstrated that the combination of the four analyzed compounds was responsible for the odor impression reminiscent of wild strawberries.

KEYWORDS: *aroma dilution analysis, dynamic headspace, side stream, black currant pomace*

INTRODUCTION

Due to their enormous biological versatility, fungi represent ideal candidates for the fermentative production of natural flavor compounds. Besides their characteristic C_8-compounds, aroma-active terpenes, aldehydes, methyl ketones, and sulfur-containing and aromatic compounds, including, for example, vanillin and raspberry ketones, have been described for various fungal species.[1,2] Especially, mushrooms from the division Basidiomycota are excellent candidates for flavor production from agricultural side streams since many of these species are edible and able to grow on a large spectrum of substrates including lignocelluloses.[3]

The juice industry extensively produces pomace as a side stream of juice pressing. The main constituents of pomace are pulp, seeds, and the skin of the respective fruits, and major nutrients present in pomace are fibers, proteins, sugars, and fatty acids. Some pomaces, such as those obtained from black currant juice extraction, are particularly rich in anthocyanins, flavonoids, and hydroxy cinnamates.[4,5] Currently, this side stream is mostly disposed or rarely used to produce biogas as compost or as an animal feed.[6] Therefore, fermentation of this high-quality nutrient source with Basidiomycota is an alluring option for its biotechnological upcycling. In the past, a number of fermentation processes employing Basidiomycota and side streams from the juice industry have been established for the production of natural flavors.[2,3,6−8] An example for the fungal transformation of a side stream for the production of vanillin was shown by Zheng *et al.* A co-fermentation process with *Aspergillus niger* and *Pycnoporus cinnabarinus* was used for bioconversion of nutrients from rice bran.[9] Nootkatone, known for its grapefruit-like odor, can be biotechnologically produced by *Pleurotus sapidus*, raspberry ketone is produced by *Nidula niveotomentosa*, and benzaldehyde is formed by *Ischnoderma benzoinum*.[10−12] The brown-rot fungus *Wolfiporia cocos* is an edible fungus, which possesses a broad enzymatic toolbox and the ability to degrade and utilize various side streams. It is especially well known in China, where it is also used as a traditional medicinal fungus.[13] Its potential to form aroma-active compounds has been widely reported. The fermentation of vine tea (*Ampelopsis grossedentata*) led to a pleasant, fruity aroma, and cultivation on carrot peels induced the production of viola-like smelling β-ionone.[14,15]

Strawberry aroma is one of the predominantly used flavors in the food industry, and the natural strawberry flavor is thus highly sought-after. An especially intense and unique flavor is attributed to wild strawberries, which often grow in forests. As wild plants, wild strawberries are rather rare and thus expensive. Numerous compounds shape their flavor, but methyl anthranilate is especially relevant for the sweetish, fruity, flowery-like, and jasmine-like aroma.[16] Other important compounds are monoterpenes like linalool and fruit esters like ethyl hexanoate and ethyl butanoate.[16]

A well-established method to determine relevant aroma compounds within a mixture is aroma extract dilution analysis (AEDA). The flavor dilution (FD) factor indicates the highest dilution step in which the compound is still detected by means of gas chromatography−olfactometry (GC−O). Compounds with low FD factors are assumed to be less or not important

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for odor impressions.[17] However, AEDA in combination with solvent-assisted flavor evaporation is quite laborious and requires high amounts of organic solvents, which may be harmful to the environment. Apart from this, discrimination of highly volatile aroma compounds may occur.[18] To compensate for these drawbacks, solvent-free aroma dilution analysis (ADA) by solid-phase microextraction (SPME), stir bar sorption extraction (SBSE), and dynamic headspace extraction (DHS) has been established. SPME, SBSE, and DHS are performed in a semi-automated, non-destructive, and fast workflow with higher sensitivities for highly volatile compounds. Thereby, dilution is performed by variation of the GC system's split ratio (SR).[19−22]

The present study elucidates a biotechnological process for the production of wild strawberry-like flavor by *W. cocos* from black currant pomace and its analysis using the recently developed ADA by DHS.[22] Further, stable isotope dilution analysis (SIDA) and standard addition were used to quantify the key aroma compounds and to finally recombine the produced aroma.

MATERIALS AND METHODS

Chemicals. Media components were purchased from the following producers: NH_4NO_3 (98%) from Acros Organics B.V.B.A. (Geel, Belgium); sodium L-aspartate monohydrate (99%) from abcr GmbH (Karlsruhe, Germany); agar-agar Kobe I, asparagine·$1H_2O$ (≥99%), glucose·$1H_2O$, KH_2PO_4 (≥98%), $MgSO_4·H_2O$ (≥99%), $MnSO_4·1H_2O$ (≥99.9%), and yeast extract (for bacteriology) from Carl Roth GmbH & Co. KG (Karlsruhe, Germany); $FeCl_3·6H_2O$ (98%), Na_2H_2EDTA (p.a.), $ZnSO_4·7H_2O$ (p.a.), and $CuSO_4·5H_2O$ from AppliChem GmbH (Darmstadt, Germany); and malt extract from Sigma-Aldrich (St. Louis, USA).

Liquid−liquid extraction was performed using the following chemicals: methanol (UHPLC-grade) from J. T. Baker (Schwerte, Germany), NaCl and Na_2SO_4 (≥99%) from Carl Roth GmbH & Co. KG, diethyl ether (99.9%) from Prolabo Chemikalien (Sion, Swiss), and pentane (99.9%) from Julius Hoesch GmbH & Co. KG (Düren, Germany).

The following authentic standards were obtained from the indicated commercial sources: geraniol (99%), (*R*)-(−)-linalool (95%), methyl anthranilate (99%), *β*-myrcene (90%), 2-nonanone (99%), and 2-undecanone (99%) from Acros Organics B.V.B.A.; isobutyl acetate (98%), 2-octanol (98%), and 2-tridecanone (>98%) from Alfa Aesar (Haverhill, USA); benzaldehyde (for synthesis) from AppliChem GmbH; isopropyl dodecanoate (98%) from chemPUR GmbH (Karlsruhe, Germany); 2-aminobenzaldehyde (>95%) from Fluorochem Ltd. (Hadfield, UK); 2-heptanone (99%), linalool (97%), linalool oxide (≥97%, furanoid isomeric mixture), 1-octen-3-one (96%), and *γ*-terpinene (97%) from Sigma-Aldrich; 6-methyl-5-heptene-2-one (>98%) from Th. Geyer GmbH & Co. KG (Renningen, Germany); linalool oxide (>98%, pyranoid isomeric mixture) and thymol (>99%) from TCI GmbH (Heuchelheim, Germany); and 2-dodecanone (≥95%) from VWR (Radnor, USA). The deuterated standards d_2-geraniol, d_5-linalool, and d_3-methyl anthranilate were obtained from aromaLAB GmbH (Planegg, Germany).

Fungus and Substrate. *W. cocos* (no. 279.55) was obtained from CBS Fungal Biodiversity Centre (Utrecht, Netherlands). The Department for Pomology Geisenheim University (Germany) provided black currant pomace of the cultivar *Ribes nigrum* L. cv. Titania.

Fermentation and Media Optimization. For screening, 23 fungi were grown on black currant leaves and pomace as a sole source of nutrients. The strains were maintained on malt extract agar (MEA; 20 g L^{-1} malt extract, 15 g L^{-1} agar-agar) or SNS-agar (30 g L^{-1} glucose·$1H_2O$, 15 g L^{-1} agar-gar, 4.5 g L^{-1} asparagine·$1H_2O$, 3 g L^{-1} yeast extract, 1.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4·H_2O$, 400 *μ*g L^{-1} ethylenediaminetetraacetic acid (EDTA), 90 *μ*g L^{-1} $ZnSO_4·7H_2O$, 80 *μ*g L^{-1} $FeCl_3·6H_2O$, 30 *μ*g L^{-1} $MnSO_4·1H_2O$, 5 *μ*g L^{-1} $CuSO_4·5H_2O$, pH 6.0). For inoculation, an agar plug (a 0.4 cm diameter) was transferred from a freshly overgrown plate to agar plates containing either 15 g L^{-1} leaves (moist mass, with 20 g L^{-1} agar-agar) or 15 g L^{-1} pomace (moist mass, with 60 g L^{-1} agar-agar). Cultivation was performed at 24 °C in the dark for up to 14 days (d). Growth conditions for *W. cocos* on black currant pomace were optimized based on a minimal medium described by Fraatz *et al.* for *P. sapidus*.[23] The concentrations of Titania pomace were varied (15, 30, 60 g L^{-1}); the pH was adjusted to 6.5; and KH_2PO_4 (1.5 g L^{-1}), sodium L-aspartate monohydrate (Asp, 6.24 g L^{-1}), NH_4NO_3 (2.4 g L^{-1}), and trace elements (8 ng L^{-1} $FeCl_3·6H_2O$, 9 ng L^{-1} $ZnSO_4·7H_2O$, 3 ng L^{-1} $MnSO_4·H_2O$, 0.5 ng L^{-1} $CuSO_4·5H_2O$, 40 ng L^{-1} Na_2H_2EDTA) were supplemented to the media. The final optimized medium named PoA + Asp consisted of 30 g L^{-1} (wet weight) pomace, 6.24 g L^{-1} Asp, and 30 g L^{-1} agar-agar. Agar was autoclaved separately in 70% of the required amount of water. As reference media, MEA, MEA + Asp (20 g L^{-1} malt extract, 6.24 g L^{-1} Asp, 15 g L^{-1} agar-agar), and PoA (30 g L^{-1} pomace, 30 g L^{-1} agar-agar separately autoclaved) were used for surface cultivation of *W. cocos*.

Monitoring of Growth and Sensory Analysis. Growth of *W. cocos* was monitored by measuring the diameter of the fungal mycelium and by measuring the pH value. The pH was measured after adding 15 mL of ultrapure water to the surface culture and homogenizing it with an ULTRA TURRAX (IKA-Werke, Staufen, Germany; 10,000 rpm, 1 min; $n = 3$). Further, on culture days 2, 4, 7, 10, and 14, sensory analysis was performed to detect aroma changes. To evaluate the flavor generated by *W. cocos* on the different culture media, the flavor attributes fruity, currant, cherry, wild strawberry, mandarin, peach, grape, citrus, tropical fruits, flowery, lavender, sweetish, marzipan, grain, malty, bread, mushroom, yeast, musty, tartly, sourish, and metallic were assessed on a scale from 0 (no odor) to 5 (highly intense odor) by 10 trained panelists (22−32 years old, non-smokers, 7 women, 3 men) for cultures grown on the 4 different media.

ADA Using DHS Extraction. *Method Validation.* For method validation, 50 *μ*L of a standard mixture of linalool, linalool oxides (furanoid, isomeric mixture), benzaldehyde, 2-undecanone, geraniol, and methyl anthranilate (135.8, 12.0, 6.9, 3.4, 3.1, and 13.8 mg L^{-1}, respectively) dissolved in purified water was added to a 20 mL headspace vial filled with 1.00 g agar plugs (30 g L^{-1} agar-agar). After storage at −20 °C, 4 *μ*L of internal standard (IST) thymol (39.5 mg L^{-1} dissolved in water) was added to the defrosted agar plugs prior to extraction. Gas chromatography−mass spectrometry−olfactometry (GC−MS−O) measurements were performed on an Agilent 7890B GC equipped with an Agilent 7977B MSD (Agilent Technologies, Santa Clara, USA), and an ODP 3 (GERSTEL GmbH & Co. KG, Mülheim a.d. Ruhr, Germany). The GC system was fitted with a CIS 4, a TDU 2, a DHS module (all GERSTEL GmbH & Co. KG), and a VF-WAXms column (30 m, i.d. 250 *μ*m, film thickness 0.25 *μ*m; Agilent Technologies). For DHS extraction, a Tenax TA liner (GERSTEL GmbH & Co. KG) was used as the sorbent material. Sample incubation was performed for 10 min at 30 °C, followed by an extraction step where the sample was vented with 750 mL of N_2 to trap the aroma compounds on the Tenax TA liner (50 mL min^{-1} at 30 °C) and a drying phase to remove water from the liner (650 mL N_2, 100 mL min^{-1}). Desorption was performed with 40 °C (0.5 min)/120 °C min^{-1}/250 °C (10 min) and cryogenic focusing at −70 °C (0.5 min)/12 °C s^{-1}/250 °C (5 min). Different SRs were used to bisect the concentrations of the injected substances. According to Trapp *et al.*, SRs of TDU and CIS were modified.[19,22] Splitless measurements were performed with 30 mL min^{-1} purge flow to split vent at 2 min in CIS and the splitless mode in TDU. The carrier gas was helium 5.0 (Nippon Gases GmbH, Hürth, Germany) with a constant flow of 1.56 mL min^{-1}. The gas flow was split 1:1 between the ODP port and MS detector. The oven temperature was 40 °C (3 min)/5 °C min^{-1}/240 °C (12 min). The MS source temperature was 230 °C, and the mass spectrometer was operated in the scan mode (m/z 33−300, 70 eV).

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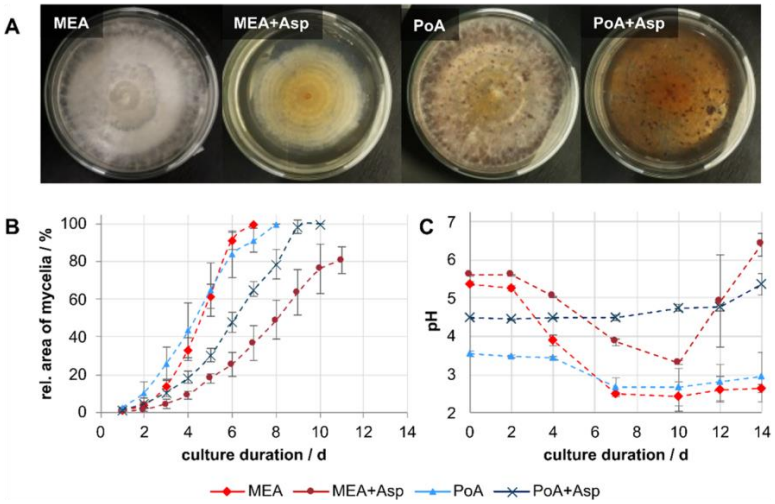

Figure 1. 10 day old surface cultures of *W. cocos* (A); growth rate of *W. cocos* as a function of the relative area covered by the mycelium and the culture day (B); pH of the medium (C) on different agar media; MEA, MEA supplemented with sodium aspartate (MEA + Asp), pomace agar (PoA), and PoA supplemented with Asp (PoA + Asp) shown in light red, dark red, light blue, and dark blue, respectively.

For determination of peak areas, the following mass fragments were used: linalool (m/z 71), (E)- and (Z)-linalool oxides (furanoid) (m/z 94), geraniol (m/z 69), 2-undecanone (m/z 58), benzaldehyde (m/z 106), methyl anthranilate (m/z 151), and thymol (m/z 135).

The samples were analyzed in a random order, and linear regression analysis of the logarithmic plots of the peak areas in extracted ion count (EIC) against the SRs was performed with Excel for determination of regression coefficients (R^2), slope (m), and intercept. Averages and standard deviations were determined with Excel. Replications were $n = 3$, $n = 4$, or $n = 10$ as indicated above. All replications were performed using individually grown fungi. Trained panelists performed the sensory evaluations.

ADA of W. cocos Cultures. To investigate the aroma of PoA + Asp, 100 equal samples were prepared. Therefore, *W. cocos* was cultivated in eight surface cultures (Petri dishes) for 10 days. Overgrown agar was cut in plugs, and approximately 125 mg per plate was weighted in 20 mL headspace vials. The pooled samples with 1000 ± 10 mg each were stored at −20 °C and analyzed as described above. Three participants (20−28 years, non-smokers, two females, one male) performed ADA. As blanks, DHS extraction was performed without the sample material, and odors perceived were not considered.

Compound Identification and Quantitation. Compounds were identified by comparison of recorded mass spectra and retention indices (RIs) according to van den Dool and Kratz[24] and perceived odors with those of authentic standards on two columns of different polarities (VF-WAXms and DB-5ms columns, both 30 m, 250 μm, 0.25 μm; Agilent Technologies) as well as with literature data.

Linalool, methyl anthranilate, and geraniol were quantified using SIDA. Response factors (RFs) were determined according to Steinhaus *et al.*[25] Liquid−liquid extraction was performed according to Kleofas *et al.* combining 4 g of overgrown agar, 16 mL of methanol, 16 mL of saturated NaCl solution, and 0.5 mL of deuterated standards (7.18 μg mL^{-1} d_3-methyl anthranilate, 3.17 μg mL^{-1} d_5-linalool, and 1.22 μg mL^{-1} d_2-geraniol, each dissolved in methanol).[27] After homogenization with an ULTRA TURRAX homogenizer (10,000 rpm, 0 °C, 2 × 30 s), the suspension was extracted three times with 25 mL of freshly distilled pentane/diethyl ether (1/1.12, v/v) for 30 min at room temperature in a closed flask with a stirring rate of 150 rpm. The extracts were combined, dried over anhydrous Na_2SO_4, filtered, and concentrated to approx. 1 mL on a Vigreux column at 45 °C. RFs were determined in methanol with the following concentrations: linalool (3.19, 9.60, 16.02, 22.39, and 28.81 μg mL^{-1}), methyl anthranilate (6.66, 12.50, 18.52, 24.19, and 30.04 μg mL^{-1}), and geraniol (0.32, 0.69, 1.01, 1.33, and 1.65 μg mL^{-1}). Extracts were measured using a 7890B GC equipped with a 5977B MSD (Agilent Technologies) and a split/splitless liner with the same parameters as described for ADA. The following fragments were used as quantifiers: linalool (m/z 93), d_5-linalool (m/z 98), geraniol (m/z 93), d_2-geraniol (m/z 95), methyl anthranilate (m/z 151), and d_3-methyl anthranilate (m/z 154).

Enantiomeric excess of linalool was determined according to Brescia *et al.*[22] Extraction was performed according to SIDA without addition of a deuterated standard using 85 g of the sample material, 100 mL of sat. NaCl solution, 100 mL of methanol, and 50 mL of pentane/diethyl ether (three times). Oven 1 (VF-WAXms 30 m, 250 μm, 0.25 μm; Agilent Technologies) was heated with 40 °C (3 min)/10 °C min^{-1}/220 °C (9 min), and oven 2 (Hydrodex-β-TBDAc 25 m, 250 μm; Macherey Nagel, Düren, Germany) was heated with 40 °C (3 min)/5 °C min^{-1}/85 °C (27.5 min).

2-Aminobenzaldehyde (m/z 121) was quantified by standard addition. Therefore, extraction was performed according to SIDA with the following modifications: 24 g of overgrown agar was homogenized, the pH was adjusted to 9.6, methanol/saturated NaCl solution (1/1, v/v) was added to a total volume of 200 mL, and 33.33 mL, corresponding to 4 g of the sample material, was pipetted into five flasks. To each flask, 0.5 mL of a 2-aminobenzaldehyde standard solution (0.00, 2.10, 4.21, 6.31, and 8.42 μg mL^{-1} dissolved in methanol) and 40 μL of IST (thymol, 750 mg L^{-1} dissolved in pentane/diethyl ether) were added. Odor activity values (OAV) were obtained by dividing the determined concentrations (c) by the respective odor threshold (OT) (eq 1)

$$\mathrm{OAV} = c \cdot \mathrm{OT}^{-1} \quad (1)$$

Recombination Experiments. 227.6 μg mL^{-1} (*R*)-linalool, 264.7 μg mL^{-1} methyl anthranilate, 16.6 μg mL^{-1} geraniol, and 92.5 μg mL^{-1} 2-aminobenzaldehyde were dissolved in purified water, and the pH was adjusted to 4.75. Brown-glass vials labeled with a randomized three-digit code were filled with 6 g of agar plugs (30 g L^{-1} agar-agar in water adjusted to pH 4.75), and 50 μL of the standard solution was added. After 2 h incubation, the samples and the reference of 6 g of overgrown PoA + Asp on d10 with 50 μL of water (pH 4.75) were presented to a trained panel (n = 10; 25−33 years, non-smokers, six women, four men). Panelists evaluated the olfactory impressions according to the sensory analysis described above.

Statistics. Statistical analysis of the recombination experiments was performed applying a two-sample t-test for dependent samples with $\alpha = 0.05$ to compare each attribute between the recombinate and the fermented sample.

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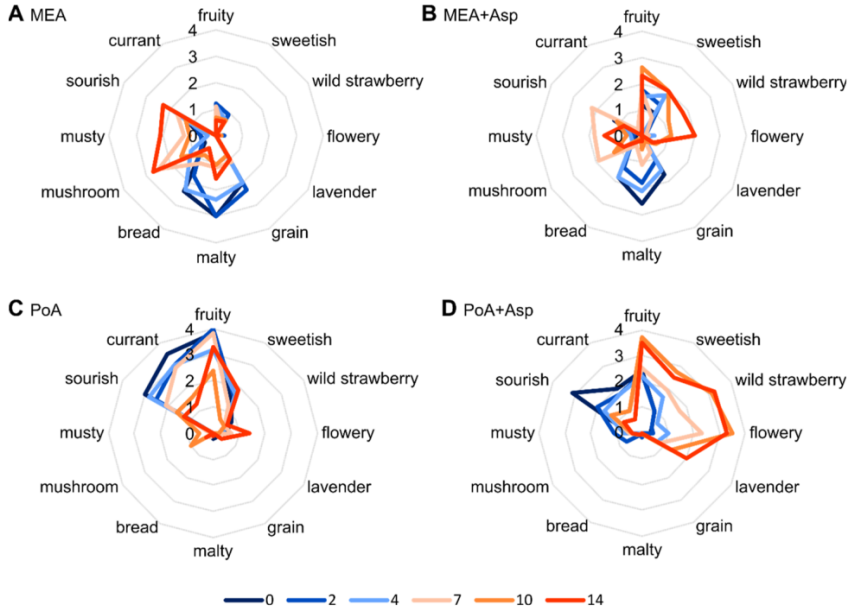

Figure 2. Sensory evaluation from 0 (no odor) to 5 (intense odor) of defined attributes for *W. cocos* grown on four media; MEA, MEA supplemented with sodium aspartate (MEA + Asp), pomace agar (PoA), and PoA supplemented with sodium aspartate (PoA + Asp) on days 0, 2, 4, 7, 10, and 14 shown in dark blue, blue, light blue, light orange, orange, and red, respectively (n = 10).

RESULTS

Medium Optimization and Sensory Analysis. In an initial screening, pomace and leaves of black currant served as the sole carbon and nitrogen source for 23 fungi. During the culture period of up to 3 weeks, plenty of interesting odor impressions were observed for several fungus−substrate combinations (Supporting Information, Table S1). The sensory impressions ranged from savory, onion-like for *Mycetinis scorodonius* grown on leaves to lemon-like for *Gloeophyllum odoratum* grown on pomace or leaves and fruity and flowery for the *W. cocos*-leaf combination. As *W. cocos* grew fast and produced a highly pleasant aroma, the influence of the medium composition and of various supplements on the flavor formation was investigated in detail. The addition of NH_4NO_3 in combination with sodium L-aspartate changed the aroma significantly. Separate evaluation of the two compounds revealed L-aspartate to be responsible for this change. Finally, the medium PoA + Asp led to an attractive odor impression reminiscent of wild strawberries.

To evaluate the influence of pomace and sodium L-aspartate on flavor generation, four media were designed: PoA, PoA + Asp, MEA, and MEA + Asp. Varying growth rates of *W. cocos* were observed on the different media, and the fungus showed different appearances on each medium (Figure 1A,B). On MEA, the mycelium of *W. cocos* spread as a fluffy, colorless filamentous structure. The growth was fast, and the surface of the plate was fully overgrown on culture day 7. On MEA + Asp, the mycelium was yellow and not fluffy, and the growth of the mycelium stagnated after 80% of the surface area was covered. The mycelium of *W. cocos* grown on PoA was fluffy and colorless and completely covered the surface after 8 days. The color of PoA changed from intense red before cultivation to light red to orange. On PoA + Asp, the mycelium was flat, almost invisible with few mycelia reaching the border of the plate after 9 culture days. The color of the agar changed from intense orange red to brown. Besides their different appearances, the pH values of the cultures differed between the four media during the cultivation (Figure 1C). In contrast to the other media, the pH value of PoA + Asp was nearly constant between 4.4 and 4.9 over the entire culture period. On MEA and PoA, the pH decreased to 2.5 after 7 days. On MEA + Asp, the pH dropped to 3.5 after 10 days and increased afterward to 6.5.

The odor attributes of the agar plates during fermentation with *W. cocos* were evaluated by a trained panel. Figure 2 shows the development of odor impressions over the cultivation period from inoculation (day 0) to day 14 for the four media. The cultures grown on MEA were described as malty, bread- and grain-like at the beginning of the fermentation and developed a musty, sourish, and mushroom-like odor during cultivation. The cultures on MEA + Asp also smelt like bread and malt on day 0 but developed an odor which was described as fruity, sweetish, flowery, and wild strawberry-like on one hand and musty, sourish, and mushroom-like on the other hand. The smell of the non-inoculated media PoA and PoA + Asp was described as fruity, sourish, and black currant-like. Throughout the fermentation, these attributes diminished, and a characteristic wild strawberry-like flavor without off-flavors was observed on PoA + Asp. A culture period of 10 days was found to be ideal for the production of a wild strawberry flavor on the medium PoA + Asp.

Identification of Aroma Compounds. In the GC−MS chromatogram (Figure 3), linalool **9** represented the most intense peak. Furthermore, (*Z*)-linalool oxide (furanoid) **7**, benzaldehyde **8**, 2-undecanone **10**, (*E*)-linalool oxide (pyranoid) **11**, and methyl anthranilate **16** were prominent compounds. The (*E*)- and (*Z*)-isomers of linalool oxides were tentatively identified according to literature data.[26,28] The identified compounds with RI on both polar and non-polar columns and the methods used for their identification are

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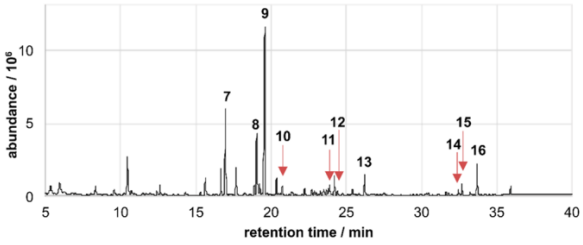

Figure 3. Gas chromatography–mass spectrometry measurement by means of dynamic headspace of overgrown pomace agar supplemented with sodium aspartate with *W. cocos* on day 10 with the labeled aroma compounds (numbers according to Table 1) (*Z*)-linalool oxide (furanoid) **7**, benzaldehyde **8**, linalool **9**, 2-undecanone **10**, (*E*)-linalool oxide (pyranoid) **11**, geraniol **13**, 6,7-epoxy linalool oxide **12**, 2-aminobenzaldehyde **14**, thymol (IST) **15**, and methyl anthranilate **16**.

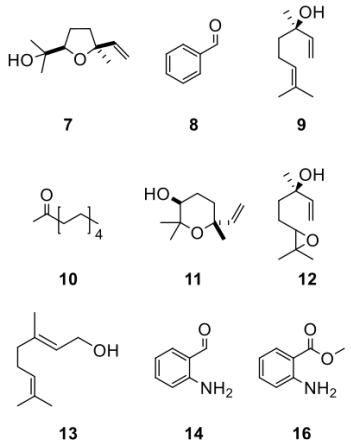

Figure 4. Structural formulae of compounds with characteristic flowery and fruity odors, (*Z*)-linalool oxide (furanoid) **7**, benzaldehyde **8**, (*R*)-linalool **9**, 2-undecanone **10**, (*E*)-linalool oxide (pyranoid) **11**, 6,7-epoxy linalool oxide **12**, geraniol **13**, 2-aminobenzaldehyde **14**, and methyl anthranilate **16**.

summarized in Table 1. Overall, 16 odorants were perceived, of which 14 compounds could be identified. Enantioselective analysis of the liquid extract revealed linalool to be the pure (*R*)-enantiomer. The odor of (*R*)-linalool **9** was described as flowery, citrus-, and lavender-like. The characteristic sweetish aroma reminiscent of wild strawberry was predominantly ascribed to 2-aminobenzaldehyde **14** and methyl anthranilate **16** (Figure 4). The three linalool oxides, namely, the (*Z*)-isomer of the furanoid linalool oxide **7**, the (*E*)-isomer of the pyranoid linalool oxide **11**, and 6,7-epoxy linalool oxide **12** were described as flowery, citrus, earthy, and rose-like. Some compounds showed a high signal but only weak olfactory impressions, for example, 2-nonanone, benzaldehyde, and (*E*)-linalool oxide (furanoid).

Validation of ADA for Solid Samples Using DHS. As the investigated sample was solid, the DHS ADA technique, developed by Brescia *et al.* for liquid samples, was first validated using a model system.[22] The split vents of TDU and CIS were sequentially combined to dilute the sample without the use of solvents. Linear correlations of the SR with the peak area of the EIC were shown for linalool, methyl anthranilate, and thymol (Figure 5A) as well as for (*E*)-, (*Z*)-linalool oxide, benzaldehyde, and geraniol (Supporting Information Figure S1A). Sequentially splitting in the TDU and CIS system showed a high linearity from SR 4 to 4096.

ADA of Surface Cultures of *W. cocos* by means of DHS. After validation of ADA using DHS for solid samples, the wild strawberry-like aroma of *W. cocos* grown on PoA + Asp was further investigated. High linearity was observed from SR 4 to 4096 with slopes of −0.9418 to −1.045 and R^2 values of 0.9799 to 0.9935 for linalool, geraniol, thymol (Figure 5B), geraniol, (*Z*)-, (*E*)-linalool oxides (furanoid), and benzaldehyde (Supporting Information Figure S1B). The highest FD of 2048 was determined for (*R*)-linalool **9**, followed by methyl anthranilate **16** (1024), 2-aminobenzaldehyde **14** (32),

Table 1. Identified Compounds with RIs, Odor Impression, the FD Factor, and Parameters for Identification

		RI				
	substance	VF-WAXms	DB-5ms	identification[a]	odor impression	FD factor
1	isobutyl acetate	1016	790	MS, RI, O, STD	sweetish, mushroom, musty	4
2	n.i.[b]	1070[c]			sweetish, peach, pastry	4
3	n.i.[b]	1088[c]			ethanol, sweetish	4
4	2-heptanone	1188	897	MS, RI, O, STD	sweetish, pastry, fruity	4
5	γ-terpinene	1247	1061	MS, RI, O, STD	coniferous forest, mushroom, tartly	4
6	1-octen-3-one	1309		MS, RI, O, STD	mushroom, *Agaricus*, cucumber	32
7	(*Z*)-linalool oxide (furanoid)	1449	1074	MS, RI, O, STD	earthy, mushroom, citrus	16
8	benzaldehyde	1534	968	MS, RI, O, STD	marzipan, sweetish	4
9	(*R*)-linalool	1556	1105	MS, RI, O, STD	citrus, lavender, flowery	2048
10	2-undecanone	1606	1293	MS, RI, O, STD	flowery, earthy, spicy	8
11	(*E*)-linalool oxide (pyranoid)	1770	1177	MS, RI, O, STD	rose, flowery, citrus	4
12	6,7-epoxy linalool oxide	1822		MS	rose, flowery, citrus	4
13	geraniol	1855	1252	MS, RI, O, STD	flowery, earthy, rose	32
14	2-aminobenzaldehyde	2176	1222	MS, RI, O, STD	sweetish, peach, wild strawberry	32
15	thymol (IST)	2189	1290		thyme, herbal	32
16	methyl anthranilate	2245	1344	MS, RI, O, STD	wild strawberry, sweetish, fruity	1024

[a]Parameters for identification: comparing mass spectra of the sample with the NIST database (MS), RI, and olfactory impression (O) with published data, and confirmation of all parameters with an authentic aroma standard (STD). [b]Compound not identified (n.i.). [c]RI calculated with the odor impression time.

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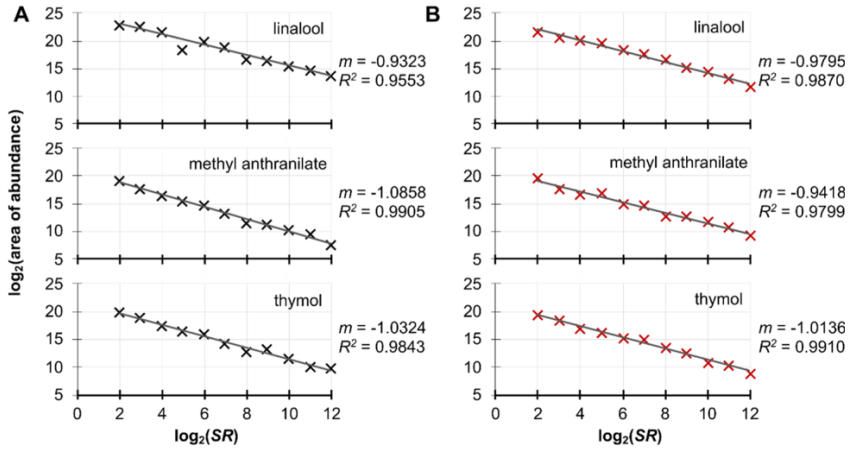

Figure 5. Log_2 of the area of the extracted ion chromatogram dependent on log_2 of the split ratios (SR) for linalool, methyl anthranilate, and thymol are shown for the standard mixtures in (A) and fermentation of *W. cocos* on PoA + Asp on d10 in (B). The slope (m) and coefficient of determination (R^2) are shown for the linear regression.

geraniol **13** (32), and 1-octen-3-one **6** (32). For all odor impressions with FD > 4, the corresponding compounds were identified.

Quantitation of Key Aroma Compounds. The compounds with the highest FD factors, namely, linalool, geraniol, methyl anthranilate, and 2-aminobenzaldehyde, were quantified. Linalool, geraniol, and methyl anthranilate were quantitated by SIDA (Figure S2). As no labeled standard was commercially available for 2-aminobenzaldehyde, this compound was quantitated by means of standard addition. Linear regressions are shown in Supporting Information (Figure S3). Concentrations, OTs, OT in water taken from the literature, and calculated OAV for minimum and maximum OT are shown in Table 2. As pure (*R*)-linalool was detected,

Table 2. Concentration *c*, Odor Threshold OT, and Odor Activity Value OAV of Aroma-Relevant Compounds: [Y] from Stable Isotope Dilution Analysis, [Z] from Standard Addition

	$c/\mu g\ kg^{-1}$	$OT/\mu g\ kg^{-1}$	OAV
(*R*)-linalool	1897 ± 1216[Y]	2.2[25]	862 ± 553
methyl anthranilate	2206 ± 923[Y]	7.73[41]	285 ± 119
2-aminobenzaldehyde	771 ± 548[Z]	11[42]	70 ± 50
geraniol	138 ± 61[Y]	3.2[43]	43 ± 19

the OT of this enantiomer was used.[29] The OAVs of linalool, methyl anthranilate, 2-aminobenzaldehyde, and geraniol were 862, 285, 70, and 43, respectively. Therefore, all quantified components most likely influenced the overall odor with OAVs higher than 1.

Recombination Experiments. For the recombination experiments, a model system was developed and evaluated by a trained panel. In Figure 6, the averages of the odor intensities of the recombinate and surface cultures of *W. cocos* grown on PoA + Asp (day 10) are displayed. Averages, standard deviations, *t*-statistics, and *P*-values of the perceived odors are listed in Supporting Information, Table S2. Odor impressions including flowery, fruity, wild strawberry-like, sweetish, and lavender dominated the flavor of the cultures (day 10) with average values of 3.2, 3.1, 3.1, 2.7, and 1.8, respectively. The intensities of the odors perceived from the recombinate model were well comparable with an average of

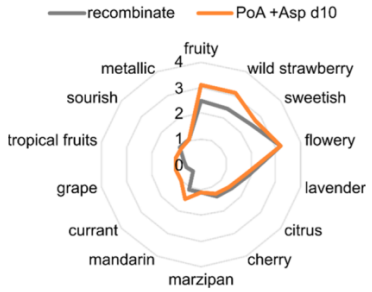

Figure 6. Sensory analysis of *W. cocos* cultivated on pomace agar supplemented with sodium aspartate (PoA + Asp) for 10 days (orange) and a recombinate consisting of agar, methyl anthranilate, (*R*)-linalool, 2-aminobenzaldehyde, and geraniol in measured concentrations at pH 4.75 (gray) assessed by a panel from 0 (no odor) to 5 (very intense) for defined attributes with a value ≥1 (n = 10).

3.2 for flowery, 2.5 for fruity, 2.4 for wild strawberry, 2.5 for sweetish, and 1.9 for lavender. The other odors showed average values < 1.5.

DISCUSSION

In the present study, a screening of fungi on side streams of the black currant juice production was performed to find a suitable candidate for the biotransformation of the agricultural byproducts. Finding a higher fungus as a biocatalyst could lead to a more efficient use of bioresources as pomace and leaves are currently mostly discarded.[6] A highly pleasant aroma was observed for cultures of *W. cocos* grown on black currant leaves as well as on pomace of black currant supplemented with aspartate. The cultivation of *W. cocos* on black currant pomace thus represents a unique fungus−substrate combination for the sustainable and cost-efficient production of the wild strawberry-like flavor.

Medium optimization experiments revealed that sodium L-aspartate had a negative impact on the growth of *W. cocos* but positively affected its ability to produce the wild strawberry-like odor. *W. cocos* has been described as a potent producer of aroma compounds previously.[14,30,31] Brown-rot fungi, like *W.*

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cocos, release organic acids into their surrounding environment to decrease the pH, which is necessary for cellulose depolymerization and absorption of nutrients.[13,32] A higher pH value in L-aspartate-supplemented surface cultures may thus explain the decelerated growth of *W. cocos*. Furthermore, the pleasant aroma of PoA + Asp correlated with the constant pH of the surface cultures. Interestingly, the intense wild strawberry-like flavor was solely produced in surface cultures of *W. cocos* and not in parallel grown submerged cultures (data not shown). The wild strawberry-like flavor production by *W. cocos* was triggered in both aspartate-containing media, but PoA + Asp lacked the unpleasant, sourish, and musty odor impressions that were perceived with the MEA + Asp medium. Therefore, pomace is essential for the pleasant overall wild strawberry-like impression. In wild strawberries, methyl anthranilate has been described as the key aroma compound, and it is not found in cultivated strawberries.[16]

For detailed analysis of the wild strawberry-like aroma emitted by surface cultures of *W. cocos*, flavor analysis was performed by DHS. DHS showed a very high selectivity for extracting aroma-active compounds, without extracting fatty acids and long-chain esters. Apart from this, it is an easy, quick, solvent-free, and targeted tool for flavor analysis.[22] It allows for the simultaneous extraction of terpenes and aromatic compounds, for example, 2-aminobenzaldehyde.

Since the samples were solid agar plates covered with fungal mycelium, it was assumed that the aroma compounds were not uniformly distributed, and thus, the production of homogenous samples was challenging. Therefore, 100 equal samples were prepared, requiring 8 surface cultures. To compensate for biological variations between the plates used, the sample material from all plates was systematically distributed among the samples. To conclude, the produced sample material showed the same intensities and a very high linearity in ADA. Therefore, ADA by means of DHS is a suitable technique for solid samples. Furthermore, it combines the advantages of being sensitive, quick, and easy.

Quantification of the aroma-relevant compounds revealed high standard deviations of up to 71% of the average concentration. As the experiments were performed in biological triplicates that were grown during a period of several weeks, the high standard deviations most likely reflect the biological variations of the fungal cultures. Linalool was detected in comparatively high amounts, and chiral analysis revealed the presence of enantiopure (*R*)-linalool. This is well in agreement with the literature as so far, only an enantioselective synthase for (*R*)-linalool has been described for Basidiomycota.[29] Besides the well-known (*E*)- and (*Z*)-furanoid and (*E*)- and (*Z*)-pyranoid linalool oxides, 6,7-epoxy linalool oxide was also detected, and all these oxides have been described in different foods, plants, and fungi, for example, coriander, *Calocybe gambosa*, and *Cortinarius odorifer*.[27,33,34] The biosynthesis of linalool oxides starting from (*R*)-linalool has been investigated in *A. niger*, *Botrytis cinerea*, and *Corynespora cassiicola*.[35] The citrus-like and flowery odor of (*R*)-linalool appeared to strongly influence the overall aroma of the cultures with an OAV of 862 ± 552. Linalool is a known compound in strawberries.[16] Another detected monoterpene was geraniol with its characteristic flowery odor. The biosynthesis of linalool and geraniol by *W. cocos* has been described previously for cultures grown on green tea, carrot peels, wine press cake, and potato dextrose broth supplemented with vitamin B_1 and corn liquor.[30,36]

Methyl anthranilate is known to exclusively occur in wild strawberries and not in cultivated ones.[16] As it has an odor impression that is highly reminiscent of wild strawberries and it has the highest FD factor, it has a major impact on the overall aroma. The biosynthesis of methyl anthranilate has not been investigated in detail for fungi, but biotransformation of dimethyl anthranilate or anthranilic acid has been suggested.[37] Zorn *et al.* and Rigling *et al.* revealed *W. cocos* as a potent producer of methyl anthranilate on different substrates, for example, green tea infusion, strawberry sieving residues with aspartate, raspberry sieving residues with aspartate, and wine pomace with aspartate.[30,31]

2-Aminobenzaldehyde with its characteristic wild strawberry-like odor has rarely been described as an aroma compound. Mostly, 2-aminobenzaldehyde occurs in flowers, fruits, or fungi. For instance, it has been detected in the flowers of false acacia (*Robinia pseudoacacia*) and broom (*Spartium junceum*), the mobola plum (*Parinari curatellifolia*), and the fungus *Hebeloma sacchariolens*.[38−40] Often, 2-aminobenzaldehyde and methyl anthranilate occur together. Biosynthetic studies were performed for the fungus *H. sacchariolens*. This study revealed anthranilic acid to be the biosynthetic precursor, which is directly reduced to the aldehyde. Anthranilic acid might thus be the precursor for both methyl anthranilate and 2-aminobenzaldehyde. Further investigations will be necessary to gain knowledge about the genes and enzymes involved in fungal biosynthesis of 2-aminobenzaldehyde and other anthranilic acid derivatives. Due to the high tendency to form polymers and its easy protonation under acidic conditions, extraction of 2-aminobenzaldehyde is challenging, but dynamic headspace proved to be a suitable technique.[40] 2-Aminobenzaldehyde showed an OAV of at least 20. Its sweet odor reminiscent of wild strawberries and peach intensifies the wild strawberry-like odor of the cultures. Comparing the aroma profile of *W. cocos* cultivated on green tea and on black currant pomace supplemented with aspartate, 2-aminobenzaldehyde appears to play an important role for the odor reminiscent of wild strawberries. Rigling *et al.* (2021) described the overall flavor impression of tea fermented with *W. cocos* as floral, jasmine-like, and slightly citrus-like. In their study, the OAV of methyl anthranilate was 802, followed by linalool (190), 2-phenylethanol (165), and geraniol (118). Different from the fermentation of tea, no acetic acid, fatty acids like nonaic acid, and additional aromatic compounds (2-phenylethanol, methyl phenyl acetate, *p*-anisaldehyde) were detected in this study for the cultures grown on black currant pomace supplemented with aspartate. Furthermore, Rigling *et al.* did not detect linalool oxides or methyl ketones, which have been identified in this study. This well illustrates the influence of the substrate as a carbon and nitrogen source as well as of the culture system (submerged cultures *vs* surface cultures) on the formation of the overall flavor profile during fermentation.[30]

The calculated OAVs of methyl anthranilate, linalool, 2-aminobenzaldehyde, and geraniol were >1, suggesting that all of them had an impact on the perceived odor of the cultures. The reconstituted aroma model reflected the odor of the sample very well. Thus, the identification and quantitation of all of the main aroma compounds was clearly confirmed. Linalool, geraniol, methyl anthranilate, and 2-aminobenzaldehyde were responsible for the wild strawberry flavor of *W. cocos* grown on PoA+Asp.

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To conclude, this study showed that *W. cocos* grown on black currant pomace and aspartate were a promising fungus–substrate combination for the biotechnological production of the wild strawberry-like flavor. ADA by means of DHS for solid samples as a fast, selective, and precise method was reported and validated here for the first time. GC–O analysis detected 16 compounds, including the highly reactive aroma compound 2-aminobenzaldehyde. Quantifying the four compounds with the highest FD factors, methyl anthranilate, linalool, 2-aminobenzaldehyde, and geraniol and recombining them in the determined concentrations showed their relevance for the perceived odor. Due to their characteristic wild strawberry-like odor, methyl anthranilate and 2-aminobenzaldehyde are especially important for the odor. Using *W. cocos* as a biocatalyst, the side stream black currant pomace was converted to a highly pleasant aroma mixture, which may be industrially used as natural flavoring.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c05770.

> Strains included in the initial screening for aroma production; two-sample *t*-test of dependent samples comparing recombinate and sample materials; correlations of split ratios and peak areas for geraniol, linalool oxides, and benzaldehyde; RFs for SIDA; and quantitation of 2-aminobenzaldehyde by use of standard addition (PDF)

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Notes

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■ ABBREVIATIONS

ADA, aroma dilution analysis; AEDA, aroma extract dilution analysis; Asp, mono sodium L-aspartate monohydrate; *c*, concentration; CIS, cold injection system; DHS, dynamic headspace; EIC, extracted ion count; FD, flavor dilution; GC–MS–O, gas chromatography–mass spectrometry–olfactometry; IST, internal standard; *m*, slope; MEA, malt extract agar; MEA + Asp, malt extract agar supplemented with Asp; n.i., not identified; NIST, National Institute for Standards and Technologies; OAV, odor activity value; ODP, olfactory detection port; OT, odor threshold; PoA, black currant pomace agar; PoA + Asp, black currant pomace agar supplemented with Asp; R^2, coefficient of determination; RI, retention index; rpm, rounds per minute; σ, standard deviation; SAFE, solvent-assisted flavor evaporation; SIDA, stable isotope dilution analysis; SR, split ratio; STD, standard; TDU, thermal desorption unit; TIC, total ion count; *W. cocos*, *Wolfiporia cocos*

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5 Chapter 3

Upcycling of black currant pomace for the production of a fermented beverage with *Wolfiporia cocos*

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6 Chapter 4

Odor characteristics of novel non-canonical terpenes

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Odor Characteristics of Novel Non-Canonical Terpenes

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Abstract: Several non-canonical, methylated terpenes have been described as products of genetically modified *Escherichia coli* recently, and the aroma properties of 28 odor-active methylated derivatives of prenol, isoprenol, bornane, camphene, carene, citronellol, fenchol, geraniol, limonene, linalool, terpineol, and farnesol were characterized for the first time in the current study. Twelve methylated monoterpenes exhibited a particularly intense and pleasant odor and were therefore chosen for the determination of their respective odor thresholds (OTs) in comparison to their non-methylated equivalents. In addition to the determination of OTs based on the literature value for the internal standard, (2*E*)-decenal, the threshold values of the compounds with individually determined OTs of the participants were calculated. This enabled a more precise identification of the OTs. Among the non-canonical terpenes, the lowest OTs in the air were found for 2-methyllinalool (flowery, 1.8 ng L^{-1}), 2-methyl-α-fenchol (moldy, 3.6 ng L^{-1}), 2-methylgeraniol (flowery, 5.4 ng L^{-1}), 2-methylcitronellol (citrus-like, 7.2 ng L^{-1}), and 4-methylgeraniol (citrus-like, 16 ng L^{-1}). The derivatives of geraniol, linalool, and citronellol showed very pleasant odor impressions, which could make them interesting for use as flavoring agents in the flavor and fragrance industry.

Keywords: methylation; odor threshold; terpenoids; (2*E*)-decenal; terpene; flavor

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1. Introduction

Isoprenoids are flavor compounds, which are known for their great structural diversity and their intense odor impressions. Most isoprenoids are formed from the C_5-prenyl pyrophosphate precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The repeated appearance of isoprene units in terpene structures was enunciated as the isoprene rule [1]. Completed isoprenoid structures contain one or more isoprene units and differ in the occurrence of double bonds, carbonyl, carboxyl, keto, and hydroxyl groups. Aliphatic structures are named terpenes, whereas structures with functional groups are called terpenoids. Both terpene and terpenoid structures have been detected as secondary metabolites in plants, animals, and microorganisms [2]. Especially, short-chain terpenoids are relevant as aroma compounds, including hemi- (C_5), mono- (C_{10}), and sesquiterpenoids (C_{15}). A common example for hemiterpenoids is prenol, which occurs, e.g., in hop or ylang-ylang flowers [3]. Monoterpenoids and monoterpenes include highly odor-active compounds such as linalool with a citrus- and lavender-like scent, thymol with a thyme-like flavor, and limonene with a fresh, orange-like odor of the (*R*)-enantiomer and a pine-like flavor of the (*S*)-enantiomer. Farnesol and (*S*)-nerolidol are examples of sesquiterpenoids that are associated with a flowery scent [2].

Exceptions to the isoprene rule are terpenes whose biosynthesis differs from the sequential condensation of the C_5 units, therefore generating structures with a number of carbon

atoms different from a multiple of five. These terpenes are called non-canonical terpenes. Non-canonical terpenes have been studied since the formulation of the isoprene rule itself, e.g., carotenoid degradation products. More recent studies have addressed the synthesis of non-canonical terpenes by means of methyl transferases. These enzymes catalyze the addition of methyl groups to the prenyl pyrophosphate precursors, thereby changing the final number of carbon atoms of the terpenoid structures. The methylated monoterpene 2-methylisoborneol **1** has been described, for example, in *Streptomyces* and *Actinomyces* species with an unpleasant muddy flavor and an extremely low odor threshold of 0.042 µg L^{-1} in water [4]. Furthermore, 2-methyl-2-bornene **2**, 1-methylcamphene **3**, and 2-methylenebornane **4** have been described in forest soil [5], and the methylated monoterpenes and monoterpenoids 2-methylgeraniol **5**, 2-methyllinalool **6**, 2-methyllimonene **7**, and 2-methyl-α-terpineol **8**, have been identified as products of *Nannocystis exedens* [6] (Figure 1).

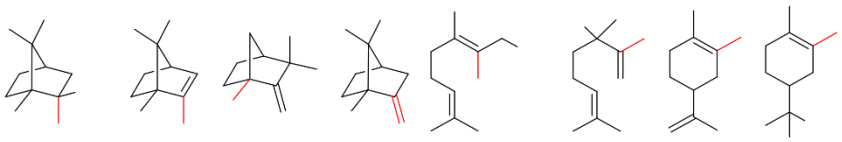

Figure 1. Structures of methylated terpenes, which have been identified in the environment with red-labeled bonds to the additional methyl group: 2-methylisoborneol **1**, 2-methyl-2-bornene **2**, 1-methylcamphene **3**, 2-methylenebornane **4**, 2-methylgeraniol **5**, 2-methyllinalool **6**, 2-methyllimonene **7**, and 2-methyl-α-terpineol **8**.

Harms et al. investigated methylated sesquiterpenes such as iso-β-elemene and isogermacrene, which were synthesized with a sesquiterpene synthase and showed potential as flavor compounds. Both have a citrus-like odor impression [7]. Kschowak et al. transformed *Escherichia coli* for the microbial production of novel C_{11} compounds, and Ignea et al. modified *Saccharomyces cerevisiae* to produce C_{11} terpenoids [8,9]. The genes encoding terpene synthases, including 2-methylisoborneol synthase from *Streptomyces griseus* subsp. *griseus*, 2-methylisoborneol synthase from *Streptomyces coelicolor*, 2-methylene bornane synthase from *Micromonospora olivasterospora*, and 2-methylene bornane synthase from *Pseudomononas fluorescens*, together with a geranyl pyrophosphate methyl transferase from *Streptomyces coelicolor*, were transferred. The *E. coli* strains in the above-mentioned study also included genes encoding an isopentenyl pyrophosphate (IPP) isomerase, the enzymes for the mevalonate pathway, and a geranyl pyrophosphate synthase. Kschowak et al. analyzed volatile compounds with solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) and detected several C_{11} compounds, of which 15 were identified [9]. For example, the study identified 6-methylfarnesol **9** and methylated monoterpenes such as 2-methylgeraniol **5**, 2-methyllinalool **6**, 2-methyllimonene **7**, 2-methyl-α-terpineol **8,** 2-methyl-α-fenchol **10**, 2-methylcitronellol **11**, and 2-methylnerol **12** (Figure 2).

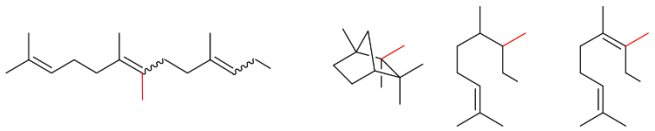

Figure 2. Structures of methylated terpenes described by Kschowak et al. with red-labeled bonds added to the additional methyl group: 6-methylfarnesol **9**, 2-methyl-α-fenchol **10**, 2-methylcitronellol **11**, and 2-methylnerol **12**.

Furthermore, Drummond et al. investigated the *S*-adenosyl methionine (SAM)-dependent IPP methyltransferase from *Streptomyces monomycini* and transferred the respon-

sible genes in *E. coli* [10]. This enabled the formation of the methylated precursors (*E*)-, (*Z*)-4-methyl-IPP, 4-methyl-DMAPP, 4,4-dimethyl-IPP, and 4,4-dimethyl-DMAPP, which were released in the form of C_6 and C_7 alcohols. Some of these methylated precursors were accepted by a native *E. coli* farnesyl pyrophosphate (FPP) synthase, and the corresponding C_{11}, C_{12}, C_{16}, and C_{17} compounds were formed. Examples of terpene alcohols identified in the mentioned study include (*Z*)-4-methylisoprenol **13**, (*E*)-4-methylisoprenol **14**, (*E*)- and (*Z*)-4-methylprenol **15** and **16**, 4,4-dimethylprenol **17**, 4,4-dimethylisoprenol **18**, 4-methylgeraniol **19**, 8-methylgeraniol **20**, and 4-methylfarnesol **21** (Figure 3). The biotechnological production using *E. coli* enabled the generation of a wide range of novel compounds, which have not been analyzed regarding their flavor properties so far. Due to their similarity to potent odor-active terpenes, they exhibited interesting flavor characteristics.

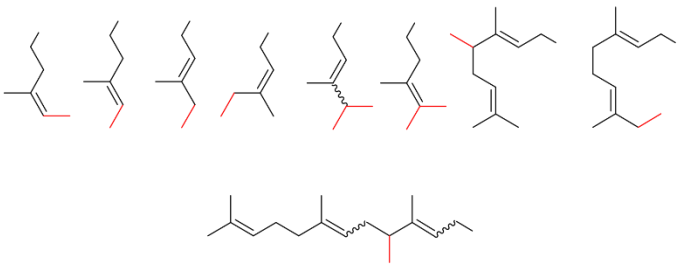

Figure 3. Structures of methylated terpenes described by Drummond et al. with red-labeled bonds to the additional methyl groups: (*Z*)-4-methylisoprenol **13**, (*E*)-4-methylisoprenol **14**, (*E*)-4-methylprenol **15**, (*Z*)-4-methylprenol **16**, 4,4-dimethylprenol **17**, 4,4-dimethylisoprenol **18**, 4-methylgeraniol **19**, 8-methylgeraniol **20**, and 4-methylfarnesol **21**.

Odor perception depends on the volatility of the compounds and the molecule geometry, which determines the interaction of the odotopes with the corresponding olfactory receptor proteins. Individual perceptions may differ between panelists, and the odor threshold (OT) values are not predictable so far by computational simulation [11,12]. Furthermore, fragrance impressions typically differ between the enantiomers. For instance, the mean OT of (+)-nootkatone is approximately 800-fold higher compared to that of its (–)-enantiomer (0.6–1.0 µg L^{-1} and 400–800 µg L^{-1} in water) [13]. The odor impression of (*S*)-carvone reminds one of caraway, whereas (*R*)-carvone has a minty odor [14]. The methylation of aroma compounds may also lead to aroma changes and different aroma thresholds. For example, ethyl vanillin smells vanilla-like but has an OT that is four times lower than that of vanillin [15]. 2-Nonanone has a fresh, sweetish, green, and weedy flavor, whereas 2-decanone is perceived as orange, peach-like, floral, and fatty [16,17].

The determination of OTs in water (OT_w) is often performed according to Czerny et al., where the component is diluted in water and evaluated in descending concentrations in a triangle test in comparison to blanks that do not contain the aroma compound [18]. Teranishi et al. used the air to water partition coefficient to calculate the corresponding OT in air (OT_{air}). According to their theory, the OT in air is proportional to the threshold in water, only depending on the relative portions of the flavor compound in the air and dissolved in water [19]. Ullrich and Grosch established a method to determine the OT in air using gas chromatography-olfactometry (GC-O) and an internal standard (IS) [20]. The standard needs to be pure, chromatographically separated from the target compound, and must have a known OT in the air. In recent studies (2*E*)-decenal became the most commonly used IS [21–24].

In this study, 28 methylated hemi-, sesqui-, and diterpenes were analyzed to determine whether they are odor-active. Especially interesting compounds were investigated by means of GC-O to determine the OT_{air} of the methylated compounds in direct comparison

to those of their non-methylated analogs. To investigate whether the published OT_{air} of the IS (2*E*)-decenal is representative of the participants, the OT in water was determined for every participant and used to calculate the individual OTs in the air.

2. Results

2.1. Determination of Purities, Response Factors, and Mass Spectra of the Methylated Compounds

As some of the synthesized non-canonical terpene standards available contained both of the respective (*E*)- and (*Z*)-isomers, their diastereomeric purities were determined. The isomers were separated by means of gas chromatography with the help of two columns of different polarity (Table 1).

Table 1. (**a**) Retention indices on a polar VF-WAXms column and a nonpolar DB-5ms column, ratios of (*E*/*Z*) isomers, ratios of (*R*)- and (*S*)-enantiomers, and enantiomeric excess (*ee*). (**b**) Retention indices on a polar VF-WAXms column and a nonpolar DB-5ms column, ratios of (*E*/*Z*) isomers, ratios of (*R*)- and (*S*)-enantiomers, and enantiomeric excess (*ee*).

		(a)		
	Compound	**RI**	**Ratios/%**	***ee*/%**
1	2-methylenebornane **4**	VF-WAXms: 1120 DB-5ms: 1017	- -	-
2	(*S*)-1-methylcamphene **3**	VF-WAXms: 1075 DB-5ms: 985	- (*R*/*S*): 0/100	100%
3	4-methyl-3-carene **22**	VF-WAXms: 1229 DB-5ms: 1091	- -	-
4	2-methylcitronellol **11**	VF-WAXms: 1824, 1834 DB-5ms: 1301, 1305	(*E*/*Z*): 64/36 $^{\#}$ (*R*/*S*): 65 $^{+}$/35 $^{+,\#}$	30%
5	4-methylfarnesol **21**	VF-WAXms: 2348 DB-5ms: 1749	- -	-
6	6-methylfarnesol **9**	VF-WAXms: 2380, 2430 DB-5ms: 1790	- -	-
7	(*S*)-2-methyl-α-fenchol **10**	VF-WAXms: 1606 DB-5ms: 1199	- (*R*/*S*): 0/100	100%
8	2-methylgeraniol **5**/2-methylnerol **12**	VF-WAXms: 1843 ^{Z,*}, 1884 ^{E} DB-5ms: 1299 ^{Z,*}, 1317 ^{E}	(*E*/*Z*): 50/50 -	-
9	4-methylgeraniol **19**/4-methylnerol **23**	VF-WAXms: 1807 ^{Z,*}, 1857 ^{E} DB-5ms: 1265 ^{Z,*}, 1293 ^{E}	(*E*/*Z*): 8/14 (*R*/*S*): 100/0 $^{\#}$	100%
10	8-methylgeraniol **20**/8-methylnerol **24**	VF-WAXms: 1919 ^{Z,*}, 1923 ^{E} DB-5ms: 1334 ^{Z,*}, 1341 ^{E}	(*E*/*Z*): 75/25 -	-
11	2-methylisoprenol **25**	VF-WAXms: 1283 DB-5ms: 812	- -	-
12	5-methylisoprenol **26**	VF-WAXms: 1348 DB-5ms: 842	- -	-
13	(*E*)-4-methylisoprenol **14**	VF-WAXms: 1363 DB-5ms: 861	- -	-
14	(*Z*)-4-methylisoprenol **13**	VF-WAXms: 1374 DB-5ms: 856	- -	-

Table 1. *Cont.*

	(b)			
	Compound	**RI**	**Ratios/%**	***ee*/%**
15	2,4-dimethylisoprenol **27**	VF-WAXms: 1393, 1401 DB-5ms: 916, 924	(*E*/*Z*): 86/14 # -	-
16	2,5-dimethylisoprenol **28**	VF-WAXms: 1378 DB-5ms: 904	- -	-
17	4,4-dimethylisoprenol **18**	VF-WAXms: 1477 DB-5ms: 958	- -	-
18	4,5-dimethylisoprenol **29**	VF-WAXms: 1441, 1467 DB-5ms: 944, 949	(*E*/*Z*): 63/37 # -	-
19	5,5-dimethylisoprenol **30**	VF-WAXms: 1400 DB-5ms: 906	- -	-
20	2-methyllimonene **7**	VF-WAXms: 1299 DB-5ms: 1122	- (*R*/*S*): 50/50	0%
21	2-methyllinalool **6**	VF-WAXms: 1620 DB-5ms: 1190	- (*R*/*S*): 50/50	0%
22	2-methylprenol **31**	VF-WAXms: 1407 DB-5ms: 877	- -	-
23	(*Z*)-4-methylprenol **16**	VF-WAXms: 1393 DB-5ms: 866	- -	-
24	(*E*)-4-methylprenol **15**	VF-WAXms: 1416 DB-5ms: 881	- -	-
25	2,4-dimethylprenol **32**	VF-WAXms: 1467, 1478 DB-5ms: 951, 956	(*E*/*Z*): 50/50 -	-
26	4,4-dimethylprenol **17**	VF-WAXms: 1448, 1470 DB-5ms: 929, 944	(*E*/*Z*): 13/87 # -	-
27	4,5-dimethylprenol **33**	VF-WAXms: 1487 DB-5ms: 959	- -	-
28	2-methyl-*α*-terpineol **8**	VF-WAXms: 1785 DB-5ms: 1286	- -	-

* = (*Z*)-isomer of methyl-geraniol is called methyl-nerol, + = enantiomeric ratio of both (*E*/*Z*) isomers; and # = only relative portions are available, no assignment to (*R*) or (*S*) and (*E*) or (*Z*); ratios are listed according to their retention times on VF-WAXms for (*E*/*Z*) or chiral column for (*R*/*S*).

The standards of 2,4-dimethylisoprenol (line 15), 4,5-dimethylisoprenol (line 18), 4,4-dimethylprenol (line 26), 2,4-dimethylprenol (line 25), 2-methylcitronellol (line 4), 2-methylgeraniol (line 8), and 4-methylgeraniol (line 9) contained isomers that could be separated on a VF-WAXms column. The ratios of 8-methylgeraniol and 8-methylnerol (line 10) were determined on a DB-5ms column. The GC-MS spectra are listed in the Supplementary Materials (Table S1). Furthermore, the ratios of the enantiomers were measured using two different chiral columns. 2-Methyllinalool (line 21), 2-methyllimonene (line 20), and 2-methylcitronellol (line 4) represented mixtures of both enantiomers (Table 1). 4-Methylgeraniol (line 9), (*R*)-camphene, (*S*)-2-methyl-*α*-fenchol (line 7), (*R*)-*α*-fenchol, and (*S*)-1-methylcamphene (line 2) were found to be pure enantio.

2.2. Odor Description of Methylated Hemi-, Mono-, and Sesquiterpenes

The odor impressions of methylated hemi-, mono-, and sesquiterpenes (Table 2) and of their analogous non-methylated compounds (Supplementary Materials Table S2) were described independently by 15 participants. All methylated compounds except for 6-methylfarnesol (line 6) were described with the same attributes by at least three participants. Only seven participants noted a weak odor impression for 6-methylfarnesol,

whereas the others did not smell anything. The comparison of the methylated prenol derivatives with prenol and methylated isoprenol derivatives with isoprenol indicated that the position of the methyl group had an influence on the respective odor quality (Tables 2 and S2).

Table 2. Odor descriptions of pure methylated hemi-, mono-, and sesquiterpenes, which were given by at least three participants, with the number of mentions in parentheses (n = 15).

	Substances	Odor Impression	Intensity
1	2-methylenebornane **4**	earthy (4), coniferous forest (3), resinous (3)	0.9 ± 0.8
2	(*S*)-1-methylcamphene **3**	resinous (10), coniferous forest (9), woody (3), fruity (3)	3.3 ± 1.0
3	4-methyl-3-carene **22**	fruity (7), coniferous forest (7), resinous (6), sweetish (4), pepper (4), mint (3), citrus (3)	3.5 ± 0.8
4	2-methylcitronellol **11**	flowery (8), citrus (6), rose (4), sweetish (3), ethereal (3), fruity (3)	3.9 ± 0.8
5	4-methylfarnesol **21**	citrus (5), resinous (5), green (3)	1.7 ± 1.0
6	6-methylfarnesol **9**	- #	0.7 ± 0.8
7	(*S*)-2-methyl-α-fenchol **10**	earthy (13), moldy (9), moss (3), beetroot (3)	4.8 ± 0.4
8	2-methylgeraniol **5**/2-methylnerol **12** *	flowery (8), citrus (5), resinous (4), rose (4), sweetish (3)	2.9 ± 1.4
9	4-methylgeraniol **19**/4-methylnerol **23** *	citrus (8), lemon (3), lemon peel (3)	3.7 ± 0.8
10	8-methylgeraniol **20**/8-methylnerol **24** *	flowery (8), resinous (6), sweetish (5), citrus (4), varnish (4)	2.5 ± 0.8
11	2-methylisoprenol **25**	resinous (5), sweetish (3), coniferous forest (3), fruity (3)	1.6 ± 1.2
12	(*E*)-4-methylisoprenol **14**	green (8), grass (4), herbal (4), coniferous forest (3), apple (3)	2.5 ± 0.8
13	(*Z*)-4-methylisoprenol **13**	flowery (9), green (6), fruity (5), apple (4)	3.1 ± 1.0
14	5-methylisoprenol **26**	pungent (6), solvent (6), glue (4), varnish (3)	4.6 ± 0.8
15	2,4-dimethylisoprenol **27**	coniferous forest (5), green (4), resinous (4)	2.5 ± 0.7
16	2,5-dimethylisoprenol **28**	resinous (9), coniferous forest (7), mint (3), green (3), varnish (3)	3.2 ± 1.2
17	4,4-dimethylisoprenol **18**	green (6), citrus (4), flowery (3), soapy (3), grass (3)	2.5 ± 1.1
18	4,5-dimethylisoprenol **29**	resinous (4), woody (3), coniferous forest (3)	1.5 ± 1.1
19	5,5-dimethylisoprenol **30**	coniferous forest (10), resinous (8), woody (3)	3.4 ± 1.1
20	2-methyllimonene **7**	resinous (6), terpene (4), mushroom (4)	3.7 ± 0.8
21	2-methyllinalool **6**	flowery (11), citrus (9), sweetish (8), fruity (6), bergamot (5), blueberry (4), lavender (3)	3.7 ± 0.5
22	2-methylprenol **31**	plastic (3), terpene-like (3)	1.5 ± 0.9
23	(*Z*)-4-methylprenol **16**	plastic (3), terpene-like (3), chemical (3)	1.7 ± 0.8
24	(*E*)-4-methylprenol **15**	sweetish (5), flowery (5), green (5), citrus (3), fresh (3), resinous (3)	2.3 ± 1.4
25	2,4-dimethylprenol **32**	resinous (4), woody (3), coniferous forest (3), glue (3), sweetish (3)	2.8 ± 1.3
26	4,4-dimethylprenol **17**	sweetish (6), fruity (3)	2.1 ± 1.3
27	4,5-dimethylprenol **33**	woody (6), resinous (3), plastic (3)	3.1 ± 1.1
28	2-methyl-α-terpineol **8**	sweetish (4), green (3)	1.1 ± 1.1

* Mixture of (*E*)- and (*Z*)-isomers. # No impression was named by ≥3 participants.

Apart from 2-methyl-α-terpineol and 2-methylenebornane (line 1, 28), which exhibited only a relatively weak odors, the methylated monoterpenes showed intense aroma impressions. Methyl-α-fenchol (line 7) was evaluated with the highest intensity, but also, 1-methylcamphene (line 2); 4-methyl-3-carene (line 3); 2-methyllimonene (line 20); 2-methyllinalool (line 21); 2-, 4-, and 8-methylgeraniol (line 8–10) had intense flavors. Therefore, these compounds were chosen for the determination of their respective OT_{air}.

2.3. Odor Threshold of (2E)-Decenal in Water and Air

For comparison with the literature, the detection and recognition thresholds of (2*E*)-decenal were determined in triplicate. For further usage, the concentration at which at least two replicates were correctly identified was defined as the odor threshold. The

participants had different detection thresholds (DT): participant 1: DT = 0.9 ± 0.3 µg L^{-1} (0.9, 0.9, and 1.8 µg L^{-1}); participant 2: DT = 0.5 ± 0.1 µg L^{-1} (0.2, 0.5, and 0.9 µg L^{-1}); and participant 3: DT = 1.8 ± 0.6 µg L^{-1} (0.5, 1.8, and 1.8 µg L^{-1}). The recognition thresholds (RT) also differed among the participants: participant 1: RT = 3.6 ± 1.2 µg L^{-1} (1.8, 3.6, and 3.6 µg L^{-1}); participant 2: RT = 0.9 ± 0.3 µg L^{-1} (0.9, 0.9, and 0.9 µg L^{-1}); and participant 3: RT = 1.8 ± 0.6 µg L^{-1} (0.4, 1.8, and 1.8 µg L^{-1}). The DT was used to calculate the OT_{air}:

1. $OT_{air,IS}$ (participant 1) = 8.0 ± 2.7 ng L^{-1};
2. $OT_{air,IS}$ (participant 2) = 4.0 ± 1.3 ng L^{-1};
3. $OT_{air,IS}$ (participant 3) = 16.0 ± 5.3 ng L^{-1}.

2.4. Odor Thresholds in Air

The OT_{air} of the methylated compounds were determined in comparison to their non-methylated counterparts, which were commercially available. To each terpene mixture, (2*E*)-decenal was added as the internal standard. The D-values of the internal standard and the other compounds slightly differed between participants. The D-value is defined according to the literature as the dilution factor in which the compound can be smelled in the lowest concentration [20] (Supplementary Materials Table S3). Odor descriptions of the participants during GC-O were similar to the descriptions of the dilutions of the standards in propandiol (Tables 2 and S4). The OT_{air} were determined with the help of the literature value of (2*E*)-decenal of 2.7 ng L^{-1} and, additionally, with the individually determined OT_{air} of each participant as described above (Figure 4) [17]. The thresholds of (2*E*)-decenal determined for the participants were higher than those reported in the literature. The OT_{air} values of 1-methylcamphene, 4-methyl-3-carene, 2-methylcitronellol, 2-methylgeraniol, 4-methylgeraniol, and 2-methyllimonene were comparable to those of their non-methylated equivalents. 8-Methylgeraniol, 2-methylnerol, and 2-methyllinalool showed higher OTs than the respective corresponding C_{10} compounds.

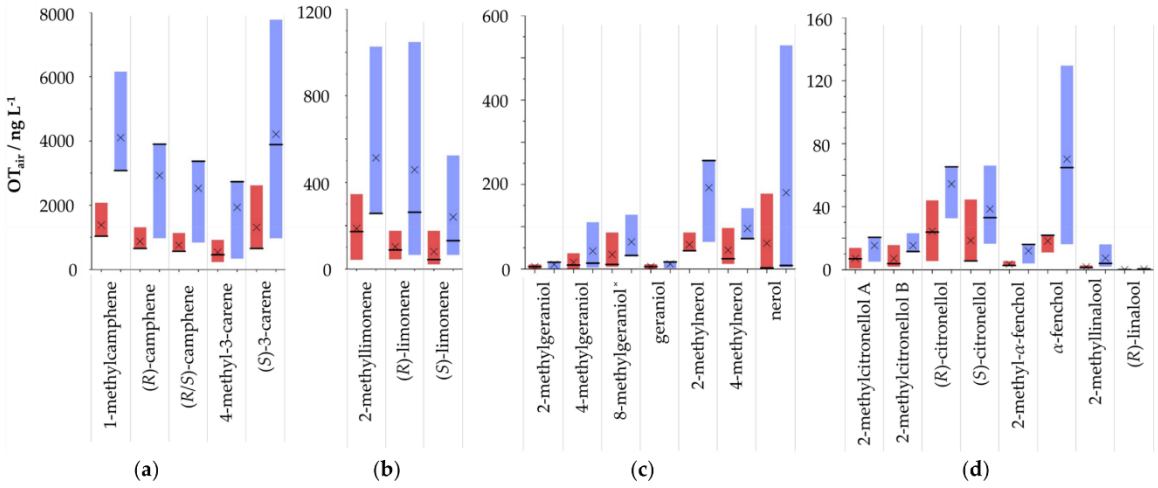

Figure 4. Ranges of the odor thresholds in the air from the three participants with the averages marked with a cross and the median labeled with a line for methylated and non-methylated compounds (**a**) camphene and carene derivatives; (**b**) limonene derivatives; (**c**) geraniol derivatives; (**d**) citronellol, fenchol, and linalool derivatives, according to Teranishi et al. in blue and the threshold determined with the individual determined threshold of (2*E*)-decenal in red, * = mixture of (*E*) and (*Z*) isomers.

3. Discussion

The odors of 28 methylated terpenes were described. All of the studied C_6-, C_7-, and C_{11} compounds were perceived as aroma-active, but the methylated farnesol derivatives had only weak odors. The odor impression of a substance depends on different factors.

Besides the air-to-water partition coefficient, the individual associations to known odor impressions and the interactions with the receptors in the olfactory epithelium are essential for the individual perception of the substances [25]. The descriptions of the odor characteristics varied among the participants, but the panel agreed on a set of attributes that represented the respective essential characteristics.

Methylated aliphatic monoterpenes and methylated monoterpenoids showed the most intense odor impressions. They have molecular masses close to those of other highly odor-active compounds and high structural similarity to monoterpenes, which are well-descripted aroma compounds. Furthermore, the odor of a compound depends on the distribution between hydrophilic and lipophilic structure elements [26,27]. This matches the observation that the terpenoids had marginally lower OTs than the aliphatic terpenes.

The human nose has approximately 430 different types of receptors [25]. The odor impression of a compound is the result of their interaction with different odotopes, which creates a pattern of signals, associated with a familiar odor. Thereby, the odor impression is dependent on the individual receptors of the nose, the association based on memories of the flavor, the health status, the age, and on other individual factors. Therefore, odor descriptions may differ between persons, and the individual thresholds can vary significantly [25,28].

Some of the non-canonical terpenes imparted especially interesting odor impressions. Methylcitronellol exhibited a very pleasant, intense aroma, which combined a citrus odor with intense flowery flavors. It may thus represent an interesting fragrance ingredient for cosmetics. Furthermore, the odor of methylcarene was described as fruity, sweetish, and coniferous forest-like, whereas (*S*)-carene has a resinous odor, resembling a coniferous forest. The influence of the position of the methyl group and of the stereochemistry was shown for geraniol and nerol. Geraniol with a double bound in the (*E*)-configuration has a citrus-like and flowery odor, whereas its isomer nerol, with the double bound in the (*Z*)-configuration, has a resinous, citrus-like, and flowery odor. The methylation of both compounds led to changes in the odor descriptions. The methylation of geraniol in position 8 led to a more resinous odor, the methylation in position 4 to a lemon-like odor, and the methylation in position 2 did not change the odor impression. All nerol derivatives showed a citrus-like, fruity odor but had slightly different odors. While nerol was described as resinous, flowery, citrus, and terpene-like, 2-methylnerol was sweetish, flowery, fresh, citrus, and orange-like. In contrast, 4-methylnerol was ascribed as green, fruity, flowery, and citrus-like. Furthermore, the flavor of (*R*/*S*)-methyllinalool stood out as very pleasant, similar to linalool but with notes of lemon and bergamot. Several synthetic terpenoids were developed to meet the rising need for flavoring agents. Some have intensive and highly pleasant aroma properties. For instance, the derivatives of ionone Iso E Super Plus® (CAS 140194-26-9) and (–)-georgywood® (CAS 828933-31-9) have odor thresholds of only few pg L^{-1} and are widely used in the cosmetics industry [29,30]. According to their odor properties, the novel geraniol and linalool derivatives could also be interesting flavoring agents, especially considering the fact that linalool and geraniol are two of the most often used flavor compounds in cosmetics, deodorants, and showering agents [31,32].

OTs_{air} of several monoterpenes have been determined in previous studies according to the method of Ullrich et al. [20]. Nevertheless, it is necessary to determine both odor descriptions and OTs by the same panelists to directly compare methylated and non-methylated equivalents. Overall, similar odor descriptions and OTs values as those reported in the literature have been determined in this study for monoterpenoids, but deviations were found for some compounds (Table 3).

Table 3. Comparison of odor thresholds in the air (OT) reported in the literature and determined in this study.

	Compound	OT (Literature)/ng L^{-1}	OT (This Study)/ng L^{-1}
1	(*R*/*S*)-citronellol	11 [33]	n.d.
2	(*R*)-citronellol	1.1 [34]	24 ± 19
3	(*S*)-citronellol	0.57 [34]	19 ± 23
4	geraniol	0.067 [34], 11.5 [33]	5.7 ± 5.4
5	nerol	61 [34], 68 [35]	61 ± 100
6	(*R*)-limonene	135 [35]	100 ± 67
7	(*S*)-limonene	270 [35]	81 ± 84
8	(*R*/*S*)-linalool	0.26 [34], 3.2 [33]	n.d.
9	(*R*)-linalool	0.036 [35]	0.098 ± 0.064

n.d. = not determined.

In particular, the OTs of the two enantiomers of citronellol were 20-fold higher than those reported by Schoenauer and Schieberle [34] but were comparable to the values determined by Elsharif and Buettner [33,34]. The individual human perception of odors varies greatly in terms of quality, threshold, pleasantness, and intensity, as it depends, e.g., on the health status, genetics, age, gender, and aroma compound [36]. Nevertheless, the panel was sensitive for all of the analyzed compounds.

The comparison of the OTs of non-canonical terpenes with those of their canonical equivalents revealed some significant differences. While similar OTs were determined for methylated carene, nerol, limonene, and 2-methylgeraniol, the thresholds of methyllinalool and 4-methylgeraniol were higher than those of their non-methylated counterparts. Surprisingly, the OT of methylcitronellol, which had a similar odor impression as citronellol, was lower than that of citronellol.

According to Teranishi et al., the OT in the air is directly proportional to the OT_W, only depending on the air-to-water partition coefficient [19]. Two of the panelists could detect the odor of (2*E*)-decenal during GC-O in all dilutions up to 1:64 and one participant up to 1:128. The panelist who perceived the odor up to 1:128 dilution also had the lowest OT_W (0.5 ± 0.1 µg L^{-1}). The OT in water of the two participants who detected the odor until a dilution of 1:64 were 0.9 ± 0.3 and 1.8 ± 0.6 µg L^{-1} in water. The thresholds determined in water of all participants differed from the literature threshold of (2*E*)-decenal (0.3 µg L^{-1}) [19]. The lowest concentration at which the participants could detect the odor during GC-O was proportional to the individual OT in water. Using the threshold from the literature leads to a less precise determination of the OTs by GC-O as the same value was taken for all participants even if their OTs differed and they perceived the odor until different dilution steps. Furthermore, the sensitivity of the human nose can also be different for different compounds [36]. Accordingly, the determination of individual OTs of the IS is as important as the individual determination of the thresholds of the new compounds.

For some compounds, the OT adopted for the internal standard did not significantly influence the calculations of the OTs. However, there was a strong influence observed for some of the evaluated compounds. For instance, the standard deviations calculated for 2-methylcitronellol, citronellol, 8-methylgeraniol, 4-methylnerol, and geraniol were multiple-fold smaller, with the individually determined OTs compared to those using the fixed literature OT. On the other hand, for α-fenchol, (*R*)-limonene, and 2-methyllinalool, the standard deviations were higher with the individually determined thresholds. Overall, the method proposed here is more precise, and the calculated thresholds of the analyzed compounds were higher when the individually determined OTs of (2*E*)-decenal were used for calculation. The individual OTs of an IS may be used to determine OTs for additional ISs, which could be more similar to the analyzed compounds, as suggested by Ullrich and Grosch [20]. If the air-to-water partition coefficient or the Henry constant are known, every substance could be used to calculate the threshold in the air.

4. Materials and Methods

4.1. Chemicals

Pure solvents were purchased: 1,2-propandiol (99,5%) from Carl Roth GmbH & Co. KG (Karlsruhe, Germany), dichloromethane (≥99.9%) and ethanol (≥99,9%) from Chemsolute (Renningen, Germany), and methanol (≥99.8%) from J. T. Baker (Deventer, The Netherlands). Authentic standards of non-methylated terpenoids were obtained from commercial sources: geraniol (99%) and (*R*)-(–)-linalool (95%) from Acros Organics B.V.B.A (Fair Lawn, NJ, USA); (+)-fenchol (96%), (*E*,*E*)-farnesol (97%), isoprenol (97%), and (*S*)-(–)-limonene (97%) from Alfa Aesar (Kandel, Germany); linalool (97%), (+)-camphene (80%), (*R*)-(+)-citronellol (97%), (*R*)-(+)-limonene (97%), and (–)-α-terpineol (≥96%) from Sigma Aldrich (St. Louis, MO, USA); and (±)-camphene (>78.0%), (+)-3-caren (>90%), (*S*)-(–)-citronellol (>98%), nerol (>98.0%), and prenol (>98.0%) from TCI Deutschland GmbH (Eschborn, Germany). The internal standard (2*E*)-decenal (95%) was obtained from Alfa Aesar.

Methylated terpenes were synthesized by Enamine Ltd. (Riga, Latvia): 2-methylene bornane **4** (95%), 1-methylcamphene **3** (95%), 4-methyl-3-carene **22** (95%), 2-methylcitronellol **11** (95%), 4-methylfarnesol **21** (95%), 6-methylfarnesol **9** (95%), (1*S*)-2-methyl-α-fenchol **10** (95%), (*E*/*Z*)-mixture of 2-methylgeraniol **5** and 2-methylnerol **12** (95%), (*E*/*Z*)-mixture of 4-methylgeraniol **19** and 4-methylnerol **23** (95%), (*E*/*Z*)-mixture of 8-methylgeraniol **20** and 8-methylnerol **24** (95%), 2-methyllimonene **7** (95%), 2-methyllinalool **6** (95%), 2-methylisoprenol **25** (95%), 5-methylisoprenol **26** (95%), 2,4-dimethylisoprenol **27** (95%), 2,5-dimethylisoprenol **28** (99%), 4,4-dimethylisoprenol **18** (95%), 4,5-dimethylisoprenol **29** (99%), 5,5-dimethylisoprenol **30** (95%), 2-methylprenol **31** (95%), (*Z*)-4-methylprenol **15** (≥95%), (*E*)-4-methylprenol **16** (≥95%), 2,4-dimethylprenol **32** (95%), 4,4-dimethylprenol **17** (95%), 4,5-dimethylprenol **33** (95%), and 2-methyl-α-terpineol **8** (95%) (Figures 1–3 and 5). (*Z*)-4-Methylisoprenol **13** (≥95%) and (*E*)-4-methylisoprenol **14** (≥95%) were purchased from AKos GmbH (Lörrach, Germany).

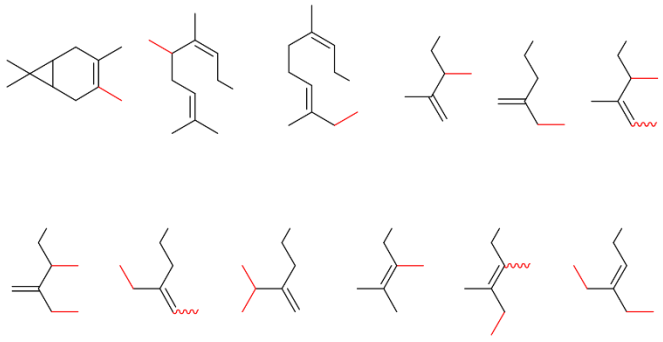

Figure 5. Structures of methylated terpenes with red-labeled bonds to the additional methyl groups: 4-methyl-3-carene **22**, 4-methylnerol **23**, 8-methylnerol **24**, 2-methylisoprenol **25**, 5-methylisoprenol **26**, 2,4-dimethylisoprenol **27**, 2,5-dimethylisoprenol **28**, 4,5-dimethylisoprenol **29**, 5,5-dimethylisoprenol **30**, 2-methylprenol **31**, 2,4-dimethylprenol **32**, and 4,5-dimethylprenol **33**.

4.2. Sensory Analysis

Fifteen participants (eight women, seven men, 23–34 years) described odors of the pure compounds, dissolved in 1,2-propandiol, freely. Therefore, 1 µL or 0.95 mg were dissolved in 200 µL of 1,2-propandiol, and 4 µL of the solutions were placed on a filter paper strip and marked with a three-digit code. The intensity of each odor impression was evaluated from 0 (no odor) to 5 (very intense odor).

4.3. Gas Chromatographic Analysis

The retention indices of the analytes on a polar column and their respective mass spectra were measured with a gas chromatography-mass spectrometry (GC-MS) sys-

tem. An Agilent 7890A GC, together with an Agilent 7000B MS triple Quad (Agilent Technologies, Santa Clara, CA, USA) equipped with a VF-WAXms column (30 m, ID 250 µm, film thickness 0.25 µm; Agilent Technologies), were used. Helium 5.0 (Nipon Gasses GmbH, Hürth, Germany) was used as the carrier gas with a constant flow rate of 1.56 mL min^{-1}. The gas flow was split 1:1 between the MS and the ODP port (ODP 3, GERSTEL GmbH & Co. KG, Mülheim a.d., Ruhr, Germany). One microliter of the sample solution was injected in a splitless liner at 250 °C. The oven was heated to 40 °C (3 min)/5 °C min^{-1}/240 °C (12 min). The mass spectrometer was equipped with an electron ionization source (230 °C, 70 eV) and operated in scan mode (m/z 33–300).

The retention indices on the non-polar DB-5ms column (30 m–320 µm–0.25 µm) were determined by means of a gas chromatography-flame ionization detector system (GC-FID) with a 7890 A GC (Agilent technologies). Measurements were performed as indicated above, except for the following parameters: the carrier gas was hydrogen 5.0 (Nipon Gasses GmbH) with a flow rate of 2 mL min^{-1}, and the oven was heated with the same ramp to 320 °C (12 min). The FID was heated at 250 °C. Retention indices (RI) were calculated according to van den Dool and Kraatz [37].

Chiral analyses were performed using a GC-FID 6890A (Agilent Technologies) equipped with a Hydrodex β-6-TBDM column (25 m–250 µm, Macherey Nagel GmbH & Co. KG, Düren, Germany). One microliter was injected in a splitless liner, which was heated to 250 °C. The GC oven was heated at 80 °C (0 min)/2 °C min^{-1} to 150 °C/20 °C min^{-1} to 250 °C (5 min). The pressure was constant at 0.8 bar, with nitrogen as the carrier gas.

Enantiomeric distribution of methylcitronellol was measured with a Shimadzu GC-MS QP2010 SE on an Astec CHIRALDEX β-DM (Supelco Inc., Bellefonte, PA, USA; 30 m–250 µm, and 0.12 µm). The injection volume was 1 µL, the column flow was 0.84 mL min^{-1}, and helium was used as the carrier gas. The source temperature was 180 °C, and molecular masses were scanned from m/z 40–400. The oven was heated with 40 °C (6 min)/5 °C min^{-1} to 120 °C (40 min)/10 °C min^{-1} and to 180 °C (1 min).

The ratios of (*E*) and (*Z*) isomers were calculated with Formula (1).

$$r_{EZ} = \frac{peak\ area\ (isomer)}{peak\ area\ (E) + peak\ area\ (Z)} \quad (1)$$

The ratios of (*R*) and (*S*) enantiomers and (R_{RS}) were determined according to Formula (2). Enantiomeric ratios of methylcitronellol were calculated with Formula (3) because the compounds could not be baseline-separated. Enantiomeric excess (*ee*) was calculated with Formula (4).

$$r_{RS} = \frac{peak\ area\ (enantiomer)}{peak\ area\ (R) + peak\ area\ (S)} \quad (2)$$

$$r_{RS,Citronellol} = \frac{peak\ hight\ (enantiomer)}{peak\ hight(R) + peak\ hight\ (S)} \quad (3)$$

$$ee = \frac{|r_R - r_S|}{r_R + r_S} \cdot 100\% \quad (4)$$

4.4. Odor Thresholds of the Internal Standard (2E)-Decenal

The odor threshold of the internal standard (2*E*)-decenal in water ($OT_{W,IS}$) was determined in pure water, as described by Hammer et al. [21]. The initial concentration was 38 µg L^{-1}, and the solution was diluted 1:2 (v/v) nine times. The tests were carried out in triplicate by each of the three participants, who also performed the GC-O analyses.

The corresponding individual odor threshold in the air of the internal standard ($OT_{air,IS}$) was then calculated for each participant according to Teranishi et al. with the help of the previously determined odor threshold in water ($OT_{W,IS}$) and the air-to-water partition coefficient K_W with Formula (5) [19].

$$OT_{air,IS} = OT_{W,IS} \cdot K_W = OT_{W,IS} \cdot \frac{c_{air}}{c_W} \quad (5)$$

4.5. Odor Thresholds in Air

The analyses were done according to Hammer et al., with adapted oven temperature ramps [21]. The compounds were dissolved in methanol, and the concentrations were chosen individually according to their respective aroma potency. The compounds were analyzed in four mixtures (Table 4).

Table 4. Composition of the four mixtures used for the GC-O analysis for determination of the odor thresholds in the air.

	Compounds
Mixture 1	(*R*)-camphene (578 mg L^{-1}), (*S*)-limonene (310 mg L^{-1}), 2-methyllimonene (304 mg L^{-1}), (*R*)-linalool (38.0 mg L^{-1}), 2-methylfenchol (38.0 mg L^{-1}), (2*E*)-decenal (38.0 mg L^{-1}), (*R*)-citronellol (77.4 mg L^{-1}), geraniol (39.5 mg L^{-1}), and 8-methylgeraniol ((*E*/*Z*)-mixture, 152 mg L^{-1})
Mixture 2	4-methyl-3-carene (405 mg L^{-1}), (*R*)-α-fenchol (38.4 mg L^{-1}), 2-methyllinalool (38.0 mg L^{-1}), (2*E*)-decenal (38.0 mg L^{-1}), (*S*)-citronellol (78.3 mg L^{-1}), and 2-methylgeraniol ((*E*/*Z*)-mixture, 152 mg L^{-1})
Mixture 3	(*R*/*S*)-camphene (500 mg L^{-1}), (*S*)-3-carene (576 mg L^{-1}), (*R*)-limonene (621 mg L^{-1}), (2*E*)-decenal (38.0 mg L^{-1}), nerol (78.4 mg L^{-1}), and 4-methylgeraniol ((*E*/*Z*)-mixture, 152 mg L^{-1})
Mixture 4	(*S*)-1-methylcamphene (456 mg L^{-1}), (2*E*)-decenal (38.0 mg L^{-1}), and 2-methylcitronellol ((*E*/*Z*)-mixture, 152 mg L^{-1})

Mixtures 1–3 were measured with a temperature program of 40 °C (5 min)/5 °C min^{-1} to 160 °C (0 min)/20 °C min^{-1} to 240 °C (4 min) and mixture 4 with 40 °C (5 min)/5 °C min^{-1} to 140 °C (2 min)/5 °C min^{-1} to 160 °C (0 min)/20 °C min^{-1} to 240 °C (4 min). The mixtures were successively diluted 1:2 (*v*/*v*) with methanol. The determination of the OT_{air} by GC-O was done by one man and two women, which were 24–29 years old. Samples were analyzed in a random order, and each participant noted the odor individually.

The OT_{air} of the analyzed compound X ($OT_{air,X}$) was calculated with the OT_{air} of the IS (2*E*)-decenal ($OT_{air,IS}$), the initial concentration of the analyzed compound c_x, the D-value of the IS D_{IS}, the initial concentration of the IS (c_{IS}), and the D-values of the analyzed compound D_x and of the IS D_{IS} with Formula (6).

$$OT_{air,x} = \frac{OT_{air,IS} \cdot c_x \cdot D_{IS}}{c_{IS} \cdot D_x} \quad (6)$$

5. Conclusions

This study characterized 28 novel methylated terpenes regarding their odor and their chemical characteristics, including mass spectra and retention indices on two columns of different polarities. Thirteen of the evaluated non-canonical terpenes showed intense aroma impressions, and the OTs were determined for the first time in comparison to those of eleven reference terpenes. Individual determination of the OTs of the IS enabled us to determine the thresholds more precisely and to expand the options for an internal standard.

6. Patents

Parts of this study are included in a European Patent "The use of non-canonical terpenes or terpenoids as aroma chemicals", Sommer, S., Fraatz, M.A., Zorn, H. (19 May 2022, EP 22174377.6).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27123827/s1. Table S1: Gas chromatography-mass spectra of unpublished compounds with the relative intensities of the fragments. Table S2: Odor description of pure hemi-, mono-, and sesquiterpenes, which were given by at least three participants, with the number of mentions in parentheses and the average and the standard deviation of the intensity. Table S3: *D*-values and odor thresholds according to Teranishi et al. (*OT*(*Teranishi*)), odor thresholds determined with the individual odor thresholds (*OT**(*individual*)) of the three participants in the GC-O measurements. Table S4: Compounds with their respective odor descriptions determined by means of GC-O and the odor thresholds of the three participants in the air, * = mixture of (*E*) and (*Z*) isomers.

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Sample Availability: Samples of the compounds are not available from the authors.

Abbreviations

DMAPP	dimethylallyl pyrophosphate
DT	detection threshold
GC-O	gas chromatography-olfactometry
ee	enantiomeric excess
FPP	farnesyl pyrophosphate
IPP	isopentenyl pyrophosphate
IS	internal standard
n.a.	not available
ODP	olfactory detection port
OT	odor threshold
OT_W	odor threshold in water
OT_{air}	odor threshold in air
n.d.	not determined
RT	recognition threshold
SAM	S-adenosyl methionine

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7 Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of an anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ in carrying out the investigations described in the dissertation.

Date, Place Signature