Biotechnologische Erzeugung natürlicher Aromastoffe aus Submerskulturen von Asco- und Basidiomyceten

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Veröffentlichungen

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- Schimanski S., Krings U., Berger R.G. Lösungsmittelfreie Extraktion und Thermodesorptions-Analyse von Aromastoffe. Lebensmittelchemie (2012) 66(3): 61.
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- Schimanski S., Krings U., Berger R. G. From cabbage cuttings to pineapple flavour: ready to use flavours from side-stream of the food industry. 10th Wartburg Symposium on Flavor Chemistry & Biology (2013).

Vorträge:

- Rheinheimer M., Schimanski S., Krings U., Berger R.G. Biotechnologische Erzeugung von hochwertigen Aromastoffen aus Nebenströmen der Lebensmitelindustrie. Arbeitstagung 2012 des Regionalverband Nord, Lebensmittelchemische Gesellschaft, Universität Hannover; Lebensmittelchemie (2012) 66(3): 61.
- Haase-Aschoff P., Schimanski S., Linke D., Berger R.G. Aromabildung und Strukturmodifikation durch Ferulasäure-Esterasen. Arbeitstagung 2013 des Regionalverband Nord, Lebensmittelchemische Gesellschaft, Hamburg School of Food Science; Lebensmittelchemie (2013) 67(3):76.

Inhaltsverzeichnis

Abkürzungsverzeichnis

% (v/v)	Volumenprozent
% (w/w)	Massenprozent
(*E*)	*trans*-Konfiguration einer Substanz
(*Z*)	*cis*-Konfiguration einer Substanz
4-VG	4-Ethenyl-2-methoxyphenol (4-Vinylguaiacol)
ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ADH	Alkoholdehydrogenase
AEDA	Aromaextraktverdünnungsanalyse (*aroma extract dilution analysis*)
AIEX	Anionenaustauschchromatographie (*anion exchange chromatography*)
CBS	Centraalbureau voor Schimmelcultures
CC	Kohlschnitt (*cabbage cuttings*)
CHARM	*Combined hedonic aroma response measurements*
CIS	Kaltaufgabesystem (*cold injection system*)
CO	Rizinusöl (*castor oil*)
COSY	*Correlation spectroscopy*
DCM	Dichlormethan
DMF	N,N-Dimethylformamide
DMP	Dess-Martin-Periodan
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DTT	Dithiothreitol ((2*S,*3*S*)-1,4-bis(sulfanyl)butan-2,3-diol)
EC	Europäischer Rat (*European Council*)
EFSA	Europäische Behörde für Lebensmittelsicherheit (*European Food and Safety Authority*)
EtO_2	Diethylether
EtOH	Ethanol
EU	Europäische Union
EG	Europäische Gemeinschaft
FA	(*E*)-3-(4-Hydroxy-3-methoxy-phenyl)prop-2-ensäure(*ferulic acid*)
FAME	Methyl (*E*)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoat (*ferulic acid methyl ester*)
FA-PPB	Kaliumphosphatpuffer, versetzt mit Ferulasäure

FD	Aromaverdünnung (*flavour dilution*)
FaD	Ferulasäuredecarboxylase (*ferulic acid decarboxylase)*
FID	Flammenionisationsdetector
Fig.	Figur/Abbildung
g	Erdbeschleunigung: 9,81 m/s^2
GC	Gaschromatograf; Gaschromatografie
GC-MS	Gaschromatografie gekoppelt mit Massenspektrometrie
GC-O	Gaschromatografie gekoppelt mit Olfaktometrie
Hb	Hämoglobin
HMBC	*Heteronuclear multiple bond coherence*
HPL	Hydroperoxidlyase
HRGC	Hochauflösende Gaschromatografie
HRMS	Hochauflösende Massenspektrometrie
HSQC	*Heteronuclear single quantum coherence*
IEP	Isoelektrischer Punkt
Ifa	*Isaria farinosa*
ITS	*Internal transcribed spacer*
K_m	Michaeliskonstante
$KMnO_4$	Kaliumpermanganat
Li	Lithium
$LiAlH_4$	Lithiumaluminiumhydrid
LOD	Nachweisgrenze (*limit of detection*)
logP	Verteilungskoeffizient zwischen Octanol und Wasser
LOQ	Bestimmungsgrenze (*limit of quantification*)
LOX	Lipoxygenase
m/z	Masse-Ladungs-Verhältnis
MCT	Mittelkettige Triglyceride (*medium chain triglycerides*)
min	Minuten
MM	Minimalmedium
NMR	Kernspinresonanzspektroskopie (*nuclear magnetic resonance spectroscopy*)
MS	Massenspektrometrie
$NaBH_4$	Natriumborhydrid
$NaHCO_3$	Natriumhydrogencarbonat
NH_3	Ammoniak
OAV	Aromawert (*odour active value*)

ODP	Olfaktorischer Detektor (*olfactory detection port*)
OECD	Organisation für wirtschaftliche Zusammenarbeit und Entwicklung (*Organisation for Economic Cooperation and Development*)
P/E	Pentan/Diethylether (1:1,12)
PAGE	Polyacrylamid Gelelektrophorese
PAK/PAH	Polycyclische aromatische Kohlenwasserstoffe (*polycyclic aromatic hydrocarbons*)
Pbet	*Piptoporus betulinus*
PPB	Kaliumphosphat-Puffer (*potassium phosphate buffer*)
R^2	Bestimmtheitsmaß
RI	Retentionsindex
RO	Rapsöl (*rape oil*)
RPC	Rapspresskuchen (*rapeseed press cake*)
rpm	Umdrehungen in der Minute (*revolutions per minute*)
rRNA	Ribosomale Ribonucleinsäure (*ribonucleic acid*)
SAFE	Lösungsmittelunterstützte Aromaextraktion (*solvent assisted flavour evaporation*)
SBSE	*Stir-bar sorptive extraction*
SDS	Natriumdodecylsulfat (*sodium dodecyl sulphate*)
SEC	Größenausschlusschromatografie (*size exclusion chromatography*)
SNM	Standardnährlösung (*standard nutrition medium*)
SPME	Festphasenmikroextraktion (*solid phase micro extraction*)
Tab.	Tabelle
TDS	Thermodesorptionseinheit
THF	Tetrahydrofuran
TLHVD	Dünnschschichthochvakuumdestillation (*thin layer high vacuum distillation*)
TMBZ	3,3',5,5'-Tetramethylbiphenyl-4,4'-diamine (*Tetramethylbenzidine*)
V	Vanillin
V_{max}	Maximale Reaktionsgeschwindigkeit

Zusammenfassung

Die Arbeit beschäftigt sich mit der biotechnologischen Erzeugung von Aromastoffen durch Pilze. Darüber hinaus befasst sie sich mit der Analytik von Aromastoffen.
Mit dem Ziel, das biotechnologische Potential von höheren Pilzen für die Erzeugung von Aromastoffen zu nutzen, wurden 32 Basidiomyceten und ein Ascomycet in einem Screening auf Nebenströmen der Lebensmittelindustrie submers kultiviert. Es wurden einige vielversprechende Substrat-Pilz-Kombinationen gefunden. Zwei der Kombinationen stellten sich als besonders vielversprechend heraus und wurden näher charakterisiert:
Bei der Kultivierung von *Isaria farinosa* auf Rapspresskuchen wurde ein Geruchseindruck nach geräuchertem Schinken und Nelken beobachtet. Durch gaschromatografische Analyse konnte 4-Vinylguajacol (4-Ethenyl-2-methoxyphenol) als Schlüsselkomponente identifiziert werden, welches aus Ferulasäure ((*E*)-3-(4-Hydroxy-3-methoxyphenyl)prop-2-ensäure) gebildet wird. Es wurde eine Reinigungsmethode für das entsprechende Enzym entwickelt und das Enzym charakterisiert.
Bei der Kultivierung von *Piptoporus betulinus* auf Kohlschnitt stellte sich ein angenehmer Geruch nach Ananas und Honig ein. Die submerse Kultivierung wurde erfolgreich auf einen 5-Liter Bioreaktor ausgeweitet. Das Aromaprofil wurde mittels Aromaextraktverdünnungsanalyse charakterisiert, wobei die Substanzen
Octan-3-on, Essigsäure, Methylbenzoat und 4-Methoxymethylbenzoat als wichtige Komponenten identifiziert wurden. Zwei weitere für den dominanten Ananasgeruch verantwortliche Substanzen, wurden anhand ihres Massenspektrums und verschiedener Kernresonanz-Spektren als Isomere von Deca-5,7,9-trien-2-on identifiziert.
Weiterhin wurde eine Methode zur schnellen Analyse von Aromastoffen in fetthaltigen Matrices entwickelt. Sie basiert auf der Extraktion der Aromastoffe mit einem technischen Triacylglycerolgemisch und anschließender thermischer Desorption direkt in das Kaltaufgabesystem eines Gaschromatografen. Sie eignet sich sehr gut für das Monitoring von biotechnologischen Prozessen. Eine Modellmischung von zehn Aromastoffen mit unterschiedlichen chemischen und physikalischen Eigenschaften diente zur Validierung. Die Anwendbarkeit wurde durch die Aromaanalytik der submersen Kultivierung von *Fomitopsis rosea* im 5-L Rührkesselreaktor belegt.

Schlagwörter: Aromabiotechnologie, Raucharoma, Ananasaroma

Abstract

This work deals with the biotechnological production of flavours by fungi and the subsequent analysis of the corresponding flavour compounds.

With the aim to exploit the biotechnological potential of higher fungi for the production of flavourings, 32 basidiomycetes and one ascomycete were screened using submerged cultivation supplemented with side streams of food processing. Some promising substrate/fungus combinations were found. Two of the combinations turned out to be very promising and were characterised in more detail.

Cultivation of *Isaria farinosa* on rapeseed press cake resulted in an odour impression reminiscent of carnation and smoked ham. 4-Vinylguaiacol (4-ethenyl-2-methoxyphenol) was identified as the main component by means of gas chromatography. Ferulic acid ((E)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid) was identified as the precursor. A purification method for the corresponding enzyme was developed and the enzyme was characterised.

Cultivation of *Piptoporus betulinus* on cabbage cuttings resulted in a pleasant odour reminiscent of pineapple and honey. Cultivation was successfully up-scaled into a 5 L stirred tank reactor. The flavour profile was characterised by means of aroma extract dilution analysis. Octan-3-one, acetic acid, methyl benzoate and 4-methoxy methylbenzoate were identified as main components. Two substances turned out to be responsible for the dominant pineapple-like odour impression. According to mass and nuclear magnet resonance spectra the compounds were identified as isomers of deca-5,7,9-triene-2-one.

Furthermore, a method for rapid analysis of volatiles in fat-containing matrices has been developed. It was based on the extraction of the flavour substances with medium-chain-triglycerides and subsequent thermal desorption. The described method was suitable for monitoring bioprocesses. A model mixture of ten flavour compounds with different chemical and physical properties was used for validation. The applicability was demonstrated by the flavour analysis of submerged cultivation of *Fomitopsis rosea* in a 5 L stirred tank reactor.

Keywords: Flavourbiotechnology, smoke flavour, pineapple flavour

1. Einleitung

“Flavour creation is still more of an art than a science” *[Wright 2010]*. Aromen beeinflussen seit Menschengedenken unsere Emotionen. Der Duft eines saftigen Steaks vom Grill oder eine duftende Tasse Kaffee mit einem Stück frischgebackenem Kuchen löst Wohlbefinden in uns aus. Genauso auch ein Spaziergang in der Natur, die mit den verschiedensten Düften von Blumen und Pflanzen unsere Sinne umspielt. Ebenso lockt der betörende Duft der raffiniertesten Parfums zahlreiche Kunden in die Parfümerien. Jedoch haben auch unangenehme Gerüche in unserem Leben Raum – so warnt z. B. Fehlgeruch vor dem Verzehr von verdorbenen Lebensmitteln. Oder ein stechender, beißender Geruch lässt uns umsichtig in einer Gefahrensituation handeln. Auf Grund des Wissens um die Vielfältigkeit der Aromen bringt es der Flavourist John Wright, der Gerüche und Aromen komponiert, mit seinem oben genannten Zitat auf den Punkt. Es ist eine Kunst, gute Aromen zu kreieren, man benötigt Intuition und Kreativität. Um diese Kunst jedoch erfolgreich zu betreiben, ist ein gewisses *Know-how* und Handwerkszeug unerlässlich. Durch wissenschaftliche Forschungen und Erkenntnisse auf dem Gebiet der Aromen, wird den Flavouristen eine Ausrüstung in die Hand gelegt, um Gerüche zu komponieren. Hierzu gehören die Kenntnis von einzelnen Aromakomponenten und deren Geruchseindruck, das Zusammenspiel verschiedener Aromastoffe, das Bereitstellen von Einzelsubstanzen und Aromaextrakten, sowie die Wirkmechanismen der für den Geruch und Geschmack verantwortlichen Sinnesorgane. Neben der analytischen Herausforderung, wichtige Aromakomponenten aus natürlichen Quellen zu extrahieren, zu identifizieren und in entsprechender Qualität zur Verfügung zu stellen, ist es ein Anliegen der heutigen Aromaforschung, neue Quellen zur Aromaproduktion, die wirtschaftlich konkurrenzfähig sind, zu erschließen und zu etablieren. So haben biotechnologisch erzeugte Aromen einen Mehrwert in Bezug auf deren kostengünstige und nachhaltige Produktion – im Vergleich zur aufwendigen Isolierung aus natürlichen Quellen – und gelten, im Gegensatz zu synthetisch hergestellten Aromen, als natürlich im Sinne der EG-Aromenverodnung[1].

Diese Arbeit befasst sich sowohl mit der Erforschung neuer Quellen für Aromastoffe und Aromastoffgemische mit Hilfe der Biotechnologie, als auch mit der Analytik und der Charakterisierung von Aromaprofilen.

1.1. Aromastoffe

1.1.1. Definition, Bildung, Vorkommen

Laut der VO (EG) Nr. 1334/2008 ist ein Aromastoff ein „chemisch definierter Stoff mit Aromaeigenschaften“[1]. Ein Stoff besitzt Aromaeigenschaften, wenn er vom Geruchssinn wahrgenommen werden kann. In der Literatur sind über 8000 Aromastoffe beschrieben[2], die entweder genuin in Nahrungsmitteln vorkommen, oder durch Bearbeitung/Verarbeitung des Lebensmittels gebildet werden.

Typische Vorläufer für die Bildung von Aromastoffen sind Fettsäuren, Aminosäuren und Zucker. Aber auch Sekundärmetabolite wie Terpene oder Phenolsäuren können als Vorläufer für weitere Aromastoffe dienen.

Beim Zerkleinern von Obst und Gemüse z.B. kommen die zunächst in unterschiedlichen Zellkompartimenten vorliegenden Primär- bzw. Sekundärmetabolite und Enzyme miteinander in Kontakt und es entfalten sich die typischen frischen und grünen Aromen. Verantwortlich für diese enzymatische Aromabildung ist die oxidative Spaltung von Linol- und Linolensäure durch eine Lipoxygenase (LOX; EC 1.13.11.12). Es bilden sich 9-, 10- oder 13-Hydroperoxide, welche anschließend durch eine Hydroperoxidlyase (HPL) in die aromaaktiven C_6, C_9 oder C_{10} Aldehyde wie Hexanal und Hexenal gespalten werden. Diese können durch eine Alkoholdehydrogenase (ADH; EC 1.1.1.1) weiter zu den entsprechenden Alkoholen reduziert werden. Fig. 1 stellt exemplarisch den Abbau der Linolsäure zum Hexan-1-al/Hexan-1-ol dar. Bei Pilzen führt der enzymatische Abbau von Linolsäure zu (*R*)-1-Octen-3-ol und dem entsprechenden Keton. Diese C_8-Verbindungen sind für den typischen Geruch von Pilzen verantwortlich. In Arten der *Allium* Gattung (Zwiebel, Knoblauch) bestimmen schwefelhaltige Aromastoffe das Aroma von frisch geschnittenen Knollen. Diese werden durch das Enzym Alliinase (EC 4.4.1.4.) aus

[1] Verordnung (EG) Nr. 1334/2008 des Europäischen Parlaments und der Rates vom 16. Dezember 2008 über Aromen und bestimmte Lebensmittelzutaten mit Aromaeigenschaften; Art. 3 (2) b)
[2] http://www.vcf-online.nl/VcfCompounds.cfm; *Volatile Compounds in Food 15.1*; Stand: Januar 2014

unterschiedlichen Cysteinsulfoxid-Verbindungen beim Schneiden der Knollen gebildet *[Christensen et al. 2007]*, *[Belitz et al. 2001]*.

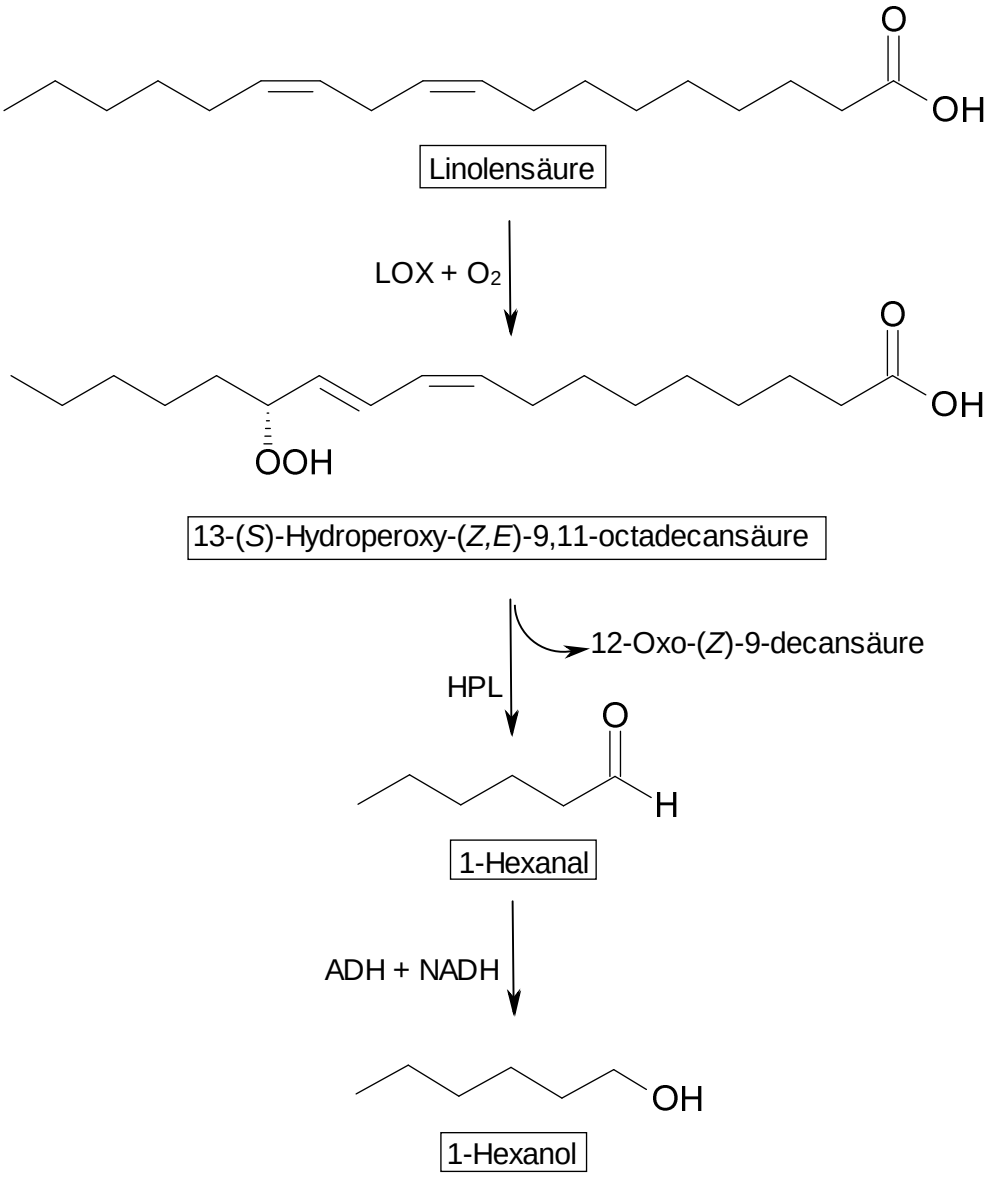


Fig. 1: Oxidative Spaltung von Linolsäure durch eine Lipoxygenase.
LOX: Lipoxygenase; HPL: Hydroperoxidlyase; ADH: Alkoholdehydrogenase
[Christensen et al. 2007]

Beim Erhitzen von Lebensmitteln kommt der *Maillard*-Reaktion eine Schlüsselrolle zu. Sie ist maßgeblich verantwortlich für den Geruch und Geschmack von gebratenen, gebackenen und gerösteten Lebensmitteln. Durch die Bildung von Melanoidinen bewirkt sie außerdem die braune Farbe dieser Lebensmittel. Es handelt sich hierbei um eine Reihe von nichtenzymatischen Reaktionen zwischen reduzierenden Zuckern und Aminosäuren bzw. Aminoverbindungen, aus denen farb- und aromawirksame Substanzen hervorgehen. Typische aromaaktive

Verbindungen der Maillard-Reaktion sind Aldehyde und Pyrazine. Aber auch Furanone, Thiole, Sulfide und Thiazole sind für den Geruch erhitzter Lebensmittel verantwortlich *[Eisenbrand et al. 2006]*.
Ein weiterer wichtiger Prozess, bei dem viele geruchs- und geschmacksaktive Verbindungen entstehen, ist die thermische bzw. enzymatische Zersetzung von Phenolsäuren. Es werden Phenole wie z.B. Guajacol, 4-Vinylguajacol, Kresol, Eugenol und deren Derivate gebildet, die für das typische Raucharoma verantwortlich sind. Näheres zum Raucharoma wird in Kapitel 1.1.2 erläutert.

Die beschriebenen Bildungswege führen zu einer Vielzahl von Aromastoffen in Lebensmitteln. So wurden z.B. in Kaffee mehr als 1000 Aromastoffe identifiziert. Allerdings kommen viele der Aromastoffe in sehr geringen Konzentrationen unterhalb ihres Geruchsschwellenwertes (Konzentration einer Verbindung, die gerade noch vom Geruchssinn wahrgenommen werden kann) vor und tragen somit nicht zum Gesamtaroma bei. Von den 1000 identifizierten, sind nur etwa 30 bis 40 Schlüsselverbindungen verantwortlich für das typische Kaffeearoma *[Grosch 2001]*. Ist nur eine Substanz maßgeblich an dem charakteristischen Aroma eines Lebensmittels beteiligt, nennt man diese Verbindung *Impact*-Verbindung. Beispiele hierfür sind das Himbeerketon (1-(*p*-Hydroxyphenyl)-3-butanon) aus Himbeeren mit einem himbeerartigem Aroma und (*R*)-1-Octen-3-ol aus Champignons mit einem pilzartigen Aroma. 2,5-Dimethyl-4-hydroxy-3(2H)-furanon (Furaneol) ist ein in vielen Lebensmitteln (v.a. Früchten) vorkommender wichtiger Schlüsselaromastoff mit einem karamellartigen, fruchtigen Aromaeindruck und einer sehr geringen Geruchsschwelle (1 ng/L in Luft *[Blank et al. 1993]*). Zum ersten Mal identifiziert wurde er von *Rodin et al. [1965]* als wichtiger Bestandteil des Ananasaromas.

1.1.2. Raucharoma

Aromastoffe mit rauchiger Note prägen den Geschmack von gerösteten und geräucherten Lebensmitteln. Durch thermische bzw. enzymatische Zersetzung werden Phenole wie z.B. Guajacol, 4-Vinylguajacol, Kresol, Eugenol und deren Derivate gebildet, die für das typische Raucharoma verantwortlich sind. Sie werden entweder aus den im Lebensmittel vorhandenen Phenolsäuren (Ferulasäure, Sinapinsäure, Zimtsäure) beim Erhitzen gebildet, oder gehen beim

Räuchern von Fisch/Fleisch oder beim Lagern von Getränken in Holzfässern in das Lebensmittel über. Sie kommen demnach u.a. in Kaffee, Bier, Weinbrand, Whiskey, gerösteten Erdnüssen und geräuchertem Fisch/Fleisch vor *[Belitz et al. 2001]*.

Die Phenole in geräucherten Produkten stammen von der thermischen Zersetzung/Pyrolyse des Lignins, welches neben Cellulose und Hemicellulose der Hauptbestandteil von Holz darstellt. Lignin ist ein dreidimensionales Polymer, dessen Struktur auf den Monomeren *p*-Cumaryl-, Coniferyl- und Sinapylalkohol basiert. Bei dem thermischen Abbau von Holz ab Temperaturen von 200°C wird die Struktur des Lignins zerstört und es entstehen ausgehend von den *p*-Cumaryl-, Coniferyl- und Sinapyleinheiten verschiedene aromaaktive Phenole. Deren Spektrum ist weniger von der Temperatur, sondern vielmehr von der Art des Holzes abhängig *[Brebu et al. 2010]*.

Um den Geschmack von geräucherten Lebensmitteln zu simulieren, werden Raucharomapräparate als Zutat für die Lebensmittelindustrie hergestellt. Diese Raucharomen werden durch kontrollierte Pyrolyse von Holz hergestellt. Der entstehende Rauch wird anschließend kondensiert, fraktioniert und für den Einsatz in Lebensmitteln aufgearbeitet *[Simon et al. 2005]*. Neben den in Raucharoma vorkommenden geschmacksgebenden Substanzen wie Phenolderivate, Carbonylverbindungen und Alkoholen *[Guillen et al. 2002]*, ist auch die Klasse der polycyclischen aromatischen Kohlenwasserstoffe (PAK) von besonderem Interesse. Einige der beim unvollständigen Verbrennen von organischem Material gebildeten Substanzen gelten als karzinogen. So gibt es festgelegte Grenzwerte für zwei Vertreter der PAKs, Benzo[a]pyren und Benzo[a]anthracen, die nicht überschritten werden dürfen[3]. In der für die Raucharomen gültigen Verordnung (EG) 2065/2003 ist weiterhin eine Prüfung aller bisher auf dem Markt verfügbaren Raucharomen (bzw. Primärprodukte für die Herstellung von Raucharomen) festgelegt. Die zuständige Behörde für die Prüfung, die Europäische Behörde für Lebensmittelsicherheit (EFSA), hat alle elf auf dem Markt verfügbaren Produkte geprüft und nur drei als unbedenklich (bei vorgesehener Anwendung) eingestuft *[Theobald et al. 2012]*. In der Durchführungsverordnung (EU) Nr. 1321/2013 zur Festlegung der Unionsliste zugelassener Primärprodukte für die Herstellung von

[3] Regelungen für Lebensmittel siehe Verordnung (EG) 1881/2006; Regelungen für Raucharomen siehe Verordnung (EG) 2065/2003

Raucharomen sind letztendlich zehn der elf Produkte gelistet und mit entsprechenden Verwendungsbedingungen zugelassen.

1.1.3. Rechtliche Grundlagen

Aromen werden auf europäischer Ebene in der Verordnung (EG) Nr. 1334/2008 geregelt. Hier sind u.a. die Begriffe Aroma, Aromastoff, natürlicher Aromastoff, Aromaextrakt, thermisch gewonnenes Reaktionsaroma und Raucharoma definiert. Die Verordnung besagt, dass bestimme Aromen nur verwendet werden dürfen, wenn sie in einer Positivliste (Anhang 1 VO (EG) Nr. 1334/2008) gelistet sind und entsprechend als sicher bewertet wurden. Ein natürlicher Aromastoff ist definiert als: „Aromastoff, durch geeignete physikalische, enzymatische oder mikrobiologische Verfahren aus pflanzlichen, tierischen oder mikrobiologischen Ausgangsstoffen gewonnen, die als solche verwendet oder mittels eines oder mehrerer der in Anhang II aufgeführten herkömmlichen Lebensmittelzubereitungsverfahren für den menschlichen Verzehr aufbereitet werden. Natürliche Aromastoffe sind Stoffe, die natürlich vorkommen und in der Natur nachgewiesen wurden;“[4] Ein Aromastoff darf als „natürlich“ deklariert werden, wenn er die Anforderungen der Definition erfüllt.
Raucharomen nehmen eine gesonderte Stellung im Aromenrecht ein. Für sie gilt die Verordnung (EG) Nr. 2065/2003. Sie sind definiert als „Erzeugnis, das durch Fraktionierung und Reinigung von kondensiertem Rauch gewonnen wird.“[5]

1.1.4. Analytik

Herausforderungen der Analytik von Aromastoffen sind die chemische Vielfalt, deren Vorkommen in meist sehr geringen Konzentrationen und die Beurteilung ihrer sensorischen Eigenschaften. Auch die Isolierung der häufig in komplexen Matrices vorkommenden Aromastoffe stellt eine nicht zu unterschätzende Schwierigkeit dar. Eine universelle Methode zur Aromaanalyse ist demnach nicht vorhanden. Es haben sich vielmehr verschiedene Standardmethoden etabliert, die ein jeweils spezifisches Abbild des zu analysierenden Aromas geben. Bei jeder Fragestellung bedarf es einer Prüfung der Analysenmethode auf Anwendbarkeit,

[4] Verordnung (EG) Nr. 1334/2008 des Europäischen Parlaments und der Rates vom 16. Dezember 2008 über Aromen und bestimmte Lebensmittelzutaten mit Aromaeigenschaften; Art. 3 (2) c)
[5] Verordnung (EG) Nr. 1334/2008 des Europäischen Parlaments und der Rates vom 16. Dezember 2008 über Aromen und bestimmte Lebensmittelzutaten mit Aromaeigenschaften; Art. 3 (2) f)

einem gegebenenfalls angepasstem Analysenprotokoll und dem entsprechendem *Knowhow*, um erzielte Ergebnisse richtig einschätzen zu können. *[Reineccius 2010]*.

Zum Isolieren der Aromastoffe gibt es verschiedene Ansätze, welche zum einen auf der Flüchtigkeit und zum anderen auf der Polarität bzw. der Löslichkeit der Substanzen beruhen. Eine der ältesten Methoden zur Isolierung von Aromastoffen ist die simultane Destillation/Extraktion nach Likens und Nickerson *[Likens et al. 1964]*. Hier werden die Aromastoffe aus einer wässrigen Probe verdampft und in einem leicht flüchtigen Lösungsmittel aufgenommen. Nachteil hierbei ist die Veränderung bzw. Bildung von Aromastoffen in der Probe aufgrund der thermischen Belastung. Eine in dieser Hinsicht schonendere Methode ist die Analytik des Kopfraums (*headspace*). Während die statische Kopfraumanalytik durch ihre meist unzureichende Sensitivität nur selten angewandt wird, ist die dynamische Methode eine gute Alternative. Die Aromastoffe werden mit einem Gas aus der Probe ausgetrieben und durch Anreicherung auf einem geeigneten Träger (z.B. Tenax) oder durch Kühlfallen isoliert. Eine neuere Methode ist die Festphasenmikroextraktion (*solid-phase-micro-extraction* (SPME)) *[Pawliszyn 1997]*, bei der die Aromastoffe aus dem Kopfraum bzw. aus der Probe direkt an einer Phase adsorbiert werden und anschließend durch thermische Desorption im Gaschromatografen analysiert werden. Die *stir-bar sorptive extraction* (SBSE) beruht auf dem gleichen Prinzip: Ein Rührfisch ist mit dem Adsorptionsmaterial überzogen, wodurch die Aromastoffe aus der Probe aufgenommen werden *[David et al. 2003]*.

Die Lösungsmittelextraktion ist eine der einfachsten und effizientesten Methoden der Aromaanalytik. Limitiert wird diese Methode jedoch vor allem durch den Lipidgehalt einer Probe. Dieser wird ebenfalls durch das unpolare Lösungsmittel extrahiert und muss in einem weiteren Schritt aufwendig von den Aromastoffen getrennt werden. Zur Isolierung von Aromastoffen aus fetthaltigen Matrices haben sich deshalb instrumentell aufwendige, aber effiziente Methoden bewährt, bei denen durch Hochvakuum die Aromastoffe aus der fetthaltigen Matrix ausgetrieben und in Lösungsmittel aufgenommen werden *[Krings et al. 2003]*, *[Engel et al. 1999]*.

Sind die Aromastoffe in ausreichender Reinheit isoliert und konzentriert, werden sie durch gaschromatografische Methoden aufgetrennt und anhand ihrer Struktur

(Massenspektrometrie, Kernresonanzspektroskopie) und ihres Geruchseindruckes (Olfaktometrie) identifiziert. Neben klassischen instrumentellen Detektionsmethoden, wie Flammenionisationsdetektor und Massenspektrometer, ist die Nase des Menschen ein unerlässlicher Bestandteil der Aromaanalytik. Der Geruchssinn als Detektor gibt Aufschluss über die sensorischen Eigenschaften einer Substanz. Aufgrund der großen Empfindlichkeit des menschlichen Geruchsinns (1.1.5) können so auch Substanzen mit sehr niedrigem Geruchsschwellenwert detektiert werden, die auf Grund ihrer geringen Konzentration von gängigen Detektorsystemen nicht erfasst werden. Um Schlüsselaromastoffe der analysierten Probe zu ermitteln, haben sich zwei Methoden etabliert. Die Aromaextraktverdünnungsanalyse (AEVA) *[Ullrich et al. 1987]* und die CHARM Methode (engl. *combined hedonic aroma response measurements*) *[Acree et al. 1984]*. Diese basieren auf dem von *Patton et al. [1957]* eingeführtem Aromawert (engl. *odour active value* (OAV)). Er beschreibt das Verhältnis der Konzentration des Aromastoffs zu seinem Geruchsschwellenwert. Der Aromaextrakt wird bei beiden Methoden so lange im Verhältnis 1:1 verdünnt, bis kein Aromastoff mehr wahrgenommen werden kann (Aromawert = 1). Die Ergebnisse können als Diagramm dargestellt werden, indem die Retentionsindices gegen die Verdünnungsfaktoren (FD-Faktor: engl. *flavour dilution*) aufgetragen werden. Bei der CHARM Methode wird zusätzlich die Dauer eines Geruchseindrucks aufgezeichnet, und CHARM-Werte berechnet *[Reineccius 2010]*, *[Belitz et al. 2001]*. Die beschriebenen Methoden werden in der Literatur hinsichtlich ihrer Aussagekraft kritisch bewertet. Es werden durch sie vielmehr die sehr wahrscheinlichen Schlüsselkomponenten ermittelt. Eine weitere sensorische Evaluation ist anzuschließen *[Audouin et al. 2001]*.

1.1.5. Geruchsinn

Die Geruchswahrnehmung kann individuell sehr unterschiedlich sein. Sie entsteht durch mentale Konstruktionen im Gehirn und ist zum Teil an Erfahrungen des Einzelnen mit bestimmten Geruchseindrücken geknüpft.

Der Mensch besitzt Millionen von Riechzellen, die sich in der oberen Nasenhöhle im Riechepithel befinden. Die Riechhärchen dieser Zellen sind eingebettet in die Riechschleimhaut und binden die unterschiedlichen Aromastoffe an spezifischen Rezeptoren. Der durch die Bindung ausgelöste Reiz wird über verschiedene G-

Protein vermittelte Kaskaden verarbeitet und in den Riechkolben (*Bulbus olfactorius*) weitergeleitet. Dieser ist eine wichtige Schaltstelle des Geruchssinns und übermittelt die Informationen zum Gehirn, wo der Geruchseindruck entsteht.
Der Geruchssinn zeichnet sich durch seine Vielfältigkeit, Spezifität und sehr hohe Empfindlichkeit aus. Menschen können Schätzungen zufolge circa 10 000 Gerüche unterscheiden. Erklärungsansätze hierfür geben uns die Vielzahl von Geruchsrezeptoren, die unterschiedlichen Kaskaden und Signalwege zur Reizverarbeitung und die Leistung des Gehirns, eine riesige Zahl an Informationen zu verarbeiten und zu einem Gesamteindruck zu vereinen. Die Wichtigkeit des Geruchssinns wird auf der genetischen Ebene deutlich. Die Genfamilie bei Säugetieren, die für die Codierung der Geruchsrezeptoren verantwortlich ist, umfasst ca. 1000 Gene. Das macht ca. 1 % aller Gene bei Säugetieren aus *[Axel 1995]*. Obwohl im menschlichen Organismus nur circa 37 % der Gene funktionell sind, zeigt diese Anzahl, dass die Natur dem Geruchsinn eine enorme Bedeutung zukommen lässt *[Glusman et al. 2001]*.

Aromastoffe erreichen die Riechzellen auf zwei unterschiedlichen Wegen: nasal und retronasal. Beim normalen Einatmen durch die Nase gelangt nur ein geringer Anteil der Aromastoffe zum Riechepithel, da der Hauptstrom der eingeatmeten Luft an diesem vorbei geleitet wird. Durch gezieltes Riechen lässt sich diese Menge allerdings erheblich erhöhen. Bei der retronasalen Geruchswahrnehmung gelangen die flüchtigen Substanzen der Nahrung durch die Verbindung zwischen Mundhöhle und Nase größtenteils direkt zu den Riechzellen. Die retronasale Geruchwahrnehmung hat demnach einen großen Anteil am Gesamteindruck von verzehrten Lebensmitteln. Dieser setzt sich, neben der Wahrnehmung der Geruchsstoffe, auch aus der Wahrnehmung der Geschmacksstoffe und dem vom Lebensmittel vermittelten Mundgefühl (Temperatur, Konsistenz, Textur) zusammen. Im Englischen verwendet man für den Gesamteindruck eines verzehrten Lebensmittels den Begriff *flavour*, der alle Wahrnehmungen beim Verzehr eines Lebensmittels vereint. *[Rehner et al. 2002]*, *[Müller et al. 2009]*, *[Christen et al. 2004]*.

1.2. Pilze

1.2.1. Einteilung und Lebensweise

Lange Zeit zählte man Pilze (*Fungi*) zum Reich der Pflanzen. Man unterteilte die Lebewesen in zwei Reiche: Tiere (*Animalia*) und Pflanzen (*Plantae*). Tiere waren definiert als Lebewesen, die fressen, zu einer bestimmten Größe heranwachsen und sich aktiv fortbewegen. Pflanzen waren definiert als Lebewesen, die nicht fressen, ein unbegrenztes Wachstum aufweisen und sich nicht aktiv fortbewegen. Auf Grund dieser übereinstimmenden Eigenschaften mit den Pflanzen, wurden die Pilze, wie auch Algen und Bakterien, dem Reich der Pflanzen zugeordnet. Mit der Weiterentwicklung der Technik und der Entwicklung biochemischer Methoden, änderte sich die bisherige Systematik der Lebewesen. Mit Hilfe von Sequenzdaten ribosomaler Ribonukleinsäuren (rRNA) wurden die Lebewesen letztendlich in zwei Domänen aufgeteilt (Fig. 2): prokaryotische Organismen (*Bacteria* und *Archaea*) und eukaryotische Organismen. Die Domäne der Eukaryoten unterteilt sich in 5 Reiche, zu denen das Reich der Pilze gehört *[Raven et al. 2006]*.

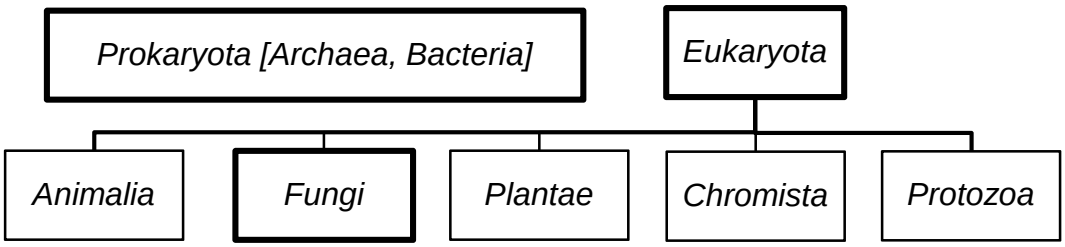


Fig. 2: Systematik der Lebewesen nach *Cavalier-Smith [1998]*

Im Reich der Pilze sind über 100.000 Arten bekannt, und es werden immer wieder neue Arten beschrieben. Aufgrund der großen Anzahl unterschiedlicher Lebensweisen und –formen, kann man von mehreren Millionen Pilzarten ausgehen *[O'Brien et al. 2005]*. Die Taxonomie der Pilze ist in Fig. 3 am Beispiel der in dieser Arbeit untersuchten Pilze dargestellt. *Isaria farinosa* (Ifa) ist in die Abteilung der Ascomyceten (Schlauchpilze) einzuordnen und ein typischer Vertreter der größten Unterabteilung der Schlauchpilze, den *Pezizomycota* (echte Schlauchpilze). *Piptoporus betulinus* ist ein Vertreter der Basidiomyceten (Ständerpilze) und in der Unterabteilung *Agaricomycotina* eingeordnet, welche die meisten bekannten Speisepilze enthält.

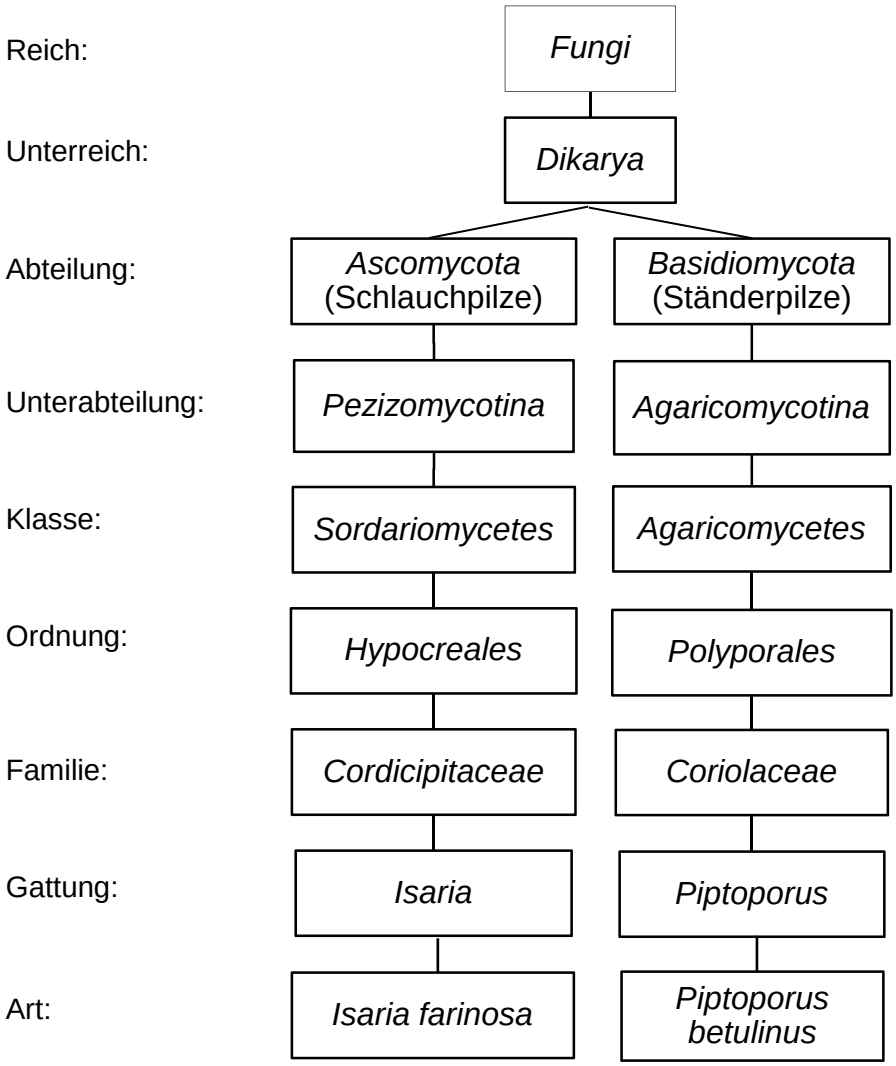


Fig. 3: Taxonomie der Pilze am Beispiel des Basidiomyceten *Piptoporus betulinus* und des Ascomyceten *Isaria farinosa* [BRENDA Tax Tree Explorer, *[Schomburg et al. 2013]*]

Pilze werden ausgehend von ihrer heterotrophen Ernährungsweise in drei Typen unterteilt: Saprophytische Pilze resorbieren ihre Nährstoffe aus totem Material, nachdem sie dieses mit einer Fülle von hydrolytischen Enzymen aufgeschlossen und in niedermolekulare Bausteine zersetzt haben. Parasitische und symbiotische Pilze hingegen beziehen ihre Nährstoffe aus anderen Organismen. Der Parasit entnimmt dem Organismus seine Nährstoffe, wodurch dieser Schaden nimmt. Der Symbiont hingegen erbringt eine vorteilhafte Gegenleistung für den Organismus. Als bekanntes Beispiel für einen saprobiotischen Pilz ist der bedeutendste Kulturpilz *Agaricus bisporus* (Kulturchampignon) zu nennen. Ein bekanntes Beispiel für einen symbiotischen Pilz ist der *Boletus edulis* (Steinpilz). Die symbiotische Interaktion zwischen Pilzen und Landpflanzen nennt man

Mykorrhiza. Das Pilzmycel steht im Kontakt mit den Wurzeln der Wirtspflanze, so dass ein Austausch von Nährstoffen aus dem Boden (Mineralstoffe, Stickstoff) einerseits und von Stoffwechselprodukten (Glucose aus der Photosynthese der Pflanze) andererseits stattfinden kann. Dies ist der Grund, warum Steinpilze nicht kultivierbar und frisch nur saisonal verfügbar sind. Der Kulturchampignon hingegen ernährt sich ausschließlich von totem Material, wodurch er – wie der Name schon verrät – in großen Mengen kultiviert werden kann und somit immer im Handel erhältlich ist *[Lüttge et al. 2010]*, *[Hall et al. 1998]*. Ein Beispiel für parasitär wachsende Pilze sind die beiden in dieser Arbeit besprochenen Pilze *I. farinosa* und *P. betulinus* (Birkenporling). Sie unterscheiden sich in ihrer Ernährungsweise anhand ihrer bevorzugten Organismen. *I. farinosa* befällt Insekten, vornehmlich Schmetterlinge, und zählt somit zu den entomopathogenen Pilzen *[Zimmermann 2008]*. *P. betulinus* befällt seinem Namen entsprechend Birken und baut deren Celluloseanteil rasch ab *[Valaskova et al. 2006]*. Er gehört zu den sogenannten Braunfäulepilzen.

Die Einteilung in Braun- und Weißfäulepilze bezieht sich auf das von den Pilzen abgebaute Material. Diese sogenannten Holzfäulepilze beziehen ihre Nährstoffe durch den Abbau der Zellwandbestandteile des Holzes (Cellulose, Hemicellulose und Lignin). Braunfäulepilze bauen Cellulose und Hemicellulose ab, so dass das im Holz verbleibende Lignin eine braune Färbung des Restes bewirkt. Weißfäulepilze bauen hauptsächlich den ligninhaltigen Teil der Pflanze ab. Es bleibt ein weißer Rest bestehend aus Cellulose und Hemicellulose zurück *[Schwantes 1996]*.

Ein weiteres Unterscheidungskriterium der in dieser Arbeit untersuchten Pilze ist die Einordnung des *P. betulinus* in die Abteilung der Basidiomyceten und des *I. farinosa* in die Abteilung der Ascomyceten.

1.2.2. Ascomyceten und Basidiomyceten

Die gängige Vorstellung von Pilzen bezieht sich auf die Abteilungen der Ascomyceten und der Basidiomyceten. Sie sind die phylogenetisch am höchsten stehende Einheit der Pilze und umfassen ca. 90 % aller bisher beschriebenen Pilzarten (30 % Basidiomyceten, 60 % Ascomyceten) *[Lüttge et al. 2010]*. Die meisten ihrer Vertreter bilden Fruchtkörper, welche im Volksmund als Pilz bezeichnet werden. Allerdings besteht der eigentliche Pilz zum größten Teil aus

einem unterirdischen Geflecht von vegetativen Hyphen, dem Mycel. Es übernimmt die Funktion der Nährstoffaufnahme und der Ausbreitung. Dieses kann sich - wie im Falle einer Art des Hallimasch (*Armillaria ostoya*) beschrieben - bis zu 8,8 km^2 ausbreiten *[Schmitt et al. 2008]*. Die meist an der Oberfläche erscheinenden Fruchtkörper bestehen aus eng miteinander verwobenen Hyphen und bilden den reproduktiven Teil des Pilzes, der für die Fortpflanzung verantwortlich ist. Anhand dieser lassen sich Ascomyceten und Basidiomyceten unterscheiden:

Bei der geschlechtlichen Reproduktion werden in den Fruchtkörpern der Ascomyceten sogenannte *Asci* (Plural von *ascus*: Schlauch) angelegt, in denen die Sporen (Ascosporen) gebildet werden. Dieser charakteristischen Schlauchbildung verdanken die Ascomyceten ihren Namen. Oft fallen die Fruchtkörper der Ascomyceten durch ihre auffällige Form und Farbe ins Auge.

Eine Besonderheit unter den Ascomyceten sind die Arten der Gattung *Tuber* (Trüffel). Diese bilden ihre Fruchtkörper unterhalb der Erdoberfläche. Die Verteilung der Sporen geschieht hier durch Tiere, die die Fruchtkörper anhand eines Lockstoffes orten, ausgraben, fressen und die Sporen mit dem Kot wieder ausscheiden. Weitere Beispiele für Ascomyceten sind Schimmelpilze wie der Schwarzschimmel *Aspergillus niger* und phytopathogene Pilze wie *Claviceps purpurea* (Auslöser des Mutterkorns). Auch Hefen (Unterabteilung: *Saccharomycotina*) wie die Bäckerhefe *Saccharomyces cerevisiae* zählen zur Gruppe der Ascomyceten.

In den Fruchtkörpern der Basidiomyceten werden die Sporen in sogenannten *Basidien* (Plural von *Basidie*: Ständer) angelegt und nach außen abgeschnürt. Es handelt sich also im Gegensatz zu den Ascosporen um exogen gebildete Sporen. Basidiomyceten lassen sich in drei Gruppen einteilen: Brandpilze, Rostpilze und echte Ständerpilze. Die meisten Speisepilze gehören der Gruppe der echten Ständerpilze an. So sind hier als Beispiel der Kulturchampignon, der Steinpilz und der Pfifferling (*Cantharellus cibarius)* zu nennen *[Raven et al. 2006]*, *[Lüttge et al. 2010]*.

1.2.3. Biotechnologisches Potential

Biotechnologie spielt schon seit Menschengedenken eine wichtige Rolle. Die Herstellung von Brot, Wein und Bier zum Beispiel mit Hilfe von Hefen, oder die Herstellung von Käse oder Joghurt durch Milchsäurebakterien sind wichtige

Bestandteile der Ernährung des Menschen. Zunächst blieben die Hintergründe dieser Verfahren ungeklärt, doch die Fortschritte im Bereich der Mikrobiologie und die Entwicklung der Molekularbiologie brachten Aufklärung und bereiteten den Weg für die heutige interdisziplinäre Biotechnologie mit breit gefächertem Anwendungsbereich in der Medizin (rote Biotechnologie), der Landwirtschaft (grüne Biotechnologie) und der Industrie (weiße Biotechnologie). Die Organisation für wirtschaftliche Zusammenarbeit und Entwicklung (OECD) definiert Biotechnologie wie folgt: *„The application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services."* [6]

Bei den von der modernen Biotechnologie genutzten Organismen spielen die Pilze eine wichtige Rolle. Pilze produzieren eine große Anzahl von unterschiedlichen Enzymen, die sich die Industrie zu nutzen machen kann. Einige Beispiele: Eine Laccase aus *Trametes versicolor* kommt in der Textil- und Papierindustrie zur Anwendung, die Protease Rennin aus *Mucor miehei* wird als Ersatz für das Lab zur Herstellung von Käse eingesetzt und Lipasen von *Aspergillus* Arten finden Anwendung in der Waschmittelindustrie *[Meyer 2008]*.

Neben der Produktion von Enzymen werden Pilze auch zur Produktion von chemischen Stoffen genutzt. Ein bekanntes Beispiel ist die Produktion des Antibiotikums Penicillin durch den Ascomyceten *Penicillium chrosogenum [Aharonowitz et al. 1981]*. Weitere Beispiele sind das Vitamin Riboflavin und die organischen Säuren Malein- und Fumarsäure aus *Candida* Arten *[Horgan et al. 2011]*. Eine weitere Stoffklasse, welche mit Hilfe von Pilzen und deren biotechnologisches Potential hergestellt werden können, sind die Aromastoffe.

1.3. Biotechnologische Aromaherstellung

Die Bildung von Aromastoffen und deren Bildungsmechanismus während der Herstellung von fermentierten Lebensmitteln ist weitestgehend bekannt. So sind Pilze, Hefen und Bakterien die Voraussetzung für den unverwechselbaren Gesamteindruck fermentierter Lebensmittel. Eine Herausforderung für die Wissenschaft ist es nun, dieses Potenzial der Mikroorganismen zur Aromabildung zu erforschen und gezielt zur industriellen Herstellung von Aromen zu nutzen.

[6] Definition der Biotechnologie laut OECD, Abruf der Seite 09.03.2014
http://www.oecd.org/sti/biotech/statisticaldefinitionofbiotechnology.htm

Die Isolierung von Aromastoffen aus Früchten oder Gewürzen ist zum Teil sehr aufwendig und eine Reinigung der gewünschten Substanzen aus ätherischen Ölen, meist komplexe Substanzmischungen, nur in den wenigsten Fällen wirtschaftlich. Die sehr viel günstigere Variante ist die Chemosynthese. So kostet Vanillin, gewonnen aus natürlicher Vanille, 1000 bis 4000 Dollar *[Serra et al. 2005]* und synthetisch hergestelltes Vanillin ca. 11 Dollar pro Kilogramm *[Schrader et al. 2004]*. Der moderne Verbraucher allerdings legt großen Wert auf natürliche Produkte. Für die Aroma- und Duftstoffindustrie stellt deshalb die heraushebende Kennzeichnung ihrer verwendeten Aromen als „natürlich“ (1.1.3) einen nicht zu unterschätzenden Marketingvorteil dar. Insbesondere bei Lebensmitteln werden chemosynthetisch hergestellte Inhaltsstoffe immer weniger vom Endverbraucher akzeptiert. Die optimale Lösung für die Lebensmittelindustrie sind demnach biotechnologisch erzeugte Aromastoffe:

- Der Preis für einen biotechnologisch hergestellten Aromastoff liegt meist im Mittelfeld zwischen natürlicher Gewinnung und Chemosynthese (biotechnologisch hergestelltes Vanillin z.B. kostet um die 1000 Dollar pro Kilogramm *[Schrader et al. 2004]*).
- Bei biotechnologischen Methoden ist die Regio- und Enantioselektivität der gebildeten Aromastoffe im Gegensatz zur Chemosynthese gewährleistet.
- Biotechnologisch hergestellte Aromastoffe können als natürlich deklariert werden (1.1.3).

Einen guten Überblick über die industrielle Anwendbarkeit biotechnologischer Herstellungsprozesse für Aromastoffe gibt *Schrader et al. [2004]*. In Tab. 1 sind einige Aromastoffe und deren industrieller Hauptsyntheseweg gelistet. Weitere Literatur gibt einen detaillierten Einblick in die aktuelle Forschung und die Fortschritte auf dem Gebiet der Aromabiotechnologie *[Berger et al. 2010]*, *[Schrader 2007]*, *[Serra et al. 2005]*, *[Menzel et al. 2007]*.

Tab. 1: Biotechnologisch hergestellte wichtige Aromastoffe *[Schrader et al. 2004]*

Aromastoffe (Geruch)	Vorstufe	Biokatalysator
Vanillin (vanilleartig)	Ferulasäure aus Reiskleie	*Amycolatopsis* sp.
γ-Decalacton (pfirsichartig)	Rizinolsäure aus Rizinusöl	*Yarrowia lipolytica*
Carbonsäuren (säuerlich, fruchtig)	Alkohole	*Acetobacteriaceae* (Essigsäurebakterien)
Ester (fruchtig)	Carbonsäuren Alkohole	Lipasen/Esterasen (immobilisiert auf Trägern)
C_6-Aldehyde/Alkohole (Grünnoten)	Linolsäure Linolensäure	Kombination aus biologischem Material (Sojamehl, Guave) und *Saccharomyces cerevisiae*

Das enorme Potenzial von Basidiomyceten zur biotechnologischen Produktion von Aromastoffen resultiert aus deren den Pflanzen meist sehr ähnlichen Bildungswegen von Aromastoffen und einem vielfältig ausgestatteten Pool verschiedenster Enzyme. Vor allem Oxidoreduktasen (Manganperoxidasen, versatile Peroxidasen) und Hydrolasen (Peptidasen, Lipasen, Esterasen), die für den Abbau der Biopolymere Lignin, Hemicellulose und Cellulose verantwortlich sind, wurden im Sekretom identifiziert *[Bouws et al. 2008]*. Das Potenzial von Basidiomyceten zur Aromabildung ist in zahlreichen Publikationen dokumentiert *[Lomascolo et al. 1999]*, *[Krings et al. 1995]*. So ist der Basidiomycet *Nidula niveo-tomentosa* in der Lage, den Schlüsselaromastoff der Himbeere, das Himbeerketon (4-(4-Hydroxyphenyl)-butan-2-on), *de-novo* zu synthetisieren *[Boeker et al. 2001]*. Durch Markierungsversuche wurde der Biosyntheseweg ausgehend von L-Phenylalanin aufgeklärt *[Zorn et al. 2003]*. Der Gemeine Schwefelporling, *Laetiporus sulphureus*, synthetisiert ausgehend von L-Isoleucin den Aromastoff Sotolon (3-Hydroxy-4,5-dimethyl-2(5H)-furanon), welcher sich durch eine geringe Geruchsschwelle und einen starken würzigen Geruch nach Brühe auszeichnet *[Lanfermann et al. 2014]*. Vertreter der Gattung *Pleurotus* sind in der Lage durch Oxyfunktionalisierung von Terpenkohlenwasserstoffen geruchsintensive Terpene zu bilden: So wird *β*-Myrcen zu Perillen *[Kruegener et al. 2009]*, *α*-Pinen zu Verbenon *[Krings et al. 2009]* und (+)-Valencen zum Schlüsselaromastoff der Grapefruit (+)-Nootkaton umgewandelt *[Fraatz et al. 2009]*. Der Kleine

Knoblauchschwindling (*Marasmius scorodonius*) ist ein weiteres Beispiel für das Konversionspotential der Basidiomyceten: durch die Spaltung von Carotinoiden bildet er norisoprenoide Aromasubstanzen, wobei *β*-Ionon dominiert *[Zelena et al. 2008]*, *[Zorn et al. 2003]*.

Auf der Suche nach vielversprechenden Methoden der nachhaltigen Aromastoffherstellung spielen Pflanzenzellsysteme in der modernen Aromaforschung eine Rolle. Aromastoffe können mit Hilfe von Zellsystemen sowohl *de novo*, als auch durch Biotransformation synthetisiert werden. Die Zellen stellen alle nötigen Cofaktoren und Transporter bereit und sind in der Lage die Enzyme zu regenerieren. Vorteile im Vergleich zur Kultivierung der Pflanzen sind u.a. die Unabhängigkeit von Wetter oder Krankheiten, die *in situ* Produktion in jedem beliebigen Labor und die höhere Ausbeute. Etablierte Systeme dienen vor allem der Herstellung von Pharmazeutika, welche mit großen Ausbeuten produziert werden können. Auch die Bildung von Aromastoffen in Zellsystemen konnte nachgewiesen werden, allerdings mit geringen Ausbeuten (Übersicht über gebildeten Aromastoffe, verwendeten Pflanzen und Gehalte siehe *Scragg [2007]*). Unter dem wirtschaftlichen Aspekt betrachtet, sind Pflanzenzellsysteme zur Aromabildung noch nicht konkurrenzfähig. Auch die Frage der Akzeptanz der auf diesem Wege hergestellten Aromastoffe beim Verbraucher bleibt abzuwarten *[Scragg 2007]*.

Bezüglich gentechnisch veränderter Organismen und dadurch hergestellter Substanzen ist die Akzeptanz des Verbrauchers sehr gering. Dennoch spielt die Gentechnik in der modernen Aromaforschung eine wichtige Rolle. Enzyme die Aromastoffe generieren, können durch heterologe Expression mit geeigneten Organismen als potente Biokatalysatoren zur Verfügung gestellt werden. Alternativ können Zellen mit Genen ausgestattet werden, die neue Biosynthesewege bereitstellen und so eine Überproduktion an wertvollen Aromastoffen initiieren. Terpenoide wie Linalool, C_6-Aldehyde, Ester und der wichtigste Aromastoff Vanillin werden u.a. mit Hilfe von gentechnischen Methoden effizient hergestellt *[Schwab 2007]*.

Es sind eine Reihe biotechnologischer Methoden zur Aromaherstellung auf dem Markt etabliert. Die mannigfaltigen Vorlagen der Natur zur Aromaherstellung sind jedoch noch lange nicht von Seiten der Biotechnologie ausgeschöpft. Im Hinblick

auf das wachsende Bedürfnis der industrialisierten Nationen nach Nachhaltigkeit und natürlichen Produkten und der schnellen Entwicklung neuer effizienter biotechnologischer Methoden ist ein Wachsen der Biotechnologie im Bereich der Aromastoffe abzusehen.

1.4. Motivation und Zielsetzung

Der moderne Verbraucher legt großen Wert auf natürliche Produkte und eine nachhaltige Produktion von Lebensmitteln. Die Lebensmittelindustrie ist demnach bestrebt, natürliche Aromastoffe/Aromaextrakte zu verwenden. Außerdem wird Augenmerk auf eine nachhaltige Firmenpolitik gelegt. Die Biotechnologie eröffnet der Aromenindustrie in dieser Hinsicht viele Möglichkeiten. Es werden bereits mehr als 100 biotechnologisch hergestellte Aromastoffe auf dem Markt vertrieben *[Schrader 2007]*. Diese werden meist ausgehend von Vorläufersubstanzen durch Biokonversion gebildet. Als Quelle solcher Vorläufersubstanzen können Nebenströme der Lebensmittelindustrie verwendet werden, die momentan hauptsächlich als Futter- /Düngemittel oder Energiequelle genutzt werden. Nebenströme enthalten jedoch auch wertvolle Präkursoren für Aromastoffe, wie z.B. Fette/Fettsäuren in Ölpresskuchen.

Ziel der Arbeit war es zunächst, eine Reihe von Pilzen (vornehmlich Basidiomyceten), deren biotechnologisches Potential zur Aromabildung bekannt ist *[Vandamme 2003]*, auf Nebenströmen submers zu kultivieren und daraus effiziente Stamm-Substrat-Kombinationen abzuleiten.
Im Anschluss an das Screening wurden die Ziele entsprechend der ausgewählten Kombinationen näher definiert:
Die Produktion von Raucharoma (4-Vinylguajacol, (4-VG)) durch einen Ascomyceten (*Isaria farinosa*) bietet die Möglichkeit, die für die Lebensmittelindustrie wichtige Produktgruppe Raucharoma ohne die als kanzerogen geltenden polycyclischen aromatischen Kohlenwasserstoffe zu produzieren *[Theobald et al. 2012]*. Das für die Konversion von 4-Vinylguajacol aus Ferulasäure verantwortliche Enzym sollte isoliert und charakterisiert werden.
Die Kultivierung eines Basidiomyceten (*Piptoporus betulinus*) auf Kohlschnitt führte zu einem angenehmen Geruch nach Ananas und Honig. Da Ananas eine der beliebtesten Südfrüchte darstellt, galt es die Kultivierung in größerem Maßstab

zu etablieren, das Aromaspektrum zu charakterisieren und für den exotischen Ananasgeruch des Extraktes verantwortliche Schlüsselsubstanzen zu identifizieren.

Weiterhin sollte eine Schnellmethode zur Analyse von Aromastoffen in fetthaltigen Matrices entwickelt werden, um ein effektives Screening gewährleisten zu können.

2. Vorwort zu: „Biotechnological production of natural flavour compounds”

Basidiomyceten besitzen ein enormes Potential zur Bildung von Aromastoffen. Für die industrielle Produktion von Aromastoffen kommen allerdings gegenwärtig in erster Linie biotechnologische Verfahren zum Einsatz, die auf pro- und eukariotische Mikroorganismen (Bakterien und Hefen) sowie auf aus diesen isolierte Enzyme als Katalysatoren zurückgreifen (siehe Kapitel 1.3).
Um das große Potential der höheren Pilze zur Biokonversion von Aromastoffen nutzen zu können, wurde ein Screening angelegt, in dem vornehmlich Basidiomyceten auf Nebenströmen der Lebensmittelindustrie submers kultiviert wurden. Aromastoffe, die auf biotechnologischen Verfahren mit höheren Pilzen basieren, erlauben die Kennzeichnung entsprechender Produkte als "natürlich" im Sinne des Lebensmittelrechts (siehe Kapitel 1.1.3). Der Vorteil von Nebenströmen als Substrate zur Biokonversion besteht in deren Gehalt an wichtigen Vorstufen für Aromastoffe, wie z.B. Fette/Öle, Lignin und Carotinoide.

Im folgenden Kapitel werden die Ergebnisse aus dem Screening verschiedener Stamm-Substrat-Kombinationen vorgestellt.

Der Ascomycet *Isaria farinosa*, submers kultiviert auf Rapspresskuchen, ist aufgrund der Bildung eines Geruchs, der an geräucherten Schinken und Nelke erinnert, im Rahmen des Screenings aufgefallen. Eine solche biotechnologische Herstellung von Raucharoma bietet eine Alternative zur klassischen Herstellung über Rauchkondensate. Das letztgenannte Verfahren steht vor allem wegen der dabei auftretenden Bildung von polyzyklischen aromatischen Kohlenwasserstoffen und den damit verbundenen gesundheitlichen Risiken in der Kritik (siehe Kapitel 1.1.2, 1.1.3).
Es wurde im weiteren Verlauf der Arbeit demnach das Aromaspektrum der Kultivierung von *I. farinosa* auf Rapspresskuchen analysiert. Als Hauptkomponente wurde 4-Vinylguajacol identifiziert. Bei einem angeschlossenen Substratscreening konnte Ferulasäure als Vorstufe identifiziert werden. Hierbei

sind die hohe Umsatzrate und die Spezifität der Reaktion aufgefallen. Deshalb wurde die Isolierung und Charakterisierung des für die Bildung von 4-VG verantwortlichen Enzyms angeschlossen.

3. Biotechnological production of natural flavour compounds

3.1. Abstract

In total 32 basidiomycetes and one ascomycete were cultivated submerged and screened for flavour production. The culture medium was supplemented either with side streams of the food industry, such as cabbage cuttings and rapeseed press cake or castor oil and rapeseed oil. A number of interesting flavour impressions were found for several substrate / fungus combinations. Amongst them one turned out to be most promising and was analysed in more detail. Cultivation of *Isaria farinosa* (Ifa) on rapeseed press cake resulted in an aroma impression reminiscent of carnation and smoked ham. The produced aroma profile was analysed by means of gas chromatography-olfactometry and mass spectrometry. The characteristic flavour impact compound of the volatile fraction of the culture supernatant was identified as 4-vinylguaiacol (4-ethenyl-2-methoxyphenol (4-VG)), originated from the degradation of ferulic acid ((*E*)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid (FA). The corresponding enzyme was isolated using a serial combination of anion exchange followed by size exclusion chromatography. For the purified enzyme maximal activity was determined at pH 6 and 20 °C.

3.2. Introduction

The history of flavour industry was initiated by distillation of essential oil and extraction of organic material in the second half of the 19th century *[Wright 2010]*. Nearly one century later first discoveries in the field of biotechnological production of flavours cleared the way for modern biotechnology. First of all yeasts and bacterial strains were used *[Schrader 2007]*; *[Farbood et al. 1983]*. Later on also higher fungi were observed for generating flavour substances *[Berger et al. 2004]*. Representatives of these higher fungi are basidiomycetes. Therefore in this work mostly basidiomycetes were chosen because of their known bioconversion and

biotransformation potential *[Abraham et al. 1994]*; *[Krings et al. 1998]*. Although basidiomycetes are known for their flavour synthesis *de novo [Lomascolo et al. 1999]*, the focus was placed on bioconversion of side streams of the food industry to flavour substances. Selected strains were cultivated on side streams, which act as the single nitrogen source for the growth of the fungus and also as a source of flavour precursors such as lipids, carotenoids, lignin and sulphurous components. Sulphur-containing side stream cabbage cutting was mainly examined in this work. Beside mentioned side stream, rape oil and castor oil were used for supplementation, because of the well-known lactone formation originated from fatty acids *[Gatfield 1997]*. Several basidiomycetes, especially *Polyporus* spp., transform castor oil to flavour components *[Tiefel et al. 1993]*. One of the first described microbiological flavour formation was the conversion of castor oil to 4-hydroxydecanoic acid and further to 5-hexyloxolan-2-one (*γ*-decalactone) *[Farbood et al. 1983]*.

Flavours, produced by bioconversion of side streams by means of basidiomycetes, can be labelled natural. The Regulation (EC) No. 1334/2008 defines natural flavouring substance as follows: “(...) flavouring substance obtained by appropriate physical, enzymatic or microbiological process from material of vegetable, animal or microbiological origin (...). Natural flavouring substances correspond to the substances that are naturally present and have been identified in nature;”

Therefore, beside some other interesting strain-substrate combinations described in this work, one turned out to be particularly valuable: *Isaria farinosa* (Ifa) was able to transform FA to 4-VG. 4-VG naturally occurs, *inter alia*, in coffee, apple and grapes, has a smoky, clove-like odour impression *[Sterckx et al. 2011]* with a low flavour threshold of 0.4-0.8 ng L^{-1} *[Blank et al. 1989]* and is used as a flavouring substance in food categories like baked goods and meat products *[Burdock 2001]*. It is also known as an important ingredient of liquid smoke flavourings *[Guillen et al. 2002]*, *[Fiddler et al. 1970]*. Commercial used smoke flavourings are produced by thermal degradation of wood. During the pyrolysis process, polycyclic aromatic hydrocarbons (PAHs) are formed. This class of substances contains carcinogenic substances *[Theobald et al. 2012]*. Therefore, some special legal regulations exist for smoke flavourings. According to the Regulation (EU) No. 1321/2013 only three smoke flavourings comply with the safety regulations of the European Food Safety Authority (EFSA).

The “cold” production of smoke flavouring by enzymatic biotransformation, described in this work for Ifa, captivate by its lack of healthy risks and the fact, that it can be declared as natural. Different microorganisms were discussed in literature to transform FA in 4-VG *[Rho et al. 2007]*, *[Gu et al. 2011]*, *[Max et al. 2012]*. The ascomycete *I. farinosa*, used in this study, is an entomopathogenic fungus, which is well known in literature *[Zimmermann 2008]*. Nevertheless, nor the production of 4-VG neither a ferulic acid decarboxylase (FaD) has been described yet.

This work discusses the high potential of fungi for flavour production, cultivated on the side stream cabbage cutting and on natural oils. In addition, it deals with the flavour profile and the purification and characterisation of FaD derived from *I. farinosa*.

3.3. Material and methods

3.3.1. Microorganisms

The microorganisms and their origin are listed in Tab. 2. The strains were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany) and the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). Two strains were obtained from *Abteilung für Molekulare Holzbiotechnologie und technische Mykologie (*Georg-August-Universität Göttingen, Germany*)*. One strain was isolated from wood in Lower Saxony (Germany) and identified according to its internal transcribed spacer (ITS) sequence. All organisms are part of the phylum Basidiomycota, except *I. farinosa* which belongs to the phylum Ascomycota.

Tab. 2: Microorganisms used in the screening; DSMZ (German Collection of Microorganisms and Cell Cultures); CBS (Centraalbureau voor Schimmelcultures)

Organism	Vendor	Strain n.	Organism	Vendor	Strain n.
Agaricus arvensis	DSMZ	8327	*Marasmius cohortalis*	DSMZ	8257
Agrocybe aegerita	1)		*Marasmius scorodonius*	CBS	850.87
Armillaria mellea	CBS	100.12	*Nidula niveo-tomentosa*	CBS	380.80
Auriporia aurea	CBS	294.71	*Phlebia radiata*	DSMZ	5111
Auriporia aurulenta	CBS	106238	*Piptoporus betulinus*	2)	
Bjerkandera adusta	DSMZ	3375	*Pleurotus eryngii*	CBS	613.91

Organism	Vendor	Strain n.	Organism	Vendor	Strain n.
Flammulina velutipes	DSMZ	1658	*Pleurotus floridanus*	1)	
Fomitopsis pinicola	DSMZ	4957	*Pleurotus ostreatus*	DSMZ	1020
Fomitopsis rosea 2)	CBS	313.36	*Pleurotus pulmonarius*	DSMZ	5331
Gloeophyllum odoratum	CBS	444.61	*Pleurotus sapidus*	DSMZ	8266
Grifola frondosa	CBS	480.63	*Pycnoporus cinnabarinus*	CBS	353.63
Hericium erinaceus	CBS	260.74	*Trametes suaveolens*	DSMZ	5237
Isaria farinosa 3)	CBS	481.73	*Tyromyces floriformis*	CBS	232.53
Ischnoderma benzoinum	CBS	311.29	*Tyromyces sambuceus*	CBS	492.76
Kuehneromyces mutabilis	DSMZ	1684	*Wolfiporia cocos*	CBS	279.55
Laetiporus sulphureus	DSMZ	11211	*Xerula radicata*	DSMZ	4830
Lentinula edodes	CBS	225.51			

1) Obtained from: *Abteilung für Molekulare Holzbiotechnologie und technische Mykologie,* Georg-August-Universität Göttingen, Germany
2) Isolated in Lower Saxony, Germany
3) Belongs to the phylum Ascomycota

3.3.2. Substrates

Rapeseed press cake was purchased from Teuteburger Ölmühle GmbH Co KG, (Ibbenbühren, Germany); Rice bran from BENEO-Remy (Leuven-Wijgmaal, Belgium) and hemp fibre was purchased from Hock GmbH Co. KG (Nördlingen, Germany). Cabbage was derived from a local supermarket and was cut into small pieces and conserved by freeze drying. Castor and rapeseed oil were derived from a local supermarket.

3.3.3. Cultivation of microorganisms

Screening

Pre-cultures were prepared by homogenisation of a 10 × 10 mm agar plug with mycelium using an Ultra Turrax (Miccra D-9, Art, Mühlheim, Germany). Afterwards the fungi were grown submerged in modified liquid standard nutrition medium (SNM) *[Sprecher 1959]*. For main culture a C-reduced and N-free minimal medium

(MM1), supplemented with the appropriate side stream was used. Tab. 3 lists the components of used media.

Tab. 3: Composition of standard nutrition medium (SNM) and minimal media (MM)

Components	SNM	MM1	MM2
D-(+)-Glucose monohydrate [g L^{-1}]	30.0	3.0	3.0
L-Asparagine monohydrate [g L^{-1}]	4.5	--	0.45
Yeast extract granulated [g L^{-1}]	3.0	--	0.3
KH_2PO_4 [g L^{-1}]	1.5	1.5	1.5
$MgSO_4$ [g L^{-1}]	0.5	0.5	0.5
Trace element solution [mL L^{-1}] [1)]	1.0	1.0	1.0
Side stream [% (w/w)]/ oil [µL]	--	2/0.1	2

[1)] Trace element solution consists of:
0.08 g L^{-1} $FeCl_3$ x 6 H_2O, 0.09 g L^{-1} $ZnSO_4$ x 7 H_2O,
0.03 g L^{-1} $MnSO_4$ x H_2O, 0.005 g L^{-1} $CuSO_4$ x 5 H_2O,
0.4 g L^{-1} EDTA disodium salt dihydrate

Prepared medium was adjusted to pH 6.0 with 1 M NaOH and autoclaved at 121 °C and 1 bar positive pressure for 20 min. Furthermore biological (fungus in MM) and chemical (side stream in MM) controls were carried out. Main cultivation was performed for 14 days. Olfactory evaluation and sampling took place at day four, ten and 14. Furthermore there was a complete harvest at the day of maximum odour impression. Subsequent analysis of flavour compounds is described in section 3.3.6.

Cultivation of *Isaria farinosa*

To determine the pathway of 4-VG formation, main cultivation was performed in MM2 (Tab. 3), supplemented with different substrates, shown in Tab. 4. Stock solution of vanillin ((4-hydroxy-3-methoxybenzaldehyde); (V)), ferulic acid methyl ester ((methyl (E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoate); (FAME)) and ferulic acid (FA) were prepared in ethanol (EtOH).

Tab. 4: Amount of substrate and substances in main cultivation of *Isaria farinosa*

Substrate	Added to 150 mL
Hemp fibre	3 g
Rapeseed press cake	3 g
Rice bran	3 g
Lignin	0.01 g
Vanillin	0.5 mM
Ferulic acid methyl ester	0.5 mM (EtOH)
Ferulic acid	0.5 mM (EtOH)

Determination of produced 4-VG was carried out by means of olfactometry.

For enzyme isolation, further cultivation of Ifa was carried out as follows: Main culture was inoculated with a three day old pre-culture in SNM and supplemented with FA at day one. Harvesting took place at day five. Concentration of FA in cultivation medium was 0.5 mM.

Upscaling of cultivation was performed in a 5 L (working volume) stirred tank reactor (ISF 100, Infors HT, Bottmingen, Switzerland) at 24 °C with an air flow of 1 L min^{-1} through a ring sparger. The bioreactor was equipped with pitched blade impeller running at 120 rpm. Before inoculation the bioreactor was autoclaved at 121 °C and 1 bar positive pressure for 30 min, containing 4.5 L SNM. Inoculation took place with 500 mL of three day old pre-culture. FA induction of main cultivation took place at day one and the bioreactor was harvested at day four.

3.3.4. Purification of ferulic acid decarboxylase (FaD)

Activity assay was performed as described by *Rheinheimer [2013]* with minor adaption. The purification method was expanded with a second step (AIEX).

Extraction of FaD

Approximately 3 g mycelium (pre-washed with demineralised water) were mixed with 2 mL of potassium phosphate buffer (PPB; 50 mM; pH 6.5) and disrupted in a bead mill (precellys 24 peqlab, Erlangen, Germany). After incubation of disrupted cells in the phosphate buffer for approx. 1 h, enzyme solution was centrifuged at

4 °C, 4000 g for 10 min (Rotina 380, Hettich, Tuttlingen, Germany), supernatant was withdrawn and cell pellet was washed with buffer. Combined enzyme solution and washing buffer was centrifuged again at 4°C, 21188 g (Rotanta 460R, Hettich, Tuttlingen, Germany). The clear supernatant (filtration by using a 0.45 µm polyester membrane if necessary) was applied to the fast protein liquid chromatography (FPLC using a BioLogic DuoFlow System (Biorad, Hercules, California, USA)).

Freeze-drying of mycelium and further resolving in PPB was also used for cell disruption and enzyme extraction.

Precipitation with acetone

Enzyme solution derived after cell disruption and centrifugation was mixed with acetone (1:5 (v/v)) and incubated at -20°C for 1-3 hours. After centrifugation (4 °C, 4000 g for 10 min) solvent was removed from the pellet (freeze drying, nitrogen stream) and re-dissolved in PPB (10 mM; pH 6.5).

Size exclusion chromatography (SEC)

Separation of the enzyme was achieved by using a Superdex 75 10/300 GL column (GE Healthcare, Buckinghamshire, England) with PPB (10 mM; pH 6.5) as running buffer and a constant flow of 0.4 mL min^{-1}. Injection volume was 1 mL. Active fractions were pooled and concentrated by freeze drying if necessary.

Anion exchange chromatography (AIEX)

Further separation of the enzyme was achieved by using a 25 mL self-casted Q-Sepahrose FF column (XK 16/20, GE Health care) with PPB (10 mM; pH 6.5) as running buffer, PPB (10 mM, pH 6.5, 1M NaCl) as eluting buffer and a constant flow of 5 mL min^{-1}. Injection volume was between 50-100 mL. The enzyme eluted at 10 % eluting buffer (100mM NaCl). Active fractions were pooled, desalted and concentrated. Desalting and concentration was performed by dialysis (MWCO 3500, 24 h) and subsequent freeze drying or by ultra filtration (MWCO 3500, 3000 g). Enzyme solution after ultra filtration and resolved lyophilisate respectively was applied to SEC.

Gel electrophoresis

Gel electrophoresis was performed as described elsewhere *[Laemmli 1970]*. Denaturing SDS-PAGE (sodium dodecyl sulphate (SDS) - polyacrylamide gel

electrophoresis (PAGE)) was performed on a 12 % or 16 % polyacrylamide resolving gel. 30 µL of the sample were mixed with 20 µL loading buffer (0.1 M Tris/HCL (pH 6.8), 0.2 M dithiothreitol (DTT), 4 % SDS, 20 % glycerol, 0.2 % bromophenol blue) and heated for 10 minutes. Semi-native SDS-PAGE was performed on a 12 % or 16 % polyacrylamide separation gel at 4 °C. 30 µL of the sample was mixed with 20 µL loading buffer (as described above, without DTT). After electrophoresis at 15 mA and room temperature the gel was stained with Coomassie R (0.005 % Coomassie brilliant blue G-250 in demineralised water, 25 % ethanol, 10 % acetic acid) or silver *[Blum et al. 1987]*.

Fluorescence spectroscopy

Sample was labelled with fluorescence dyes (T-Dye red 310, G-Dye red 310, DyeAGNOSTICS, Halle, Germany) according to Refracting-2D Labelling Kit (DyeAGNOSTICS, Halle, Germany) and separated by means of 16.5 % Tricin-SDS-PAGE *[Schaegger et al. 1987]*. Afterwards gel was scanned with a fluorescence scanner (Typhoon, GE Healthcare, Buckinghamshire, England) to visualize separated protein bonds (excitation: 650 nm / emission: 665 nm).

Activity assay

Reaction mixture consisted of 30 µL enzyme solution and 71 µL FA-PPB (50 mM; pH 6.5; FA 3.57 mM). It was incubated for 90 min at 26 °C, while shaking with 450 rpm (thermomixer comfort, Eppendorf, Hamburg, Germany). The assay was terminated by addition of 399 µL acetonitrile. An aliquot was analysed by HPLC after centrifugation at 4°C, 17860 g for 15 min (Micro 200R, Hettich, Tuttlingen, Germany).One unit of enzyme activity was defined as 1 µmol 4-VG produced per minute.

3.3.5. Characterisation of the enzyme

To determine the pH optimum, the enzyme solution derived after described purification steps was incubated in the pH range from 3.6 to 8.0. Following buffer systems were used: 100 mM acetate buffer (3.6-5.5) and PPB (5.6-8.0). FA concentration in each buffer solution was 1.8 mM.

To determine the temperature optimum, the enzyme solution derived after described purification steps was incubated in the temperature range from 4.9 °C to

45.4 °C without shaking, using Mastercycler Gradient (Eppendorf, Hamburg, Germany).

K_m and v_{max} values were determined by incubation of the enzyme with different ferulic acid concentrations (range from 0.005 mM to 5 mM in EtOH) and calculated according to Michaelis-Menten using Origin (OriginLab, Northampton, USA).

Substrate specificity against different substrates (Tab. 5), particularly cinnamic acid ((*E*)-3-phenylprop-2-enoic acid) derivates, was carried out by incubation with different compounds dissolved in PPB (50 mM, pH 6.5). A stock solution of each substrate was prepared in EtOH.

Tab. 5: Different substrates used for determination of substrate specificity.

Substrate	[mM][1)]
(*E*)-3-Phenylprop-2-enoic acid (cinnamic acid)	1.847
(*E*)-3-(2,3,4-Trimethoxyphenyl)prop-2-enoic acid (2,3,4-trimethoxycinnamic acid)	1.847
(*E*)-3-(3,4,5-Trimethoxyphenyl)prop-2-enoic acid (3,4,5-trimethoxycinnamic acid)	1.847
(*E*)-3-(2,3-Dimethoxyphenyl)prop-2-enoic acid (2,3-dimethoxycinnamic acid)	1.847
(*E*)-3-(2,4-Dimethoxyphenyl)prop-2-enoic acid (2,4-dimethoxycinnamic acid)	0.924
(*E*)-3-(3,4-Dimethoxyphenyl)prop-2-enoic acid (3,4-dimethoxycinnamic acid)	1.847
(*E*)-3-(2,5-Dimethoxyphenyl)prop-2-enoic acid (2,5-dimethoxycinnamic acid)	1.847
(*E*)-3-(3,5-Dimethoxyphenyl)prop-2-enoic acid (3,5-dimethoxycinnamic acid)	1.847
(*E*)-3-(3-Methoxyphenyl)prop-2-enoic acid (3-methoxycinnamic acid)	1.847
(*E*)-3-(4-Methoxyphenyl)prop-2-enoic acid (4-methoxycinnamic acid)	1.847
(*E*)-3-(4-Hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid (sinapinic acid)	1.847
(*E*)-3-(4-Hydroxyphenyl)prop-2-enoic acid (4-hydroxycinnamic acid)	1.847
(*Z*)-3-(4-Hydroxyphenyl)prop-2-enoic acid (4-hydroxycinnamic acid)	1.847
(*E*)-3-(2-Hydroxyphenyl)prop-2-enoic acid (2-hydroxycinnamic acid)	1.847
(*E*)-3-(3-Hydroxyphenyl)prop-2-enoic acid (3-hydroxycinnamic acid)	1.847
(*E*)-3-(3,4-Dihydroxyphenyl)-2-propenoic acid (caffeic acid)	1.847
3-Phenylpropanoic acid	1.847
3-(4-Hydroxyphenyl)propanoic acid (phloretic acid)	1.847
3-(4-Hydroxy-3-methoxyphenyl)propanoic acid (3-methoxyphloretic acid)	1.847

Substrate	[mM][1)]
(*E*)-3-(4-Hydroxy-3-methoxyphenyl)prop-2-enoic acid (ferulic acid)	1.847
(*E*)-3-(3-Hydroxy-4-methoxyphenyl)prop-2-enoic acid (isoferulic acid)	0.914
4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	1.847
2-Phenyl acetic acid	1.847
Methyl (*E*)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoate (ferulic acid methyl ester)	1.847
Methyl (*E*)-3-phenylprop-2-enoate (cinnamic acid methyl ester)	1.847

[1)] Concentration in potassium phosphate buffer (50 mM, pH 6.5)

3.3.6. Flavour analysis

Solvent extraction of volatiles

Liquid culture broth was extracted in a separating funnel three times or via continuous liquid/liquid extraction with pentane/diethylether (1:1.12 (v/v)) (P/E). The organic phase was dried over sodium sulphate and concentrated with a Vigreux column at 40 °C. External standard was cyclopentanol, internal standard (determination of the recovery) was cyclohexanol. The derived flavour extracts were analysed by means of gas chromatography, described in 3.3.8.

Aroma extract dilution analysis (AEDA)

A flavour dilution factor (FD-factor) was determined for each sensory active compound by sniffing a dilution series. Therefore, the extract was diluted stepwise (1:1) with P/E, until odorous compounds were no longer perceived at the sniffing port.

3.3.7. High performance liquid chromatography (HPLC)

Determination of FA and 4-VG was performed using a Hewlet Packard Ti-Series 1050 system, coupled with a Hewlett Packard 1050 multiple wavelength detector (detection wavelengths: 280nm, 263 nm, 325 nm). Separation was performed using a Chromolith Performance RP-18e 100-4.6 reverse-phase column (Merck; Darmstadt; Germany) with following gradient at a flow rate of 1.5 mL min^{-1}: 20 µL sample was loaded in 90 % buffer A (0.1 % formic acid), 10 % buffer B (acetonitrile); 10-58 % buffer B in 6.0 minutes; 58-100 % buffer B in 2.0 minutes;

100 % buffer B for 1 minute; 100-10 % buffer B in 4 minutes; re-equilibrate with 10 % buffer B for 2 minutes; total run time: 15 minutes.

3.3.8. Gas chromatography

Gas chromatography-olfactometry (GC-O)

GC-O was performed using an Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled with a flame ionisation detector (FID, 250 °C) and an olfactory detection port (ODP, 250 °C; Gerstel, Mühlheim, Germany). The chromatographic conditions were: 30 m x 0.32 mm i.d. x 0.25 µm DB-WAX (J&W Scientific, Folsom, USA) with 2 mL min^{-1} hydrogen as carrier gas using the following temperature program: 40 °C (hold for 3 minutes) with a rate of 5 °C min^{-1} to 230 °C, hold for 10 minutes.

Gas chromatography mass spectrometry (GC-MS)

The volatile extracts were analysed using a Fisons GC 8000 gas chromatograph and a Fisons MD 800 mass selective detector (interface: 230 °C, ion source: 200 °C, quadrupole: 100 °C, electron impact ionisation 70 eV, scan range m/z 33 to 300 amu). Analytical conditions were as follows: 30 m x 0.32 mm i.d. x 0.25 µm DB-WAX (J&W Scientific, Folsom, USA) with 1 mL min^{-1} helium as carrier gas using the following temperature program: 40 °C (hold for 3 minutes) with a rate of 3 °C min^{-1} to 230 °C hold for 10 minutes. Identification of volatiles was achieved by comparing their linear retention indices (RI), calculated according to *n*-alkanes as external references *[Van den Dool et al. 1963]*, odour impression and mass spectra with those retrieved from digital libraries (Wiley 08/NIST 08).

3.4. Screening of basidiomycetes for flavour formation – noticeable strain-substrate combinations

In total 32 fungi, listed in Tab. 2, were cultivated on cabbage cuttings, rape oil and castor oil in order to find some potential flavour producers. Cultivation took place for 14 days, while samples were taken on day four, ten and 14. Tab. 6 shows the interesting strain-substrate combinations with their characteristic odour impression and their time course.

Tab. 6: Strain-substrate combination for flavour production.
CC: cabbage cuttings; RO: rape oil; CO: castor oil.

Basidiomycetes	Day 4	Day 10	Day 14
Auriporia aurulenta (CC)	Cabbage	Cabbage	Sweetish, fruity
Fomitopsis pinicola (RO)	--	Citric	Sourishly
Gloeophyllum odoratum (CC)	Fruity	Fruity, pear	Melon, lychee
Laetiporus sulphureus (CO)	--	--	Peach
Piptoporus betulinus (CC)	Cabbage	Cabbage, sweetish	Pineapple, honey
Polyporus floridanus (RO)/(CO)	Fresh, sweetish, flowery	Fresh, sweetish, flowery	--
Pycnoporus cinnabarinus (CC)	Concord grape	Concord grape	Concord grape
Trametes suaveolens (CC)	Almond, sweetish	Almond, sweetish	Almond, liquorice

The basidiomycete *Auriporia aurulenta* – in Germany colloquially called *duftender Goldporling*, named according to its fruity, peach like flavour and its fresh orange colour *[Kunze 2010]* – is a rare brown rot fungus. It was part of the screening, because of the described smell of fruiting bodies and the fact, that *A. aurulenta* is not known in scientific literature. 14 days of cultivation on cabbage cuttings resulted in a pleasant fruity and sweet odour impression.

The flavour of fruiting bodies of *Fomitopsis pinicola* is primarily evoked by a wide range of terpenes *[Roesecke et al. 2000]*. Submerge cultivation of this fungus supplemented either with pure rape oil or rapeseed press cake resulted in a citrus/lemon-like odour impression caused by the formation of limonene (1-methyl-4-(1-methylethenyl)-cyclohexene) *[Rheinheimer 2013]*.

Fruiting bodies of *Gloeophyllum odoratum* disseminate the flavour of anise and fennel. Beside some alcohols, ketones, aldehydes and terpenes, methyl 2-(4-methoxyphenyl)acetate was reported to be the major component *[Roesecke et al. 2000]*. This methyl ester with its sweetish, fruity and creamy flavour might be causal for the odour impression reminiscent of melon and lychee, perceived in the headspace above submerged cultures of the fungus supplemented with cabbage cuttings.

The flavour profile of both, fruiting bodies and submerged cultivated *Laetiporus sulphureus* was described in literature *[Krings et al. 2011]*, *[Abraham et al. 1993]*, *[Wu et al. 2005]*. The basidiomycete is known for its biotransformation potential. For example, L-isoleucine is transformed to sotolon (3-hydroxy-4,5-dimethylfuran-2(*5H*)-one), a seasoning-like flavour compound *[Krings et al. 2011]*. This outstanding biotransformation potential was also demonstrated during a cultivation of *L. sulphureus* on castor oil with the formation of an intense peach-like odour after 14 days of submerged cultivation. The major building block of castor oil is ricinolic acid ((9*Z*,12*R*)-12-hydroxyoctadec-9-enoic acid). It can be degraded *via* an incomplete *ß*-oxidation which yielded the ultimate precursor of *γ*-decalacton – an important flavour compound known for its peach and creamy flavour *[Gatfield et al. 1993]*.

Pycnoporus cinnabarinus is well known as a flavour producing basidiomycete. Beside *de novo* production of 1-phenylethanone *[Gross et al. 1989]*, *P. cinnabarinus* is able to transform ferulic acid to vanillin *[Lesage-Meessen et al. 1999]*. Cultivation of this strain on cabbage cuttings resulted in so called “foxy” odour (odour impression of Concord grape). By further flavour analysis per GC, methyl anthranilate (methyl 2-aminobenzoate) turned out to be responsible for this flavour impression. It was reported, that *P. cinnabarinus* is able to produce this ester either *de novo [Gross et al. 1990]* or *via* biotransformation of 2-aminobenzoic acid *[Kittleson et al. 1995]*.

Flavour profiles of both, fruiting bodies and submerged cultures of *Trametes suaveolens* were described. Major flavour components were methyl 4-methoxybenzoate and 4-methoxybenzaldehyde *[Roesecke et al. 2000]*, *[Birkinshaw et al. 1944]*. Furthermore *T. suaveolens* was able to produce benzaldehyde, *de novo* as well as along the bioconversion of L-phenylalanine *[Lomascolo et al. 2001]*. Cultivation of *T. suaveolens* on cabbage cuttings resulted in an almond-like, sweetish and liquorice odour impression. It is most likely, that the fungus used amino acids from the side stream cabbage cuttings to generate characteristic flavours.

Cultivation of *Polyporus betulinus* on cabbage cuttings resulted in a potent sweetish and pineapple-like flavour, which is discussed in section 4.

The biotechnological potential of basidiomycetes cultivated on other side streams are discussed elsewhere: Lipid containing side stream rapeseed press cake was mainly described by *Rheinheimer [2013]*, whereas carotenoid- and lignin-containing side streams were examined by project partners at University of Giessen, Germany *[Kunkel 2013]*, *[Bosse 2013]*.

3.5. *Isaria farinosa* – cold production of smoke flavour

As a part of the screening described by *Rheinheimer [2013]* one fungus proved to be particularly valuable. The ascomycete *I. farinosa* was able to transform FA to the flavour compound 4-VG. This chapter discusses the flavour profile and purification/characterisation of the enzyme responsible for the biotransformation.

3.5.1. Characterisation of the aroma profile

Cultivation of *I. farinosa* on rapeseed press cake resulted in a pleasant flavour of carnation and smoked ham. The main culture was harvested at day four and the culture broth was submitted towards solvent extraction to recover aroma components. A subsequent AEVA of the concentrated extract resulted in three main flavour impressions. Although an odour impression is rarely caused by a few selected substances, in this case 4-VG was discovered as the most important odorous component, responsible for the smoke-like aroma of the culture broth.

Important odorous substances were identified by comparison of their retention index, odour impression and mass spectrum with data from literature. They are listed in Tab. 7. With respect to the quantity, the main component of the extract was 4-VG, while guaiacol (2-methoxyphenol) amounted to 4.5 % and an unidentified substance with a flowery note to 11.8 %. Considering the odour thresholds of guaiacol (0.1 and 0.8 ng L^{-1} *[Guth et al. 1991]*) and 4-VG (0.4 and 0.8 ng L^{-1} *[Blank et al. 1989]*), the smoke flavour was mainly evoked by 4-VG.

Tab. 7: Main flavour compounds identified during a cultivation of *Isaria farinosa* on rapeseed press cake according to their flavour dilution factors

	Volatile compound [a]	RI [b]	RI Lit.	Odour	FD-factor [c]	Amount [%] [d]
A	Guaiacol	1846	1883 [e]	Smoke, flowery	64	4.5
B	4-Vinylguaiacol	2182	2198 [f]	Smoke, sweetish	256	83.8
C	Not identified	2211		Flowery	256	11.8

[a] Identified tentatively according to linear retention index (RI), mass spectrum, and odour impression of reference substances
[b] RI = linear retention index (according to *n*-alkanes *[Van den Dool et al. 1963]*)
[c] FD-factor = flavour dilution factor
[d] Relative amount of substance according to peak area
[e] *[Cullere et al. 2004]*
[f] *[Baek et al. 1997]*

Higher fungi play an important role in the degradation of plant biomass. They are equipped with an appropriate enzyme system to decompose the main cell wall components like lignin, hemicellulose and pectin *[Brink et al. 2011]*. The resulting degradation products of these polymer structures can act as precursor for some further metabolites such as 4-VG *[Ishikawa et al. 1963]*. Therefore, studies with natural substrates for fungi (Tab. 4), containing possible precursors of 4-VG, were amended. Substrates were chosen according to their composition: hemp fibre with its content of hemicellulose, pectin, lignin and rice bran because of their content of esterified ferulic acid *[Cicero et al. 2005]*, *[Norton 1995]*. Furthermore, some single compounds like ferulic acid, ferulic acid methyl ester and vanillin were used because of their precursor potential. During the screening for 4-VG formation a sensorial detection of product formation was used. Odour impression of 4-VG was perceived only during submerged cultivation of Ifa supplemented with rice bran or after direct addition of FA or FAME to the culture liquid. This confirmed the assumption, that free or bound FA was the ultimate precursor of 4-VG formation by Ifa. Biotransformation of FA to a variety of other aromatic compounds by several microorganisms was reported *[Rosazza et al. 1995]* as well. *Rahouti et al. [1989]* first described the FA metabolism of the closely related ascomycete *Paecilomyces variotii.*

3.5.2. Purification of ferulic acid decarboxylase

The formation of 4-VG requires a non-oxidative decarboxylation of FA. This can be carried out either chemically and leads, for example, to the formation of 4-VG in stored orange juices *[Ruiz Perez-Cacho et al. 2008]* or catalysed along the action

of a decarboxylating enzyme. First FaD described was reported for the yeast *Saccharomyces cerevisiae [Huang et al. 1994]* and from the bacterial strain *Bacillus pumilus [Degrassi et al. 1995]*.

To exclude a simply chemical formation of 4-VG during the cultivation of Ifa various controls were performed: FA at pH 3, at pH 6 and autoclaved mycelium, supplemented with FA, were shaken for 24 hours at room temperature. No 4-VG formation was observed.

Before starting the isolation and purification of the putative FaD of Ifa the culture parameters were optimised for maximal activity. Optimisation took place in shaking flasks. Main cultures were inoculated with pre-cultures of different cultivation days (day 3, 4, 5) in SNM. The main cultures were induced with FA at different times (day 1, 2, 3). Concentration of FA in the cultures was always 0.5 mM. Sampling took place every day, and the enzyme activity (per mg of wet biomass) was determined directly after cell disruption in the crude extract. According to FaD activity, optimised culture conditions were: Main culture prepared based on a three day old pre-culture, induced on day one (FA concentration in culture 0.5 mM) and harvested on day five (data shown in appendix: Fig. 15, Fig. 16). Afterwards, cultivation was scaled-up into a 5 L stirred tank reactor.

Previous studies by *Rheinheimer [2013]* showed that FaD is an intracellular enzyme with an IEP of 5.2. The molecular mass was determined by SEC to 29 kDa (native enzyme). Moreover, it was freeze stable. Based on these results, the purification was further optimized by modifying certain parameters and by the application of a second purification step, AIEX. Cell disruption was achieved by freeze-drying instead of using a bead mill (Precellys). Furthermore, activity assay was reduced to 90 minutes based on the results of the kinetics of 4-VG formation (data shown in appendix: Fig. 17).

FaD was purified after cell disruption using AIEX and SEC. Fig. 4 shows the chromatograms of the two purification steps. After AIEX, most of the FaD-activity was found in an approximately 50 mL large fraction. Enzyme was eluted with 100 mM NaCl solution. Confirming the native molecular mass, it was determined by SEC and amounted to 33.5 kDa, which is comparable to previous described 29 kDa. FaDs from bacterial strains have a native molecular mass in the range from 40 to 45. They were described as homodimers with monomers of 20.4 to 23 kDa *[Gu et al. 2011]*, *[Huang et al. 1994]*.

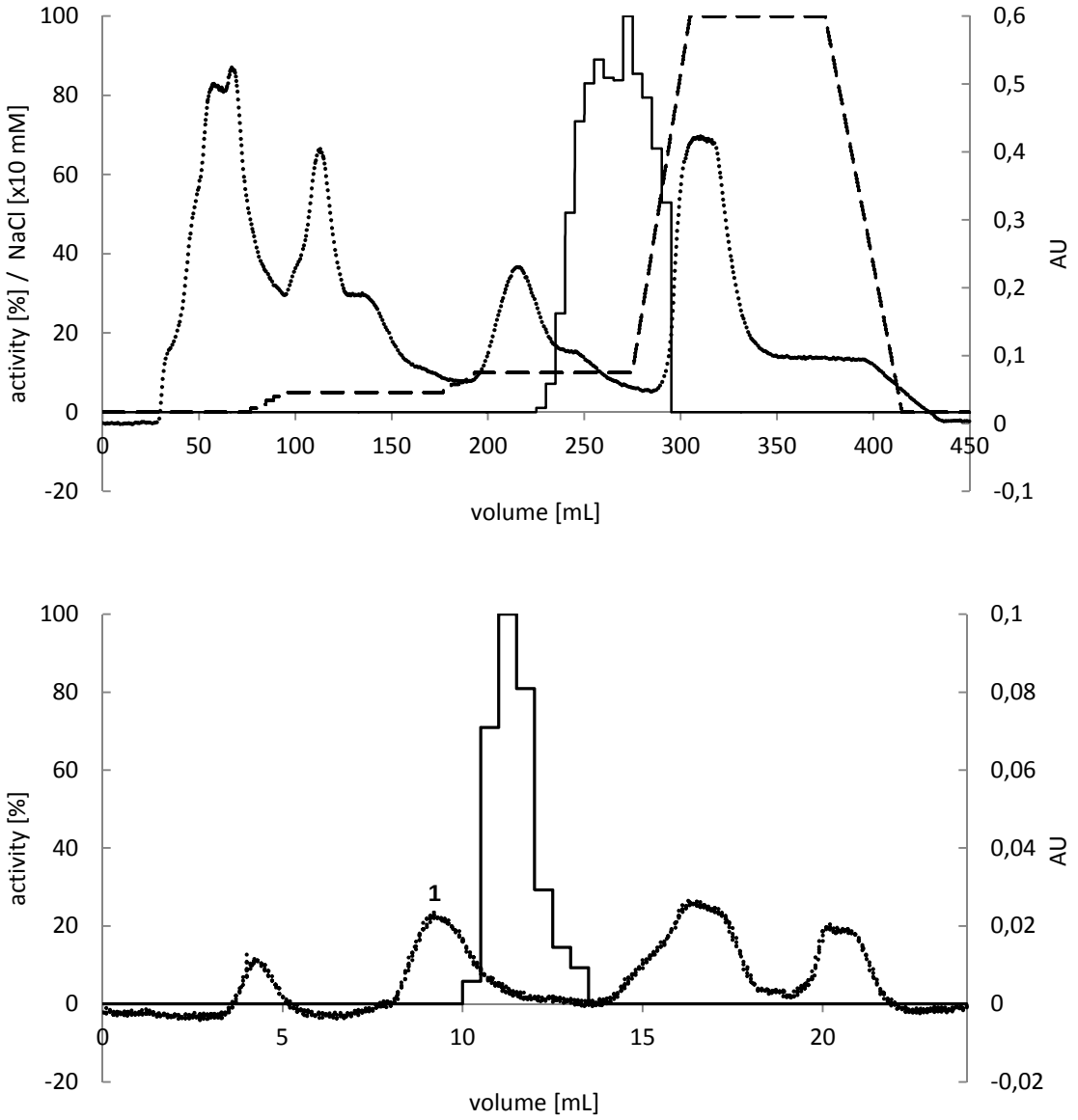


Fig. 4: Anion exchange chromatogram (top) and size exclusion chromatogram (bottom) of enzyme solution. Peak no. 1: glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
Dotted line: UV detection; solid line: relative activity, dashed line: NaCl concentration

After the two chromatographic purification steps most of the extrinsic protein in the sample was separated from the FaD (Fig. 4). However, enzyme concentration was found low, as the highest activity was measured in fractions, which eluted at a low detector signal (absorbance at 280 nm). Further efforts to improve the recovery of active enzyme did not succeed. The relative recoveries of enzyme activity after several methods applied were as follows: Ultra filtration 31 %, AIEX 32 %, precipitation with acetone 15 % and SEC 24 %. According to these results the

described method, using AIEX, followed by SEC, turned out to be the best for enzyme purification.

Finally the purity of pooled enzyme fraction was checked using SDS-PAGE. Fig. 5 shows the silver stained SDS gel. Lane three shows proteins of the active fractions after AIEX (solid line in Fig. 4 (top)). Lane one shows the proteins in a not active fraction after SEC (Peak no. 1 in Fig. 4 (bottom)). A dominant band is visible at approximately 40 kDa in both lanes. After protein sequencing this band turned out to be a glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Proteins of the active fraction after SEC (solid line in Fig. 4 (bottom)) were separated in lane two. There is no protein band visible, even not after silver staining, although a high activity was measured.

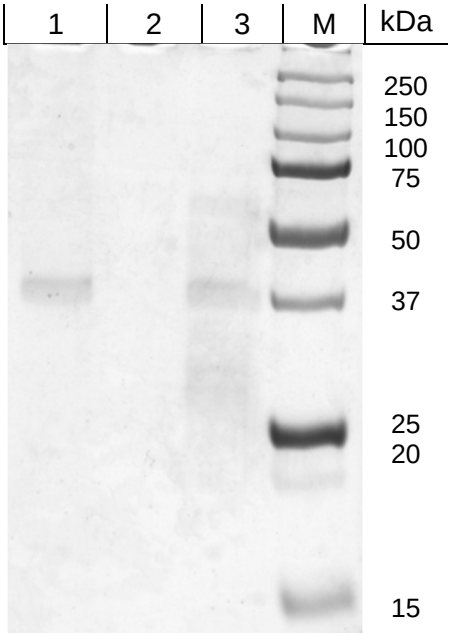


Fig. 5: SDS-PAGE (12 %) of FaD purification; silver stained.
Lane 1: not active fraction after SEC; lane 2: active fraction after SEC; lane 3: active fraction after AIEX; Lane M: marker proteins

After silver staining, up to 0.1-1 ng protein can be inspected visually *[Blum et al. 1987]*. A protein content of 0.03-0.13 ng can be detected, after labelling sample with fluorescent dyes (product information of Refraction 2D Labeling^{TM} Kit, 2012). Therefore, a concentrated sample derived after described purification procedure was labelled with red fluorescence dye. SDS-PAGE was performed and separated proteins were visualised by fluorescence emission at 665 nm. Fig. 6 shows the scan of the gel according to the emission wavelength 665 nm. Lane two and lane three were the same sample, derived after the complete purification procedure,

whereas the sample separated in lane three is concentrated by a factor of ten. Lane one shows the sample after AIEX and before SEC. After scanning the gel, protein bands were cut and analysed after tryptic digest by means of ESI-Q-TOF. The main band turned out to be the already described GAPDH. Other sequence data gave no hits for enzymes, responsible for the ferulic acid decarboxylation activity.

Therefore separation and purification were not the limiting factors in the identification of the putative FaD and further purification steps as described in literature *[Huang et al. 1994]*, *[Degrassi et al. 1995]* were thereby not considered. The focus of further work should be set on increased enzyme purification in order to get a markedly visible band on the SDS-PAGE gel to cut off a sufficient amount of enzyme for MS sequencing.

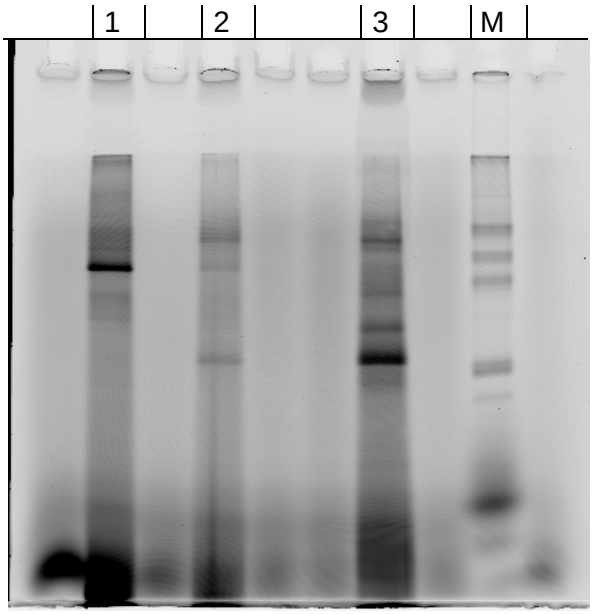


Fig. 6: SDS-PAGE (16.5 %) of FaD purification; visualisation was performed with fluorescence scanner after labelling the sample with fluorescent dye.
Scan of gel at the emission wavelength of 665 nm.
Lane 1: active fraction after AIEX; lane 2: active fraction after SEC; lane 3: concentrated sample in lane 2; Lane M: marker proteins

3.5.3. Characterisation of ferulic acid decarboxylase

Characterisation was performed using enzyme solution in PPB after purification steps described in 3.3.4. First of all, temperature and pH optimum were determined. Fig. 7 shows enzyme activity depending on temperature and pH value. Maximum activity was observed at a pH of 6.0 (68 U L^{-1}) in an optimal temperature range between 15 and 25 °C. Determination of pH optimum was also

performed in other buffer systems like citrate, Bis-Tris and Tris buffer. Activity course was similar, but significant lower activities could be detected (13 U L^{-1} at pH of 6 in Bis-Tris buffer). PPB turned out to be the optimal buffer for this decarboxylase. *Huang et al. [1994]* described an activity loss of FaD isolated from *Pseudomomas fluorescens* in amine containing buffer systems up to 30-40 %. Temperature and pH optima of FaD from Ifa were comparable to other FaDs described in literature *[Degrassi et al. 1995]*, *[Huang et al. 1994]*.

Kinetic parameters v_{max} and K_m were determined according to Michaelis-Menten Theory. K_m value was 3.4 mM for ferulic acid and v_{max} was 3.3 U/mg. K_m value of other FaD differs over a wide range in literature (between 1.03 and 7.9 mM) *[Gu et al. 2011]*, *[Huang et al. 1994]*, *[Degrassi et al. 1995]*.

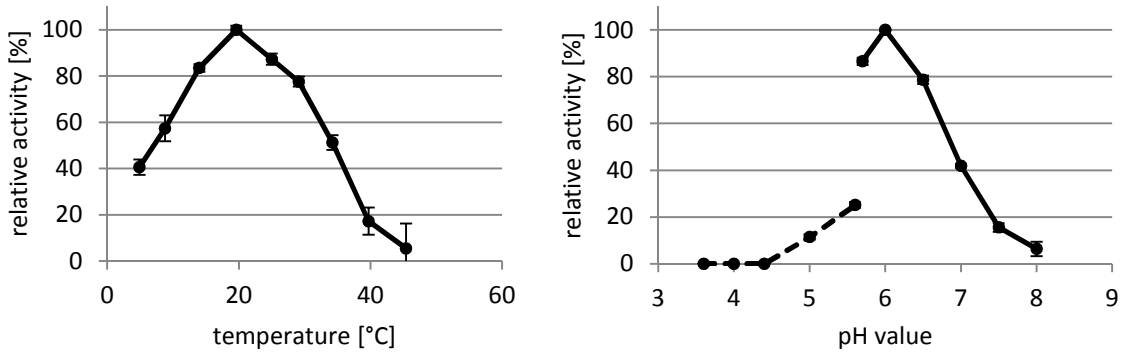


Fig. 7: Temperature optimum (left) of ferulic acid decarboxylase; pH optimum (right) of ferulic acid decarboxylase; dotted line: pH range 3.6 - 5.6 in sodium acetate buffer; solid line: pH range 5.5 - 8.0 in potassium phosphate buffer

Furthermore, to characterise the substrate specificity of FaD, some cinnamic acid derivatives (Tab. 5) were tested. 25 substrates characterised by a variety of methoxy and hydroxy substituted cinnamic acid derivates, phenylpropanoic acid derivates and structurally similar compounds were selected in order to get an idea about substrate binding and the decarboxylation mechanism. Most FaDs catalyse a non-oxidative decarboxylation of FA as described by *Huang et al. [1994]*. Among all tested substrates, only FA (87.7 % degradation) and 4-hydroxy cinnamic acid (100 % degradation) were transformed to corresponding vinyl derivatives. For substrate binding and transformation a hydroxy substituent *para* to the unsaturated C_3-side chain at the aromatic ring must be present. Therefore, proposed mechanism for the decarboxylation catalysed by the FaD from Ifa followed the pathway with a quinoide intermediate shown in Fig. 8.

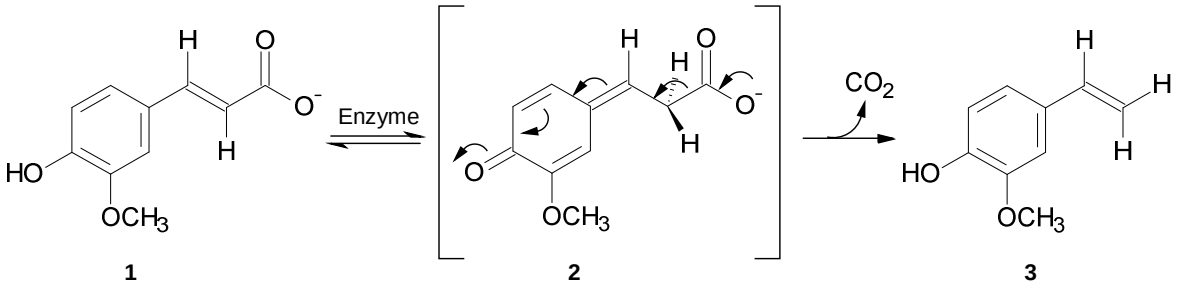


Fig. 8: Non oxidative mechanism for FA decarboxylation to 4-VG *[Huang et al. 1994]*. 1: ferulic acid; 2: quinoide intermediate; 3: 4-vinylguaiacol

3.6. Conclusion

In this work the huge metabolic potential of basidiomycetes for flavour production was discussed. Some basidiomycetes cultivated on cabbage cuttings, rape oil or castor oil generated pleasant flavour impressions reminiscent of peach, melon, lychee, pineapple, grape, honey and almond. Characteristic flavour compounds were identified.

The ascomycete *I. farinosa* produced an odour impression reminiscent of smoked ham and cloves. Main component turned out to be 4-vinylguaiacol, with ferulic acid as a facultative precursor. As the decarboxylation of FA was proven to be catalysed by an enzyme, further work was focused on the purification and characterisation of the putative ferulic acid decarboxylase. The enzyme had a temperature optimum at 20 °C and a pH optimum at 6.0. The mechanism is proposed to be a non-oxidative decarboxylation, according to the requirement for a *para*-hydroxy group in the substrate. According to its characteristics, the purified enzyme is comparable to FaDs already described in literature. Because of its high specific activity it can be assumed, that this FaD from Ifa is a potential tool to convert FA to 4-VG.

4. Vorwort zu: „Formation of pineapple-like flavour substances – Characterisation of the aroma profile generated by *Piptoporus betulinus*"

In dem in Kapitel 0 beschriebenen Screening ist die Kultivierung des Basidiomyceten *Piptoporus betulinus* auf Kohlschnitt aufgrund des angenehmen Geruchs nach Ananas und Honig aufgefallen.

Der Basidiomycet *P. betulinus* ist vor allem bekannt für seine antibakteriellen und entzündugshemmenden Eigenschaften. Auch die Bildung eines fruchtigen Aromas und die Identifizierung einiger Aromastoffe, sowohl in Fruchtkörpern, als auch bei der Kultivierung auf Agarplatten, wurde in der Literatur beschrieben. Bei der im Folgenden beschriebenen Kultivierung auf Kohlschnitt wurden jedoch noch weitere Aromastoffe detektiert, welche für das typische ananasartige Aroma der Kultur verantwortlich sind.
Ananas erfreut sich als tropische Frucht großer Beliebtheit. Ihr Aroma setzt sich aus einer Vielzahl von unterschiedlichen Aromastoffen zusammen. Vor allem Ester, Kohlenwasserstoffe und Schwefelverbindugen sind für den charakteristischen Geruch verantwortlich. Eine Schlüsselverbindung, wie sie z.B. für die Himbeere das Himberketon (1.1.1) darstellt, ist bislang nicht identifiziert worden.

Im Folgenden werden die Identifizierung der für den ananasartigen Geruch verantwortlichen Substanzen und deren Bildungskinetiken beschrieben.

5. Formation of pineapple-like flavour substances – Characterisation of the aroma profile generated by *Piptoporus betulinus*

5.1. Abstract

The basidiomycete *Piptoporus betulinus* (Pbet), cultivated on cabbage cuttings (CC), evolved a pleasant flavour reminiscent of pineapple and honey. Submerged cultivation of the basidiomycete was successfully up-scaled into a 5 L stirred tank reactor. Aroma profile was examined by means of gas chromatography-olfactometry (GC-O) and mass spectrometry. The flavour dilution factors (FD-factor) of the most potent aroma compounds were determined. Culture conditions such as pH value and D-glucose concentration were positively correlated with the kinetics of flavour formation. Sensorial relevant components are octan-3-one (FD-factor: 64), acetic acid (FD-factor: 125), methyl benzoate (FD-factor: 64) and methyl 4-methoxybenzoate (FD-factor: 64). Two sensorial relevant compounds (FD-factor: 125) turned out to be responsible for the dominating flavour impression pineapple. Several attempts were performed to identify the substances as isomers of deca-5,7,9-trien-2-one.

5.2. Introduction

The pineapple is a widespread and popular tropical fruit because of its fruity and exotic flavour. Fresh pineapple market is one of the fastest growing fruit and vegetable market in the United States and Europe. Imports of fresh pineapple have more than doubled in the years from 2000 to 2008 *[Pay 2009]*. A large number of studies dealt with the aroma of fresh pineapple, pineapple juices and concentrates. Especially the group of esters constitutes the main part of the odour active substances. However, hydrocarbons and sulphur compounds had been described as important flavour substances *[Umano et al. 1992]*, *[Elss et al. 2005]*, *[Berger et al. 1985]*, *[Montero-Calderon et al. 2010]*. Due to the great demand of

pineapple, food industry strives to create pineapple flavoured products. According to the consumer expectation, natural flavourings are essential for such products. Flavourings can be declared as natural, when they are produced by enzymatic or microbiological process from material of vegetable or microbiological origin.[7] Flavours produced by cultivation of microorganisms therefore can be regarded as natural. Recent research on flavour producing potential of microorganisms also implies higher fungi *[Berger 2008]*. Some *Pleurotus* species for example convert monoterpenes to furanoterpenoids *[Kruegener et al. 2009]* and *Laetiporus sulphureus* is able to produce numerous potent heterocyclic flavours, when degrading wheat gluten *[Krings et al. 2011]*.

A representative of the higher fungi, the basidiomycete *Piptoporus betulinus,* is basically known for medicinal effects *[Cyranka et al. 2011]*. Even the ice mummy "Ötzi" carried pieces of *P. betulinus* with him, probably because of anti-inflammatory and antibacterial properties *[Fowler 2000]*. For example, lanostanoids from *P. betulinus* were discussed to have anti-inflammatory effects *[Wangun et al. 2004]*. Also the degradation potential of birch wood and the enzymes accounting were and still are under investigation *[Valaskova et al. 2006]*, *[Szklarz et al. 1989]*. However, the flavour profile of this fungus has not been addressed in much detail. Some studies were performed according to the flavour profile of fruiting bodies, which was described with fruity notes *[Rapior et al. 1996]*. Mainly terpenes were identified in fruiting bodies *[Roesecke et al. 2000]*. *Badcock [1939]* described the odour of *P. betulinus* mycelium cultured on agar plates. He observed an apple-like flavour. *Gallois et al. [1990]* described a pineapple-like flavour impression of *P. betulinus* in liquid media, but the underlying flavour compound was not identified.

This work discusses biotechnological potential of the basidiomycete *P. betulinus* according to natural flavour formation. The aroma profile of submerged cultures supplemented with cabbage cuttings was studied. Two flavour substances, mainly responsible for pineapple-like odour impression were identified as isomers of deca-5,7,9-triene-2-one.

[7] Regulation (EC) No. 1334/2008 of the European Parlament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods

5.3. Material and methods

5.3.1. Chemicals

Adsorbent material XAD-16, a styrene-divinylbenzene copolymer, is purchased from Rohm & Haas (Philadelphia, Pennsylvania, USA). Cyclohexanol (≥ 99 %; Merck-Schuchardt, Hohenbrunn, Germany) and cyclopentanol (99 %; Sigma-Aldrich, Steinheim, Germany) were used as analytical standards in gas chromatography.

5.3.2. Cultivation of *Polyporus betulinus*

Microorganism and substrate

The fungus *P. betulinus* was isolated from wood in Lower Saxony, Germany, and identified according to its internal transcribed spacer (ITS) sequence. Cabbage was derived from a local supermarket and was cut into small pieces and conserved by freeze drying.

Cultivation conditions

For pre-cultivation, the fungus was grown submerged in modified liquid standard nutrition medium (SNM) *[Sprecher 1959]*: 30.0 g L^{-1} D-(+)-glucose monohydrate (Merck KGaA, Darmstadt, Germany), 4.5 g L^{-1} L-asparagine monohydrate (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 3.0 g L^{-1} yeast extract granulated (Merck KGaA, Darmstadt, Germany), 1.5 g L^{-1} KH_2PO_4 (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 0.5 g L^{-1} $MgSO_4$ (Carl Roth GmbH & Co. KG, Karlsruhe, Germany); 1.0 mL L^{-1} trace element solution [0.08 g L^{-1} $FeCl_3$ x 6 H_2O (Fluka Chemie AG, Neu-Ulm, Germany), 0.09 g L^{-1} $ZnSO_4$ x 7 H_2O (J. T. Baker, Deventer, Netherlands), 0.03 g L^{-1} $MnSO_4$ x H_2O (Merck KGaA, Darmstadt, Germany), 0.005 g L^{-1} $CuSO_4$ x 5 H_2O (Riedel-de Haën AG, Seelze, Germany), 0.4 g L^{-1} EDTA disodium salt dehydrate (Carl Roth GmbH & Co. KG, Karlsruhe, Germany)]; adjusted to pH 6.0 with 1 M NaOH and autoclaved at 121 °C and 1 bar positive pressure for 20 min. Pre-cultures were prepared by homogenisation of a 10 × 10 mm agar plug with mycelium using an Ultra Turrax (Miccra D-9, Art, Mühlheim, Germany). Cultivation took place in 500 mL/1000 mL Erlenmeyer flasks (250 mL/500 mL medium) and orbital shaking at 150 rpm and 24 °C (Multitron, Infors HT, Bottmingen, Switzerland) for 8 days.

Main cultivation was performed in a minimal SNM with reduced D-(+)-glucose-monohydrate concentration (3 g L^{-1}) and no nitrogen source, but supplemented with cabbage cuttings (2% (w/w)). The submerged cultivation of the fungus was finally scaled up into a 5 L (working volume) stirred tank reactor (ISF 100, Infors HT, Bottmingen, Switzerland) at 24 °C with an air flow of 1 L min^{-1} through a ring sparger. The bioreactor was equipped with pitched blade impeller running at 120 rpm. Before inoculation the bioreactor was autoclaved at 121 °C and 1 bar positive pressure for 30 min, containing 4.5 L minimal medium as described above. Cultivation was started by the inoculation with 500 mL pre-culture. The reactor was harvested after 13 days of cultivation.

Sampling

Every day about 5 mL medium of the stirred tank reactor were taken and separated from fungal cells by centrifugation. 500 mL medium were taken on day six of cultivation for comprehensive flavour analysis. Withdrawn liquid medium was replaced by fresh minimal medium.

Trapping of flavour compounds from exhaust air was performed according to *Krings et al. [2008]*. Adsorbent material XAD-16 was chosen because of its physical properties *[Krings et al. 1993]*. A glass column was filled with 7 g of XAD-16 and connected to the waste air outlet of the bioreactor. Flavour compounds were trapped continuously in the period from day two of cultivation until day twelve. Loaded adsorber columns were replaced by unloaded columns each 24 h.

Determination of glucose

Concentration of glucose was determined using the enzyme kit K-SUFRG12/12 (Megazyme, Wicklow, Ireland). The change of absorbance was monitored with a Synergy 2 Microplate reader (BioTek, Bad Friedrichshall, Germany).

5.3.3. Flavour analysis

Solvent extraction of volatiles

To monitor flavour formation a daily sample of 3 mL of the culture broth, displaced with 100 µL of internal standard solution (607 µg mL^{-1} cyclohexanol), were extracted by shaking for 2 minutes with 1 mL of dichloromethane (DCM), spiked with 100 µL external standard (569 µg mL^{-1} cyclopentanol). The organic phase was separated and dried over sodium sulphate.

Trapped volatiles on XAD-16 were extracted two times under stirring with pentane/diethyl ether (1:1.12; v/v) (P/E). After drying over sodium sulphate, the organic phase was concentrated using a Vigreux column at 40 °C to approximately 1 mL. Subsequently 100 µL external standard (569 µg mL^{-1} cyclopentanol) were added.
Both, DCM and P/E extracts were analysed by means of gas chromatography.

Fractionation of aroma extract

0.5 mL of the P/E extract of volatiles of both, culture broth and XAD-16 desorbate, were fractionated using solid phase extraction columns filled with silica gel (Chromabond SiOH, Macherey & Nagel, Düren, Germany). Three eluents with different polarities were subsequently applied: pentane/diethylether (9:1; v/v), pentane/diethylether (3:1; v/v) and diethylether. The pineapple-like flavour compound eluted in the second fraction and was subsequently delivered to further purification and accumulation by means of preparative GC. The enriched pineapple flavour compound was supplied to NMR analysis.

Aroma extract dilution analysis (AEDA)

Flavour dilution factors (FD-factor) were determined by sniffing a dilution series of respective extracts (diluted stepwise (1:1) with P/E) until odorous compounds were no longer perceived at the sniffing port.

5.3.4. Analytical methods

Gas chromatography flame ionisation detection/olfactometry (GC-FID/O)

GC-O was performed using an Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled with a flame ionisation detector (FID, 250 °C) and an olfactory detection port (ODP, 250 °C; Gerstel, Mühlheim, Germany). The chromatographic conditions were: 30 m x 0.32 mm i.d. x 0.25 µm DB-WAX (J&W Scientific, Folsom, USA) with 2 mL min^{-1} hydrogen as carrier gas using the following temperature program: 40 °C (hold for 3 minutes) with a rate of 5 °C min^{-1} to 230 °C, hold for 10 minutes.

Gas chromatography mass spectrometry (GC-MS)

The volatile extracts were analysed using a Fisons GC 8000 gas chromatograph and a Fisons MD 800 mass selective detector (interface: 230 °C, ion source: 200 °C, quadrupole: 100 °C, electron impact ionisation (70 eV), scan range m/z 33

to 300 amu). Analytical conditions were as follows: 30 m x 0.32 mm i.d. x 0.25 µm DB-WAX (J&W Scientific, Folsom, USA) with 1 mL min^{-1} helium as carrier gas using the following temperature program: 40 °C (hold for 3 minutes) with a rate of 3 °C min^{-1} to 230 °C hold for 10 minutes. Identification of volatiles was achieved by comparing their linear retention indices (RI), calculated according to *n*-alkanes as external references *[Van den Dool et al. 1963]*, odour impression and mass spectra with those retrieved from digital libraries (Wiley 08/NIST 08).

High resolution gas chromatography - high resolution mass spectrometry (HRGC-HRMS)

For determination of accurate mass of pineapple flavour compound, an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled with a double focusing sector field MS-device (AMD QuAS^{3}AR, Harpsted, Germany) was used. Conditions were as follows: interface: 250 °C, ion source: 200 °C, electron impact ionisation 70 eV. Accurate mass determination was achieved at a dynamic working resolution of R=1300 (10% valley), a scan rate of 2 scans per mass decade and integrated calibration with high boiling perfluoro kerosine (PFK) prior to and lock/shift mass calibration during analysis. The chromatographic conditions were as described for GC-MS.

Semi-preparative gas chromatography

Isolation of pineapple-like flavour compounds was performed with a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Palo Alto, USA) equipped with a cold injection system (CIS 3), a multi column switching system II (both Gerstel, Mülheim, Germany) and a HP 7673 autosampler (Hewlett-Packard, Palo Alto, USA). An Optima Wax pre-column (5 m x 0.53 mm x 2 µm) was connected to an Optima Wax preparative column (25 m x 0.53 mm x 2 µm) (both Macherey &Nagel, Düren, Germany) and hydrogen at a flow of 8.9 mL min^{-1} at 100 °C was used as carrier gas. Following temperature ramp was used: 40 °C (3 min)//3°C/min//156 °C//1°C/min//168°C //20°C/min//240 °C (15 min).

Nuclear magnetic resonance spectroscopy (NMR)

NMR experiments and data interpretation were carried out by Dr. Jörg Fohrer at the Institute of Organic Chemistry, Leibniz University Hanover. ^{1}H-, ^{13}C-NMR were performed on Bruker DRX 500 MHz spectrometer equipped with 5 mm TCI ^{1}H-

$^{13}C/^{15}N$ (Z-GRD) cryo sample head (^{1}H at 500 MHz, ^{13}C at 125 MHz). Furthermore two-dimensional COSY (correlation spectroscopy), HSQC (heteronuclear single quantum coherence) and HMBC (heteronuclear multiple bond coherence) were performed. Samples were dissolved in CD_2CL_2.

5.4. Results and discussion

5.4.1. Aroma profile

Cultivation of *P. betulinus* on the side stream cabbage cutting resulted in a pleasant odour impression, reminiscent of pineapple and honey. Upscaling of cultivation in shaking flasks was successfully performed in a 5 L stirred tank bioreactor. Flavour profile and cultivation parameters were monitored over the entire cultivation period. The flavour compounds in culture broth were extracted daily and analysed by means of GC. Comprehensive flavour analysis and AEDA were carried out on day six of cultivation (GC-FID chromatogram is shown in appendix: Fig. 18). There were nine main flavour impressions detected (Tab. 8). Octan-3-one [A], acetic acid [C], methyl benzoate [D] and methyl 4-methoxybenzoate [I] were identified according to their retention index, mass spectrum and odour impression. With respect to quantity, the most abundant component was [I], with flowery and lilac odour impression. This flavour compounds were already described as a constituent of the volatile fraction of cultivation of *P. betulinus [Gallois et al. 1990]*. Two substances were not identified. One of these compounds ([B]; FD-factor 125) emitted a green flavour impression, but the identification according to the EI-mass spectrum was not possible. Compound [E] had a high FD-factor of 256 and emitted a flavour reminiscent of red fruits. The aroma threshold is expected to be very low. Though the flavour impression at the sniff port was very intense, the physico-chemical detector (FID) did not show any peak at the respective retention time. Therefore an immediate identification of this substance was not possible. Particular attention was paid to the identification of sensorial important compounds [F] and [G] (FD-factor 125), responsible for the pineapple-like flavour impression, which turned out to be difficult. After determination of the exact mass and evaluation of mass spectrum and several NMR spectra, substance [G] could be identified as (5*E*,7*E*/*Z*)-deca-5,7,9-trien-2-one. It was assumed that substance [F] was an isomer of [G].

Another substance ([H], FD-factor 32) emitted a green and sweetish odour impression. It was assumed that [H] was one corresponding alcohol of the isomers [F] or [G]. Identification of compound [G] and confirmation of the assumptions referring [F] and [H] are described in section 5.4.2.

Tab. 8: Main flavour compounds of cultivation of *Piptoporus betulinus* on cabbage cuttings according to their flavour dilution factors

	Volatile compound [a]	RI [b]	RI Lit.	Odour	FD-factor [c]	Amount [%] [d]
A	Octan-3-one	1308	1244 [g]	Fungus	64	5.9
B	Not identified	1383		Green	125	0.5
C	Acetic acid	1454	1452 [h]	Pungent	125	15.5
D	Methyl benzoate	1631	1635 [h]	Flowery, herbal	64	13.1
E	Not identified	1676		Red fruit	256	n. d.
F	(5*E*/*Z*,7*E*/*Z*)-Deca-5,7,9-trien-2-one[e]	1842		Pineapple	125	1.0
G	(5*E*,7E/*Z*)-Deca-5,7,9-trien-2-one[f]	1863		Pineapple	125	7.2
H	(5*E*/*Z*,7*E*/*Z*)-Deca-5,7,9-trien-2-ol[e]	1976		Green, sweetish	32	4.6
I	Methyl 4-methoxybenzoate	2105	2092 [i]	Lilac	64	52.2

[a] Identified tentatively according to linear retention index (RI), mass spectrum, and odour impression of reference substances
[b] RI = linear retention index (according to *n*-alkanes *[Van den Dool et al. 1963]*)
[c] FD-factor = flavour dilution factor
[d] Relative amount of substance according to peak area
[e] Identified tentatively according to comparison of RI and mass spectrum with substance [G]
[f] Identified according to RI, mass spectrum and NMR analysis
[g] *[Fischer et al. 1987]*
[h] *[Aznar et al. 2001]*
[i] *[Binder et al. 1989]*

Culture conditions were monitored and turned out a positive correlation between flavour formation and fungal growth. The culture had an initial pH value of 4.3, caused by the supplementation of cabbage cuttings. The pH value decreased during the cultivation rapidly and levelled off to a value of approximately 2.0. *P. betulinus* is known for the acidification of the culture medium *[Osipowicz et al. 1994]*. Glucose, besides the side stream cabbage cutting, was the sole carbon source for the growth of the fungus. The concentration decreased rapidly during the first four days and was completely off on day five. Fig. 9 shows the course of

pH value, glucose concentration and flavour formation during the cultivation of *P. betulinus.*

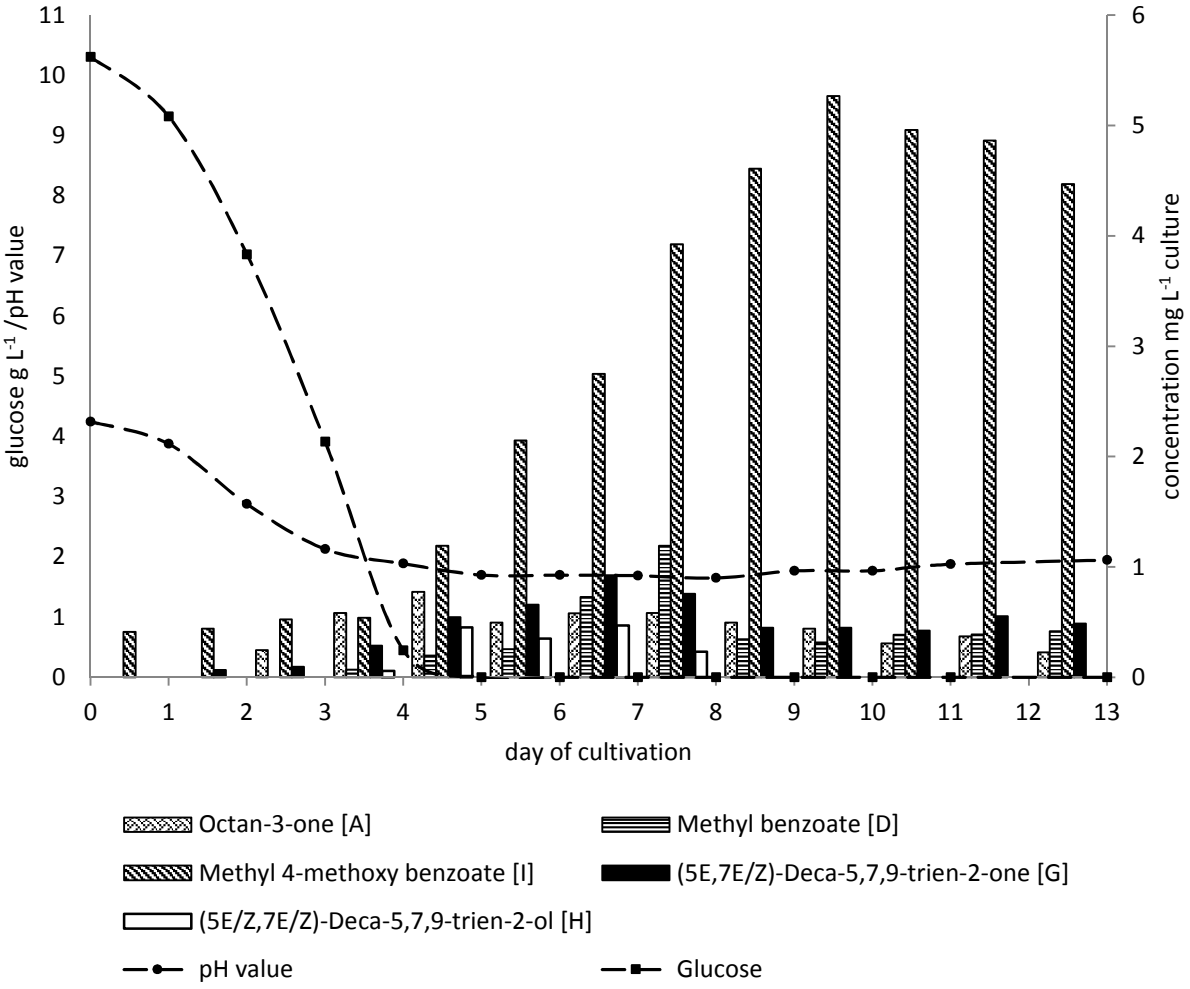


Fig. 9: Course of flavour formation during the cultivation of *Piptoporus betulinus* on cabbage cutting supplemented minimal medium in 5 L stirred tank bioreactor. Course of glucose consumption and course of pH value during the cultivation

Formation of main flavour component [I] peaked at day nine, whereby it dominated the overall flavour impression of the culture broth. Formation started on day one and was continued until the end of cultivation. The amount of [I] on day of inoculation resulted from the amount of [I] in pre-culture (eight days old). Production of pineapple flavour substance [G] started on day one and peaked at day six. Formation course of compound [F] is not shown in Fig. 9, because of its low concentrations, but the formation course is similar to compound [G]. Occurrence of substance [H] in culture broth is limited to day three to seven. Formation started on day three and peaked at day six. On day eight, substance [H]

is completely transformed. Apart from [I] all flavour compounds reached maximal concentration between day four and seven.

5.4.2. Identification of pineapple-like flavour compounds

Two compounds [F] and [G] turned out to be responsible for the pineapple odour impression. Their mass spectrum and their odour impression were identical. Retention indices differed by about 20 ([F]: 1842 and [G]: 1863 (Tab. 8)). It was assumed that [F] and [G] must be isomers. Concerning a comparison of the mass spectrum with the database, the identification of these compounds was not possible. Concentration of compound [F] in the extract was very small, so identification was focused on substance [G]. An exact mass determination of substance [G] was carried out to achieve a molecule formula and to confirm the proposed decatrienone structure, derived after interpretation of mass spectrum (Fig. 10 (top)).

Determination took place with a GC-HRMS device with the resolution of 1300 (10 % valley). Exact mass of molecule ion was 150.096 amu. A molecule formula of $C_{10}H_{14}O$ was deduced which fits well to the calculated mass of 150.104 amu. To confirm the keto group at C2, exact mass of the fragment derived after *α*-cleavage (Fig. 10 (top)) was also determined. A mass of 107.083 was detected. Calculated theoretical mass of fragment ion C_8H_{11} was 107.086. These results were consistent with the appearance of fragment *m/z* 43, which is characteristic for an acetyl group, derived after *α*-cleavage. Fragments *m/z* 77, *m/z* 79, *m/z* 91, *m/z* 93 arose from the triene-skeleton and were comparable to those of undeca-1,3,5-triene (Fig. 10 (bottom)).

For substance [H] a comparable mass spectrum (Fig. 10 (middle)) was detected by GC-MS. Molecule mass is increased to 152 amu compared to 150 amu for the compounds [F] and [G]. It was assumed, that this compound was one of the corresponding alcohols of the pineapple-like flavour compounds.*Miyazawa et al. [2009]* described the mass spectrum of several undeca-6,8,10-trienones with their corresponding alcohols. Mass spectrum of (6*E*,8*E*)-undeca-6,8,10-trien-3-one was comparable to that of substance [G] and mass spectrum of (6*E*,8*E*)-undeca-6,8,10-trien-3-ol was comparable to that of substance [H] (mass spectra of undecatrienone and undecatrienol described by *[Miyazawa et al. 2009]* are shown in appendix: Fig. 19).

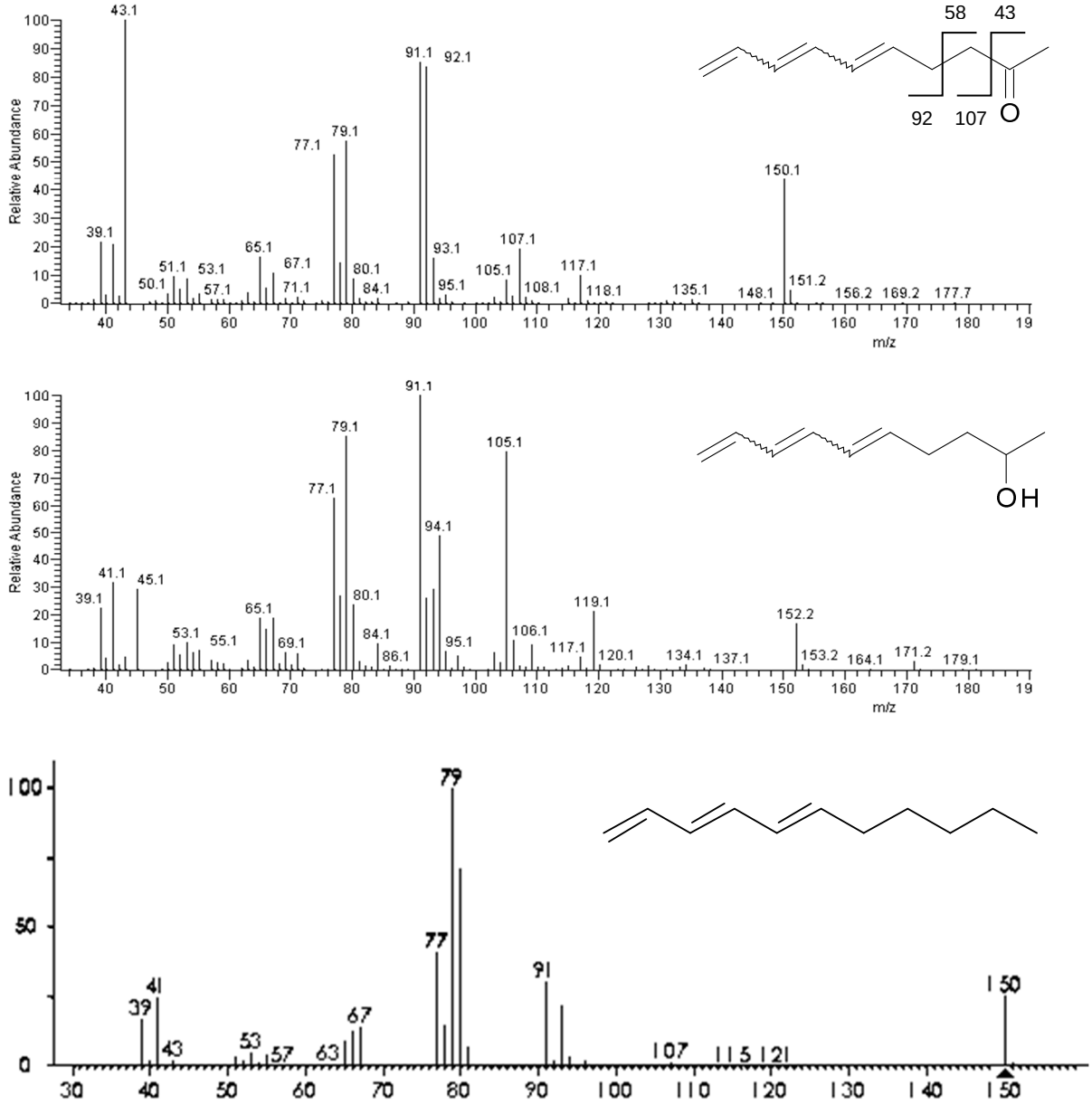


Fig. 10: Mass spectrum of supposed isomers of deca-5,7,9-trien-2-one [G] and [F] (top); Mass spectrum of supposed deca-5,7,9-trien-2-ol [H] (middle); Mass spectrum of undeca-1,3,5-triene (Wiley 08 / NIST 08)

Suspected deca-5,7,9-trien-2-one (CAS: 1221252-76-1) is mentioned only twice in literature: *Tomita [2010]* described the flavour components of Yuzu (*Citrus junos* Siebold ex. Tanaka) and its derivatives; *Nakanishi et al. [2010]* described fragrant enones having fresh and fruit-like flavour impressions, their chemical synthesis and their use for flavour and fragrance compositions. In both publications deca-5,7,9-trien-2-one is mentioned in terms of its chemical synthesis as a derivate of odour active enones. The corresponding alcohol is not known in literature.

Therefore, a confirmation of the assumed decatrienone structure was performed by several NMR analyses.

The flavour compound was isolated from cultures of *P. Betulinus* grown on cabbage cuttings. Derived P/E extract was concentrated and further fractionated on silica gel. Medium-polar fraction was used for isolating compound [G] using a preparative gas chromatograph. The substance was trapped in a glass trap, cooled down to -15 °C, and rinsed out with D_2-dichloromethane for NMR analysis. Several NMR experiments (^{1}H-, ^{13}H-NMR, COSY, HSQC and HMBC) were performed (NMR spectra are shown in appendix: Fig. 21-Fig. 25). Summarised spectroscopic data of substance [G] are listed in Tab. 9.

Tab. 9: Spectroscopic data of (5*E*,7*E*/*Z*)-deca-5,7,9-trien-2-one [G]

MS-EI [m/z (%)]:	150 (M^+, 38.5), 117 (9.2), 107 (18.6), 105 (8.5), 93 (16.0), 92 (83.5), 91 (84.4), 80 (8.7), 79 (58.7), 78 (14.3), 77 (53.8), 67 (11.6), 66 (5.8), 65 (16.6), 53 (9.5), 52 (5.3), 51 (10.0), 43 (100), 41 (24.4), 39 (24.3)
HRMS-EI:	calculated mass for $C_{10}H_{14}O$: 150.104; measured mass 150.096
^{1}H-NMR: (500 MHz, CD_2Cl_2)	d [ppm] = 6.35 (1H, *dt*, $^3J_{H,H}$ = 10.0, 10.0, 16.9 Hz, H9), 6.17 (2H, *m*, H7, H8), 6.09 (1H, *m*, H6), 5.71 (1H, *dt*, $^3J_{H,H}$ = 6.9, 6.9, 14.8 Hz, H5), 5.18 (1H, *d*, $^3J_{H,H}$ = 16.9 Hz, H10), 5.05 (1H, *d*, $^3J_{H,H}$ = 10.0 Hz, H10‘), 2.52 (2H, *t*, $^3J_{H,H}$ = 7.3 Hz, H3, H3‘), 2.34 (2H, *q*, $^3J_{H,H}$ = 7.3 Hz, H4, H4‘), 2.10 (3H, *br. s*, H1)
^{13}C-NMR: (125 MHz, CD_2Cl_2)	d [ppm] = 208.1 (*s*, C2), 137.6 (*d*, C9), 134.5 (*d*, C5), 133.7 (*d*, C7), 132.1 (*d*, C8), 131.1 (*d*, C6), 117.0 (*t*, C10), 43.3 (*t*, C3), 30.2 (*q*, C1), 27.4 (*t*, C4)

On the basis of extensive spectroscopic data, the decatrienone structure of the pineapple-like flavour compound [G] was confirmed. However, a conclusive determination of the configuration of C7 double bond was not possible.

The ^{1}H-Spectrum was examined in terms of typical chemical shift of protons (Fig. 21): Signals in the range of δ = 5.5-7.5 ppm indicated protons at unsaturated carbon atom C5 (H5: 5.71 ppm), C6 (H6: 6.09 ppm), C7/C8 (H7 and H8: 6.17 ppm), C9 (H9: 6.35 ppm) and C10 (H10: 5.18 ppm and H10´: 5.05 ppm). Signals in the range of δ = 2-3 ppm indicated two protons at saturated carbon atom C4

(H4: 2.34 ppm) and C3 (H3: 2.52 ppm), respectively. The signal at 2.10 ppm indicated three protons at C1. Examination of multiplicity and intensity agreed with the correlation of the signals to the corresponding protons. Furthermore, data derived from the two-dimensional homonuclear DQF-COSY experiment confirmed the proposed structure for [G] (Fig. 22). ^{13}C-spectrum showed five signals in the range of δ = 131-138 ppm which were attributed to unsaturated C5, C6, C7, C8 and C9. Signals in the range from δ = 27-43 ppm indicated saturated carbon atoms C1, C3 and C4. The signal at 177 ppm indicated the carbon atom C10. The signal attributed to C2 (keto group) showed a chemical shift of 208.1, but the intensity was very low (Fig. 23). Confirmation of the keto group was executed by two-dimensional HMBC experiment (Fig. 24): In this experiment, heteronuclear correlations over ranges of about 2-4 bonds were detected. X-axis showed the chemical shift of protons, y-axis the shift of carbon atoms. The spectrum showed three signals with the chemical shift of 208.1 ppm (C2), which indicated the correlation of carbon atom C2 with the proton H1 (2.1 ppm), H4 (2.34 ppm) and H3 (2.52 ppm). Another two-dimensional experiment which detects heteronuclear correlations was performed: ^{13}C-HSQC data showed the correlation between carbon atoms and protons, separated by one bond. Positive signals indicate carbon atoms with one or three protons (CH- or CH_3-groups), negative signals those with two protons (CH_2-groups). Signals attributed to C3 (43.3 ppm/2.56 ppm), C4 (27.4 ppm/2.38 ppm) and C10 (117.0 ppm/5.05 ppm/5.18 ppm) were negative and indicated CH_2-groups, while signals attributed to C1, C5, C6, C7, C8 and C9 were positive and indicated CH or CH_3 groups (Fig. 25).

Determination of the configuration of the double bond at C7 by typical coupling constants and chemical shifts for *cis*-/*trans*-protons and *cis*-/*trans*-substituted carbon atoms was not possible, because of the high similarity of C7/H7 and C8/H8 signals and overlapping effects. *Trans*-configuration of double bond at C5 was confirmed by the coupling constant of 14.9 Hz.

Possible structures of [G], [F] and corresponding alcohol [H] are summarised in Fig. 11.

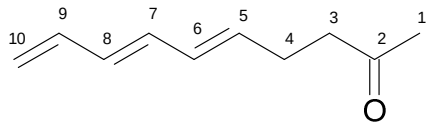
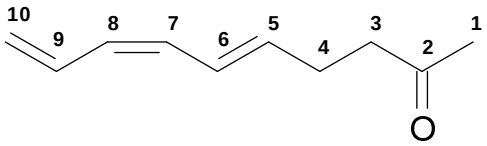
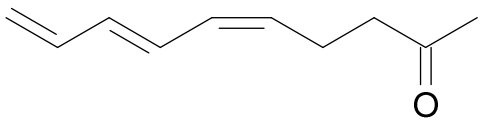
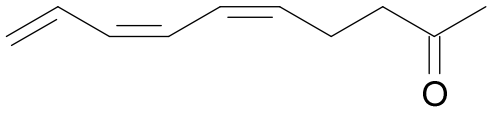
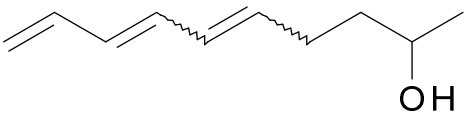
[G]: (5*E*,7*E*)-Deca-5,7,9-trien-2-one	
[G] /[F]: (5*E*,7*Z*)-Deca-5,7,9-trien-2-one	
[F]: (5*Z*,7*E*)-Deca-5,7,9-trien-2-one	
(5Z,7Z)-Deca-5,7,9-trien-2-one	
[H]: (5*E*/*Z*,7*E*/*Z*)-Deca-5,7,9-trien-2-ol	

Fig. 11: Possible structures of pineapple-like flavour compounds [G], [F] and [H]. Bold: probable structures for [G] and [F].

In contrast to the identified (5*E*,7*E*/*Z*)-deca-5,7,9-trien-2-one and its isomers, which were mentioned only twice in literature, undecatrienones were discussed in literature as key flavour compounds of Yuzu fruits and galbanum oil. Yuzunone ((6*Z*,8*E*)-undeca-6,8,10-trien-3-one) and Yuzuol ((6*Z*,8*E*)-undeca-6,8,10-trien-3-ol) for example were described as important flavour substances by *Tomita [2010]* and *Miyazawa et al. [2009]*. *Miyazawa et al. [2009]* examined such undecatrienones in galbanum oil and assumed that undecatrienones were oxygenated derivatives of the corresponding unsaturated C_{11}-hydrocarbons. Unsaturated C_{11}-hydrocarbons are important flavour components because of their low flavour threshold and their green, balsamic, pine-like odour impression. They naturally occur in some fruits and vegetables (apple, pineapple, celery, peach, hops) and their precursors are supposed to be fatty acids *[Belitz et al. 2001]*, *[Berger et al. 1985]*. *Boland et al. [1985]* described the formation of undecatrienes in algae, which act as pheromones, based on the decarboxylation of fatty acids. A possible formation pathway of undecatrienes/undecatrienones is shown in Fig. 20 (appendix) using

the example Yuzunone. Following this theory, possible precursors of pineapple-like flavour substances [G], [F] and [H] were odd-numbered fatty acids. *Rezanka et al. [1987]* found such fatty acids as minor components in *Polyporus betulinus* and other basidiomycetes. Furthermore, a wide range of other fatty acids could act as precursor of enones, because of the miscellaneous pool of enzymes produced by basidiomycetes. For example oxygenation and desaturation of fatty acids by basidiomycetes were described *[Sakai et al. 2003]*, *[Zhang et al. 2007]*, *[Del Rio et al. 2001]*.

As already mentioned, the configuration of the double bond at C7 of substance [G] could not be uniquely determined. Assuming that a biosynthesis of substance [G] was based on corresponding fatty acids, described for Yuzunone in Fig. 20, the most likely isomer was (5*E*,7*E*)-deca-5,7,9-trien-2-one.

Miyazawa et al. [2009] described the occurrence of two isomers (6*E*,8*E*)-undeca-6,8,10-trien-3-one and (6*Z*,8*E*)-undeca-6,8,10-trien-3-one (Yuzunone) in galbanum oil, whereas the odour impression of the (6*E*)-isomer was weaker. Applying this observation to the two isomers [G] and [F], the same picture arose: amount of [G] was higher than amount of [F] by a factor of seven, but FD-values were the same. Therefore, it can be expected that odour activity of [G] is weaker and thus, that it could be the (5*E*)-isomer.

In summary, pineapple-like flavour substances were identified as isomers of deca-5,7,9-trien-2-one, whereas [G] was assumed to be (5*E*,7E)-deca-5,7,9-trien-2-one and [F] was assumed to be (5*Z*,7*E*)-deca-5,7,9-trien-2-one (Fig. 11). In the future work on these interesting flavour compounds reminiscent of pineapple, the effort must be to identify further derivatives and possible precursors to clarify formation pathway and to achieve flavour production on large scale.

5.5. Conclusion

The characterisation of the aroma profile obtained during submerged cultivation of *P. betulinus* in minimal medium supplemented with cabbage cutting yielded nine flavour impact compounds, responsible for the overall pineapple-like flavour impression of the culture medium. Beside Octan-3-one, acetic acid, methyl benzoate and methyl 4-methoxybenzoate, the substances responsible for the distinguished pineapple flavour impression were identified as isomers of deca-5,7,9-trien-2-one by means of mass spectroscopy and NMR spectroscopy.

6. Vorwort zu: „Rapid analysis of volatiles in fat-containing matrices for monitoring bioprocesses“

Bei dem in Kapitel 0 beschriebenem Screening von submers kultivierten Basidiomyceten auf deren Aromabildungspotential, war die Bestimmung der Aromastoffe aus den mit Raps- und Rizinusöl supplementierten Kulturen eine analytische Herausforderung. Klassische Lösungsmittelextraktion hat den Nachteil, dass die co-extrahierten Fettbestandteile die Gaschromatographie stören bzw. das Messsystem belasten. Etablierte Methoden zur Aromaextraktion aus fetthaltigen Matrices wie die TLHVD bzw. die SAFE (siehe Kapitel 1.1.4) ermöglichen eine störungsfreie Chromatographie, allerdings sind diese Methoden mit einem hohen zeitlichen Aufwand verbunden und deshalb für ein Screening unbrauchbar. Pflanzenöle als Quellen von Fettsäuren, die bedeutende Aromastoffvorläufer (siehe Kapitel 1.1.1, 1.3) darstellen, sind allerdings wichtige Supplemente für ein solches Screening. Aus diesem Grund wurde eine schnelle und valide Methode zur Analyse von Aromastoffen aus fetthaltigen Matrices entwickelt.

Diese Methode beruht auf der Extraktion der Aromastoffe mit einem Triacylglycerolgemisch (Tegosoft CT) und anschließender Thermodesorption gekoppelt mit der gaschromatographischen Trennung. Zur Validierung der Methode wurde eine Modellmischung aus zehn Aromastoffen mit unterschiedlichen chemischen und physikalischen Eigenschaften herangezogen. Die Validität der Methode wurde in unterschiedlichen Matrices belegt: Neben dem mit Pflanzenöl bzw. fetthaltigem Rapspresskuchen supplementiertem Kulturmedium, wurde auch Milch als klassischer Vertreter einer Öl-in-Wasser Emulsion herangezogen.

Aus dem Screening hervorgehende Basidiomyceten mit einem großen Aromabildungspotential werden auf deren Robustheit bezüglich eines Up-scalings geprüft. Die Anwendbarkeit der beschriebenen Methode für das Monitoring solcher Maßstabsvergößerungen wurde anhand der Kultivierung von *Fomitopsis rosea* in einem 5 L Bioreaktor belegt.

7. Rapid analysis of volatiles in fat-containing matrices for monitoring bioprocesses

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7.1. Abstract

A rapid, single-step method for the analysis of volatile flavours based on the extraction using the medium-chain-triglyceride (MCT) Tegosoft CT and subsequent thermal desorption gas chromatography (TDS-GC), was developed. The MCT was spiked with a mixture of volatile compounds to validate the protocol. Thermal desorption was complete over a dynamic range of more than three orders of magnitude (except *γ*-dodecalactone) and linear for all constituents of the volatile mixture. The respective limits of detection and quantification were in the range between 12 and 28 mg L^{-1} MCT for *γ*-dodecalactone and 39 and 95 mg L^{-1} for isoamyl acetate. The applicability of the method was evaluated with aqueous and lipid-containing matrices which were spiked with a known amount of the volatile mixture and extracted subsequently using MCT. A complete recovery of non-polar, medium recovery of semi-polar and zero recovery of the most polar compound (maltol) showed the potential and limits of the protocol. The kinetics of volatile formation during the cultivation of the basidiomycete *Fomitopsis rosea* grown in a lipid enriched medium was monitored.

Keywords: Volatile analysis, bioprocess monitoring, TDS-GC, oil-based extraction

7.2. Introduction

High resolution gas chromatography (HRGC) coupled with mass spectrometry (MS) has become standard for the analysis of the aroma of foods. Current work aims at the reduction of measurement time and simplification of sample preparation. Aroma separation from any matrix depends on either hydrophobicity (solvent extraction) or volatility (distillation) or on combinations of both principles. Lipid-rich matrices, however, still present an analytical challenge. Steam distillation, with its risk of artefact formation, and insensitive headspace analysis suffer from inherent disadvantages. Only few options exist for an artefact-free and quantitative aroma separation *[Reineccius 2010]*. Best results were obtained with two related methods, solvent assisted flavour evaporation (SAFE) *[Engel et al. 1999]* and thin layer high vacuum distillation (TLHVD) *[Krings et al. 2003]* which are both based on solvent extraction of total lipids and volatiles, followed by a subsequent high vacuum transfer of the volatiles out of the lipid phase. Both are time consuming and require experienced laboratory staff; they are not suited for screening, monitoring and high-throughput analyses as required, for example, to track the changes of volatile profiles of a bioprocess over time. A recent exploratory study based on the extraction of volatiles using a medium-chain-triglyceride *[Abubaker et al. 2009]* promised to combine rapidness with easy handling.

Two-phase systems are common in biotechnology for creating product accumulation sites. Taking up this approach, the present work aimed at validating a food-grade protocol and at applying this to both, food samples and cell cultures. For example, cultivation of basidiomycetes, such as *Polyporus durus*, on a lipid-enriched medium stimulated the formation of natural flavours *[Berger et al. 1986]*, *[Berger 2009]*. Thus, a model volatile mixture was incorporated in matrices with different contents of lipids. The applicability of the method was demonstrated by analysing volatiles in milk as well as by monitoring the formation of volatiles during the cultivation of a basidiomycete in a lipid-enriched culture medium.

7.3. Material and methods

7.3.1. Chemicals

The model mixture consisted of ten volatile compounds representing the chemical diversity of volatile compounds with different polarities (LogP) and boiling points. Compounds were dissolved in a pentane / diethyl ether (1:1.12 (v/v)) - methanol mixture (10:1) (stock solution). Tab. 10 lists the ten reference compounds and their respective physiochemical properties. All chemicals used in the model mix were purchased from Sigma-Aldrich (Steinheim, Germany) with purities > 99 %.

Tab. 10: Physiochemical properties of constituents of the volatile model mixture *[Burdock 2001]*

Aroma compound	MM[a] [g mol^{-1}]	logP[b]	Boiling point [°C]	Stock solution [mg L^{-1}]
1-Hexanal	146.16	1.79	131	2570
2,4,5-Trimethyl-1,3-oxazole	111.14	1.09	133	2524
Isoamyl acetate	130.18	2.26	145	2592
1-Octen-3-ol	128.21	2.64	175	2500
2-Acetyl pyrazine	122.13	0.2	189	2470
2-Acetyl thiazole	127.16	0.37	212	2586
Carvone	150.21	2.55	230	2418
Maltol	126.11	0.07	285 ± 40*	2462
Vanillin	152.14	1.19	285	2470
γ-Dodecalactone	198.31	3.45	310	2481

* thermal decomposition
[a] MM = molecular mass
[b] P = partition coefficient between octanol and water

Tegosoft CT (Evonik, Marl, Germany), a medium-chain-triglyceride (MCT) containing octanoic and decanoic acid was used as the extraction solvent. Cyclohexanol (≥99 %; Merck-Schuchardt, Hohenbrunn, Germany) and cyclopentanol (99 %; Sigma-Aldrich, Steinheim, Germany) were used as analytical standards in gas chromatography.

Rapeseed press cake, the residue of rapeseed oil production, was kindly provided by Teuteburger Ölmühle (Ibbenbühren, Germany). The oil content of the press cake was determined to 22.0 % (dry matter (w/w)), Soxhlet extraction with *n*-

hexane). Bovine milk (3.5 % fat) was purchased from a local supermarket and spiked with respective volumes of the aroma mixture stock solution.

7.3.2. Evaluation of the method

Desorption of volatiles from MCT

The synthetic MCT was spiked with the volatile stock solution in different ratios to prepare the respective sample concentrations. Every sample was mixed for 30 s using a Vortex Genius (IKA, Staufen, Germany). Afterwards a volume of exact 10.0 µL of each sample was syringed into a desorption tube (Gerstel, Germany) and immediately analysed by means of thermal desorption gas chromatography (TDS-GC). Dispension of 10 µL volumes of MCT was realized by withdrawing the oil slowly into the syringe. The oil was syringed into the tube while turning it. This resulted in a thin film and a good desorbability. Reproducibility of manual syringing of 10.0 µL oil was tested by weighing (syringing 6 times 10 x 10 µL; relative standard deviation 1.79 %). The retained MCT/lipids in the desorption tube were completely removed by flushing with an adequate amount of acetone. Subsequently, the tube was dried with compressed air and re-used. As a control the synthetic oil was replaced by *n*-hexane. The completeness of desorption of volatiles from spiked extraction oil was evaluated for different temperatures and concentrations, and was shown by comparing the respective slope of the regression lines for *n*-hexane and MCT for each compound. Both, limit of quantification and limit of detection were calculated using Valoo 2.0 (analytic software, Germany).

Recovery from spiked liquid samples

To determine the extraction recovery of each spiked compound from pure water and lipid-containing samples, the aroma mixture was added either to 5 mL deionised water (without matrix effects) or to 5 mL supplemented culture medium (2 % (w/w) rapeseed press cake), 5 mL bovine milk (3.5 % fat) and to 5.5 mL of a water-rapeseed oil-emulsion (oil content: 9 %; without emulsifying agent) in different concentrations. To evaluate the minimal volume of synthetic oil required to achieve sufficient recovery at maximal sensitivity (less diluted oil samples) different ratios (v/v) of MCT to sample volume were tested. Aroma compounds were extracted from the liquid phase by adding the respective volume of MCT, followed by manual and mechanical mixing for 60 s using a Vortex Genius,

respectively. For complete separation samples were centrifuged at 4 °C, 4000 g for 5 min (Rotina 380, Hettich, Tuttlingen, Germany). Finally, an aliquot of 10.0 µL of the respective oil extract was placed into the thermal desorption tube and analysed. For comparison 2.5 mL of water spiked with model mixture was extracted with dichloromethane.

7.3.3. Application of the method: Aroma producing fungus

Fomitopsis rosea, a plant pathogenic agaricomycete, was purchased from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. The fungus was grown submerged in modified liquid standard nutrition medium *[Sprecher 1959]*: 30.0 g L^{-1} D-(+)-glucose monohydrate (Merck KGaA, Darmstadt, Germany), 4.5 g L^{-1} L-asparagine monohydrate (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 3.0 g L^{-1} yeast extract granulated (Merck KGaA, Darmstadt, Germany), 1.5 g L^{-1} KH_2PO_4 (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 0.5 g L^{-1} $MgSO_4$ (Carl Roth GmbH & Co. KG, Karlsruhe, Germany); 1.0 mL L^{-1} trace element solution [0.08 g L^{-1} $FeCl_3$ x 6 H_2O (Fluka Chemie AG, Neu-Ulm, Germany), 0.09 g L^{-1} $ZnSO_4$ x 7 H_2O (J. T. Baker, Deventer, Netherlands), 0.03 g L^{-1} $MnSO_4$ x H_2O (Merck KGaA, Darmstadt, Germany), 0.005 g L^{-1} $CuSO_4$ x 5 H_2O (Riedel-de Haën AG, Seelze, Germany), 0.4 g L^{-1} EDTA disodium salt dehydrate (Carl Roth GmbH & Co. KG, Karlsruhe, Germany)]; adjusted to pH 6.0 with 1 M NaOH and autoclaved at 121 °C and 1 bar positive pressure for 20 min.

Cultivation of the fungus

The fungus was pre-cultured for 12 days in nutrition medium described above. Pre-cultures were prepared by homogenisation of a 10 × 10 mm agar plug with mycelium using an Ultra Turrax (Miccra D-9, Art, Mühlheim, Germany). Cultivation took place in 1 L Erlenmeyer flasks (500 mL medium) and orbital shaking at 150 rpm and 24 °C (Multitron, Infors HT, Bottmingen, Switzerland).

Main cultivation was performed in a 5 L (working volume) stirred tank reactor (ISF 100, Infors HT, Bottmingen, Switzerland) at 24 °C with an air flow of 1 L min^{-1} through a ring sparger. The bioreactor was equipped with pitched blade impeller running at 120 rpm. Before inoculation the bioreactor was autoclaved at 121 °C and 1 bar positive pressure for 30 min, containing 4.5 L minimal medium with reduced D-(+)-glucose-monohydrate concentration (3 g L^{-1}) which was additionally supplemented with 20 g L^{-1} (dry matter) rapeseed press cake.

Sampling

Every day about 20 mL sample of the medium was taken from the culture broth and separated from fungal cells by centrifugation at 4 °C and 4000 g. Two samples of exact 10 mL were extracted with 1.0 mL MCT and 10 µL extract analysed as described above.

7.3.4. Analytical methods

Thin layer high vacuum distillation (TLHVD)/Gas chromatography olfactometry (GC-O)

The MCT extracts of the daily samplings were pooled, and the volatiles were isolated using TLHVD as described elsewhere *[Krings et al. 2003]*. The volatile extract obtained was further analysed by means of high resolution gas chromatography mass spectrometry (HRGC-MS) and GC-O. A flavour dilution factor (FD-factor) was determined by sniffing a dilution series. The extract was diluted stepwise (1:1) with pentane/diethylether (1:1.12 (v/v)), until odorous compounds were no longer perceived at the sniffing port. GC-O was performed using an Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled with a flame ionisation detector (FID, 250 °C) and an olfactory detection port (ODP, 250 °C; Gerstel, Mühlheim, Germany). The chromatographic conditions were: 30 m x 0.32 mm i.d. x 0.25 µm DB-WAX (J&W Scientific, Folsom, USA) with 2 mL min^{-1} hydrogen as carrier gas using the following temperature program: 40 °C (hold for 3 minutes) with a rate of 5 °C min^{-1} to 230 °C, hold for 10 minutes.

Thermal desorption gas chromatography (TDS-GC)

MCT samples (spiked oil or sample extracts) were analysed using an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled with a thermal desorption system (TDS 2), a cold injection system (CIS 4) (Gerstel, Mühlheim, Germany) and a flame ionisation detector (250 °C) with the following parameters: TDS 2: 20 °C (tube assembly) to 150 °C with 60 °C min^{-1}, hold for 2 min (tube desorption) in the splitless mode; refocusing of the volatiles on a liner filled with Tenax TA in the CIS at -10 °C. After complete thermal desorption the CIS was heated to 230 °C (12 °C sec^{-1}, 2 min) in the solvent vent mode. The chromatographic conditions were as described above.

High resolution gas chromatography mass spectrometry

The volatile extracts obtained after TLHVD were analysed using a Fisons GC 8000 gas chromatograph and a Fisons MD 800 mass selective detector (interface: 230 °C, ion source: 200 °C, quadrupole: 100 °C, electron impact ionisation 70 eV, scan range m/z 33 to 300 amu). Analytical conditions were as follows: 30 m x 0.32 mm i.d. x 0.25 µm DB-WAX (J&W Scientific, Folsom, USA) with 1 mL min^{-1} helium as carrier gas using the following temperature program: 40 °C (hold for 3 minutes) with a rate of 3 °C min^{-1} to 230 °C hold for 10 minutes. Identification of volatiles was achieved by comparing their linear retention indices, calculated according to *n*-alkanes as external references *[Van den Dool et al. 1963]*, odour impression and mass spectra with those retrieved from digital libraries (Wiley 08/NIST 08).

7.4. Results and discussion

7.4.1. Validation of the method

The conditions for a complete and artefact-free desorption of volatiles from MCT were figured out to confirm the working principle. One mL of MCT was spiked with different volumes of volatile stock solution and then desorbed at different temperatures in the TDS. The desorption increased and converged to 100 % – except *γ*-dodecalactone – and was perfectly linear ($R^2 \geq 0.9993$) up to a desorption temperature of 150 °C for all substances of the volatile mixture in the range from 10 to 750 mg L^{-1} MCT (Tab. 11). Desorption of the lactone was 66% only, as shown by the lower slope (13.2) of the regression line for the desorption from MCT compared to the desorption from the control (19.9, *n*-hexane). This is ascribed to physicochemical properties of the lactone (logP and boiling point, Tab. 10) compared to the relatively low desorption temperature of 150 °C during TDS-GC analysis. It was shown previously that the desorption of lactones from lipid-rich matrices is exceptionally low compared to other compounds with similar logP values and boiling points *[Engel et al. 1999]*; *[Krings et al. 2003]*. Nevertheless, a desorption temperature of maximal 150 °C was chosen as a reasonable compromise between complete desorption and possible thermal alteration of volatile compounds, extraction oil, or co-extracted genuine lipids of the samples.

The limit of quantification and the limit of detection were derived from the respective calibration curves. The lowest limit of detection/quantification was found for *γ*-dodecalactone, 12/39 mg L^{-1} oil, whilst the highest was calculated for isoamyl acetate, 28/95 mg L^{-1} oil (Tab. 11).

Tab. 11: Validation of the desorption of volatile compounds from medium-chain-triglyceride

Volatile compound [10-750 mg L^{-1}][a]	R^2 [b]	Slope[c] (*n*-Hexane)	Slope[c] (MCT)	LOD[d],* [mg L^{-1}]	LOQ[e],* [mg L^{-1}]
1-Hexanal	0.9996	22.3	23.1	16.2 ± 9.0	56.3 ± 8.7
2,4,5-Trimethyl-1,3-oxazole	0.9998	14.2	18.3	19.1 ± 9.9	65.6 ± 9.5
Isoamyl acetate	0.9996	12.4	19.4	27.9 ± 8.6	94.5 ± 8.3
1-Octen-3-ol	0.9997	24.0	26.3	14.2 ± 11.5	49.3 ± 11.1
2-Acetyl pyrazine	0.9997	19.2	19.9	19.9 ± 10.5	68.3 ± 10.1
2-Acetyl thiazole	0.9998	14.2	15.0	17.4 ± 7.5	60.2 ± 7.2
Carvone	0.9998	26.3	25.4	12.2 ± 13.4	42.6 ± 13.1
Maltol	0.9996	12.7	12.2	18.4 ± 8.1	63.5 ± 7.8
Vanillin	0.9998	12.9	12.0	17.2 ± 15.2	59.3 ± 14.7
γ-Dodecalactone	0.9993	19.9	13.2	11.5 ± 24.2	38.5 ± 22.8

[a] 7 data points
[b] R^2: Coefficient of determination
[c] Slope: derived from regression line for the desorption of volatile compound
[d] LOD: Limit of detection
[e] LOQ: Limit of quantification
*n = 3, ± relative standard deviation [%]

7.4.2. Recovery from spiked samples

To evaluate the applicability of MCT as an extracting agent for volatile compounds, the recovery of the constituents of the model mixture was determined from pure water, whole bovine milk (3.5 % fat) and culture medium, supplemented with rapeseed press cake. Supplemented culture medium was used to prove the applicability of this method for the extraction of volatiles from liquid lipid-rich matrices. Milk served as a prototypical food representing lipid-in-water emulsion and, thus, as an example for a possible extension of the method. A volume of at least 1 mL MCT was required for good phase separation during the extraction of aqueous samples (20% (v/v) MCT/sample). A further increase of the volume of the

extraction oil did not significantly increase the recovery but lowered the sensitivity of the method owed to sample dilution (data not shown).

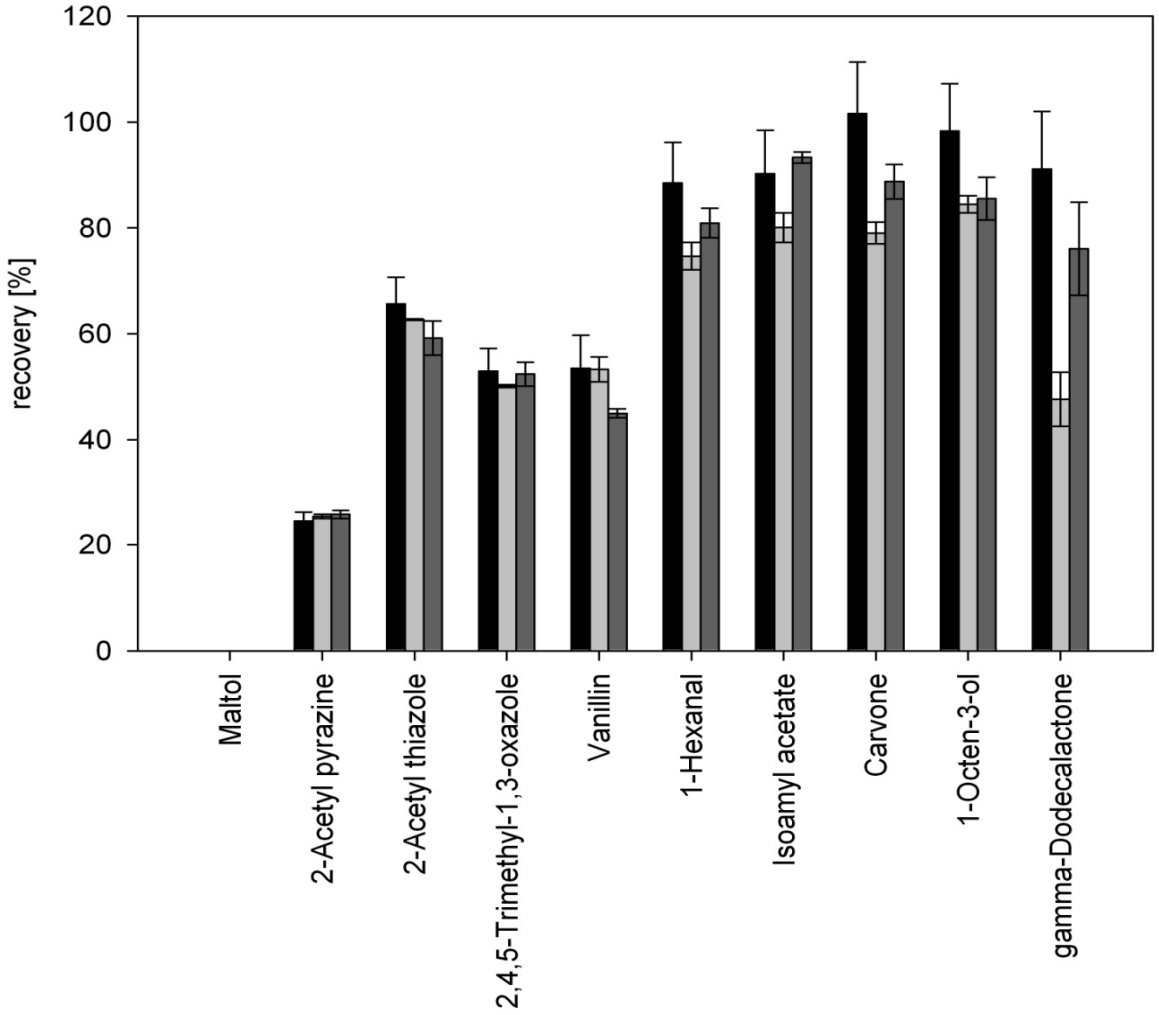

Fig. 12: Recovery of volatile flavours from different matrices using medium-chain-triglyceride. Black: water, light grey: model culture broth supplemented with 2 % rapeseed press cake, dark grey: whole bovine milk

Fig. 12 shows the recoveries of all compounds from the respective matrices. Desorption directly from MCT, spiked with equal amounts of volatile compounds (about 25 mg L^{-1}; Tab. 10) was used as the control and represented 100 % recovery. The low standard deviations (less than ± 10.8 %) of the recovery rates illustrate the good reproducibility of the method within independent repeats on three consecutive days. The recovery of volatile compounds from pure water roughly followed the principles of solvent extraction. A single distribution between water and MCT yielded recoveries comparable to a three-time repeated extraction using a non-polar solvent, such as dichloromethane, where the recovery is in the

range of 70.4 % to 101.5 %. Substances with a low polarity/high logP value, such as carvone, 1-octen-3-ol, isoamyl acetate, and *γ*-dodecalactone showed almost complete recovery (90.2 % to 101.6 %). Substances characterised by a higher polarity/low logP value, such 2-acetyl thiazol and 2-acetyl pyrazine were extracted partially, and the most polar compound, maltol, with a logP of 0.07 was not recovered showing the limits of this approach.

Furthermore, for lipid containing matrices the recovery of less lipophilic compounds with a logP ≤ 1.79 showed no significant difference in comparison to the recovery for pure water. Slightly lower recovery rates were found for compounds with a logP ≥ 1.79, in particular for culture medium supplemented with rapeseed press cake. The adsorption of volatiles on tissue particles of the supplemented rapeseed press cake may have led to this loss. Although the lipid content was different (3.5 % for milk and 0.5 % for the supplemented culture medium) the recovery rates for most substances were similar for both. This was expected, as the genuine lipids of the samples were extracted with MCT as well and, thus, did not retain non-polar volatile compounds in the sample. Still, compared to the more laborious high vacuum transfer reference methods for volatile analysis of lipid matrices, the direct extraction of volatile compounds with MCT yielded comparable or, in the case of *γ*-dodecalactone, even significantly higher recoveries *[Engel et al. 1999]*; *[Krings et al. 2003]*. The sensitivity of the method could be further improved by either increasing the volume of the sample or by injecting more than 10 µL oil into the TDS device. However, the optimal volumetric ratio between MCT and sample in terms of recovery, reproducibility, phase separation and handling was found in the range from 1:5 to 1:10 (data not shown). The flavour release from lipids is affected by the molarity of the lipid phase *[Rabe et al. 2003]*. Lipids of respective samples were co-extracted together with the volatiles. Therefore, the composition of the final extract depended on the lipid type and concentration in the sample. In order to assess the effect of these compositional changes, desorption of volatiles from different phases was determined. MCT, rape seed oil and a mixture of both (1:1) were selected.

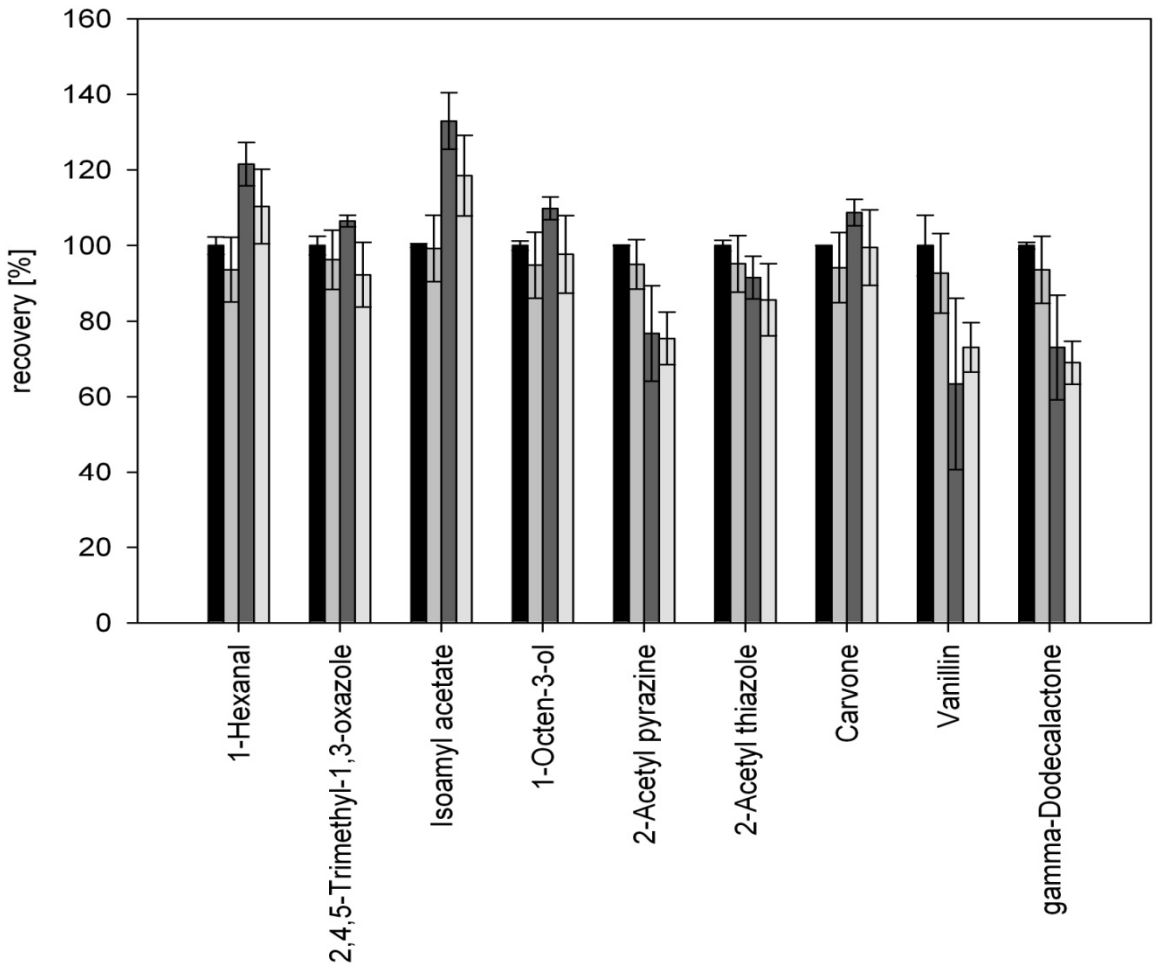

Fig. 13: Desorption of aroma components from different extraction agents spiked with aroma mixture; black: desorption from *n*-hexane (set to 100 %), light grey: desorption from medium-chain-triglyceride, dark grey: desorption from rape oil, white: desorption from the mixture medium-chain-trigylceride and rape seed oil (1:1)

Fig. 13 shows the various desorption rates. Desorption from *n*-hexane (as the control) and MCT showed only small differences. Desorption from rape seed oil and the mixture of rape seed oil and MCT, respectively, showed larger differences. However, the desorption rates for every compound followed the same trend.

As a consequence, accurate quantifications from high lipid samples will require external standards dissolved in the same MCT to sample lipid ratio as the sample extracts to be measured.

7.4.3. Applications

The example of spiked milk proved the qualification of the method as an alternative for the analysis of the volatiles of liquid lipid-rich matrices. The

comparison of the volatile profile of cream cheese analysed after SAFE and MCT extraction showed this as well *[Abubaker et al. 2009]*. A further field of application is the monitoring of volatile formation during the cultivation of volatile producing microorganisms. As fatty acids are common precursors of volatiles, formation of aroma is often observed in lipid supplemented culture medium. The MCT method was applied to monitor the kinetics of formation of volatiles during a 5 L batch cultivation of the basidiomycete *F. rosea* in a liquid minimal medium supplemented with rapeseed press cake. *F. rosea* produced a large number of odorous compounds. The odour impression of the culture broth after twelve days of cultivation was characterized by pleasant flowery and fruity notes. Applying the MCT method enabled the identification and quantification of volatiles over the entire cultivation period. Tab. 12 shows the main compounds produced over the cultivation period together with their odour impressions and their corresponding FD-values. One Peak (no. 7) featured a characteristic odour impression, comprising green, fruity, sweet and floral notes. Three co-eluting compounds responsible for the peculiar flavour were identified.

Tab. 12: Volatiles produced by *Fomitopsis rosea* cultivated in 5 L stirred tank bioreactor

No.	Volatile compound[a]	RI[b]	RI Lit.	Odour	FD-factor[c]
1	3-Octanone	1254	1244[d]	Fresh, mint	
2	1-Octene-3-one	1297	1305[e]	Mushroom	64
3	Methyl octanoate	1393	1389[f]	Dull, green	128
4	Cyclohexanol (Internal Std.)	1405	1403[g]		
5	1-Octen-3-ol	1458	1465[d]	Mushroom	64
6	1-Octanol	1564	1565[e]	Herbal, green	
7	4-Methylbenzaldehyde	1603	1642[h]	Cherry	
	2-Octen-1-ol	1607	1618[i]	Green, citrus	
	1-Phenylethan-1-one	1610	1650[h]	Almond, floral	

[a] Identified tentatively according to linear retention index (RI), mass spectrum, and odour impression of reference substances
[b] RI = linear retention index (according to *n*-alkanes *[Van den Dool et al. 1963]*)
[c] FD-factor = flavour dilution factor
[d] *[Zawirska-Wojtasiak et al. 2007]*
[e] *[Cullere et al. 2004]*
[f] *[Varming et al. 2004]*
[g] *[Fu et al. 2002]*
[h] *[Alasalvar et al. 2003]*
[i] *[Weckerle et al. 2001]*

Quantitative evaluation of the daily samples is shown in Fig. 14.The concentration of different compounds peaked at different point in time as suggested by their stepwise formation. The initial product of linoleic acid peroxidation, 1-octene-3-one, showed low and constant levels indicating a dynamic equilibrium of de novo formation and rapid reduction to 1-octene-3-ol and, more slowly, to 3-octanone. Monitoring the evolution of volatiles, thus, not only allowed gaining insights into biogenetic pathways, but also identifying metabolic bottlenecks and optimal separation time for a specific volatile target compound.

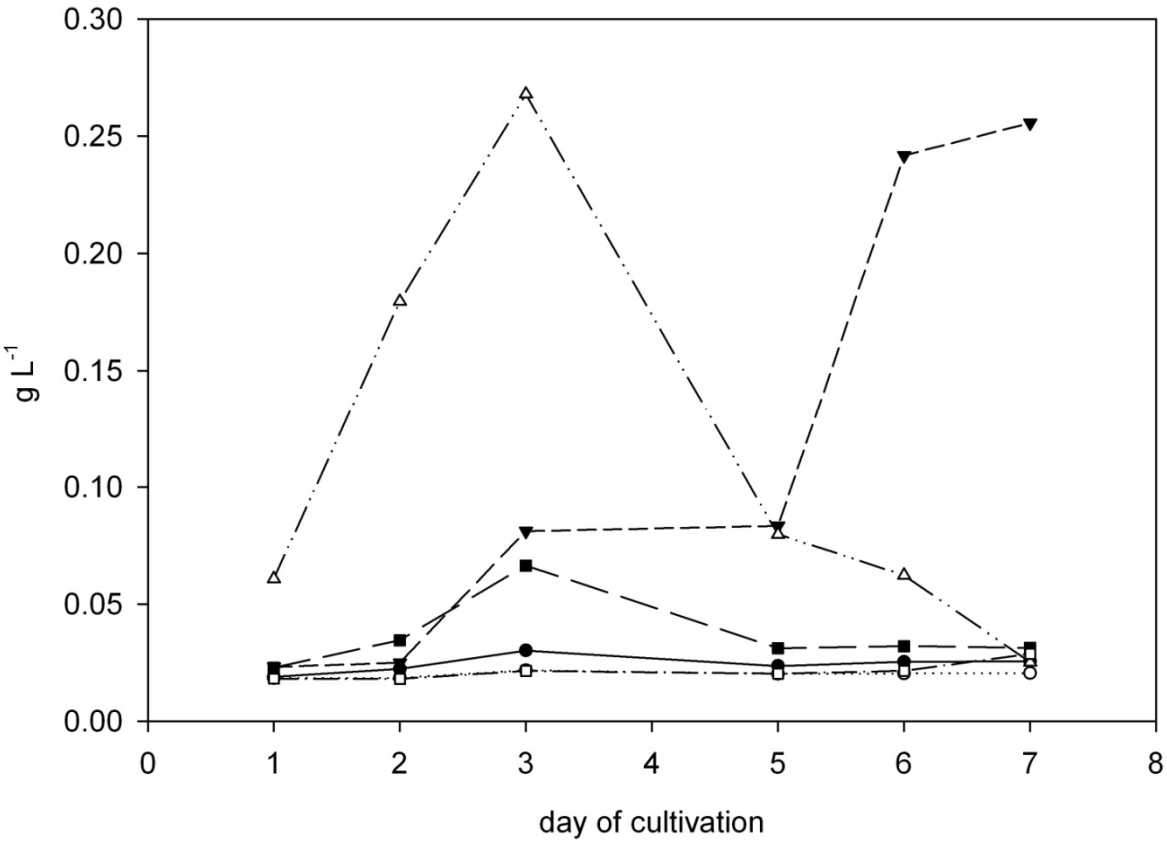

Fig. 14: Course of volatile formation during the cultivation of *Fomitopsis rosea* on rapeseed press cake supplemented minimal medium in 5 L stirred tank bioreactor [g L^{-1} culture broth]. ● 3-Octanone, o 1-Octene-3-one, ▼ Methyl octanoate, Δ 1-Octene-3-ol, ■ 1-Octanol, □ 4-Methylbenzaldehyde, 2-Octen-1-ol, 1-Phenylethan-1-one

7.5. Conclusion

A distribution-coefficient based protocol using the medium-chain-triglyceride Tegosoft as the extraction agent was developed to monitor volatile formation in lipid-rich matrices. The volatile-loaded MCT was directly introduced into a TDS-GC. Limits of quantification and limits of detection of the method, as well as

desorption and recovery rates from a model mixture were investigated. The kinetics of formation of volatiles during the cultivation of *F. rosea* grown in a lipid enriched culture medium were also successfully monitored. The rapid and easy handling of the analytical method came along with acceptable rates of recovery of analytes.

7.6. Acknowledgement

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9. Anhang

9.1. Ergänzende Daten zu Kapitel 2:

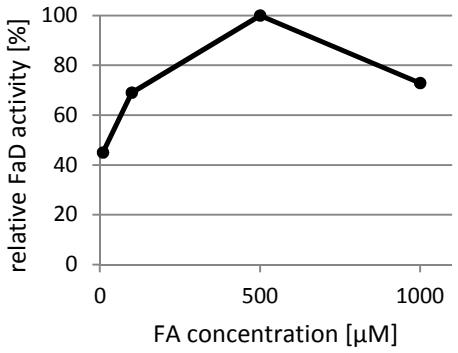

Fig. 15: Abhängigkeit der relativen Aktivität der FaD von der Konzentration an Ferulasäure in der induzierten Kultur
Die Optimierung der Konzentration der Ferulasäure nach Induktion der Kultur wurde auf Grundlage der hier dargestellten Daten auf 500 µM festgelegt.

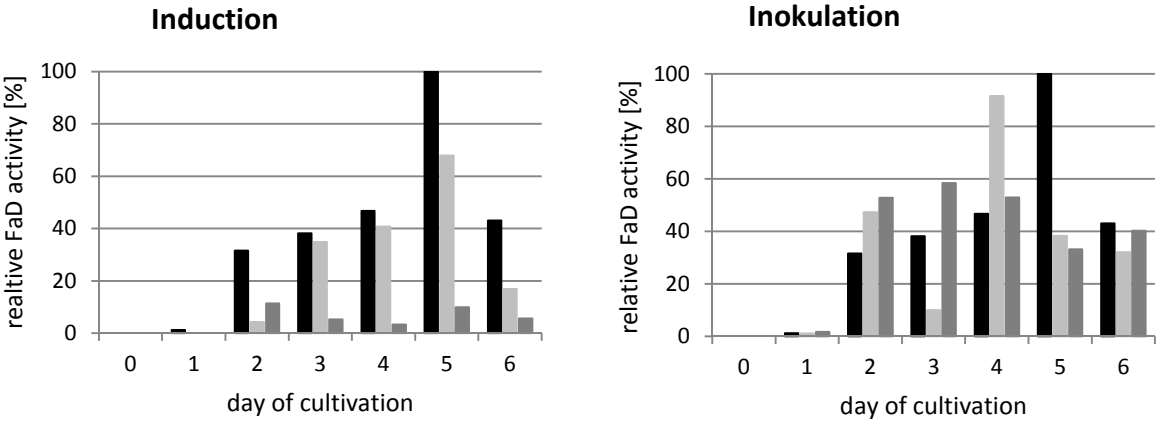

Fig. 16: <u>Links:</u> Abhängigkeit der relativen Aktivität der FaD vom Zeitpunkt der Induktion der Hauptkultur (Inokulation jeweils nach 3 Tagen Vorkultur). Schwarz: Induktion an Tag 1; Hellgrau: Induktion an Tag 2; Dunkelgrau: Induktion an Tag 3. <u>Rechts:</u> Abhängigkeit der relativen Aktivität der FaD vom Zeitpunkt der Inokulation (Induktion jeweils an Tag 1). Schwarz: Inokulation nach 3 Tagen Vorkultur; Hellgrau: Inokulation nach 4 Tagen Vorkultur; Dunkelgrau: Inokulation nach 5 Tagen Vorkultur
Die Optimierung der Kultivierungsbedingungen bezüglich des Zeitpunkts der Inokulation, der Induktion und der Ernte der Hauptkultur, wurden auf Grundlage der hier dargestellten Daten wie folgt festgelegt: Inokulation nach 3 Tagen Vorkultur, Induktion der Hauptkultur an Tag 1, Ernte an Tag 5.

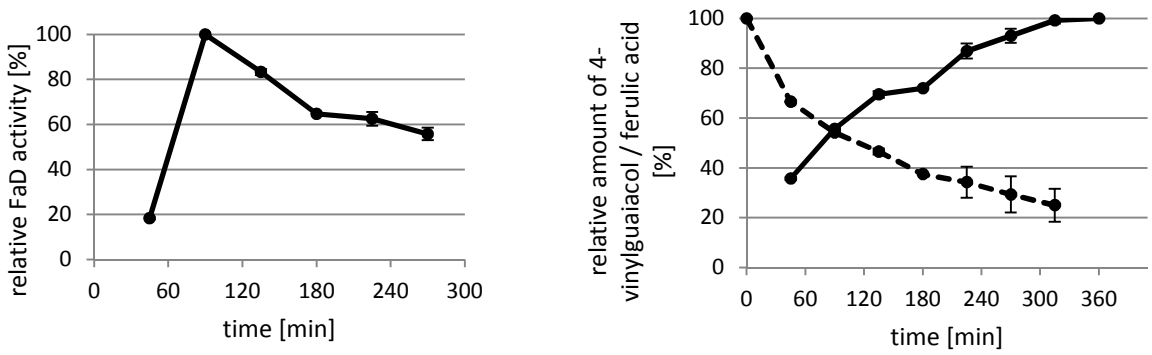

Fig. 17: <u>Links:</u> Zeitverlauf der relativen Aktivität der FaD im Aktivitätsassay. <u>Rechts:</u> Bildung 4-Vinylguajacol (durchgezogene Linie) bzw. Abbau Ferulasäure (gestrichelte Linie) im Aktivitätsassay

Die Optimierung des Aktivitätsassays bezüglich der Inkubationszeit wurde auf Grundlage der hier dargestellten Daten auf 90 Minuten festgelegt.

9.2. Ergänzende Daten zu Kapitel 3: Formation of pineapple-like flavour substances – Characterisation of the aroma profile generated by *Piptoporus betulinus*

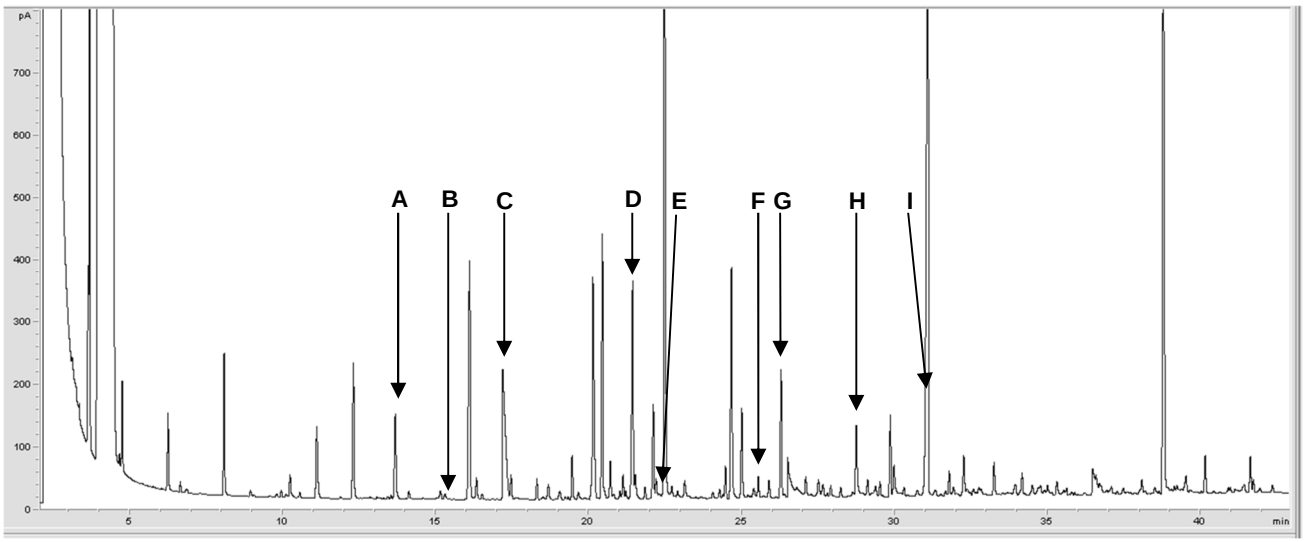

Fig. 18: GC-FID Chromatogramm des P/E Extraktes von 500 mL Kulturbrühe an Tag 6 der Kultivierung im Bioreaktor (AEDA). A: Octan-3-on; B: nicht identifiziert; C: Essigsäure; D: Methylbenzoat; E: nicht identifiziert; F: (5*E*/*Z*, 7*E*/*Z*)-Deca-5,7,9-trien-2-on; G: (5*E*, 7*E*/*Z*)-Deca-5,7,9-trien-2-on, H: (5*E*/*Z*, 7*E*/*Z*)-Deca-5,7,9-trien-2-ol; I: Methyl 4-methoxybenzoat

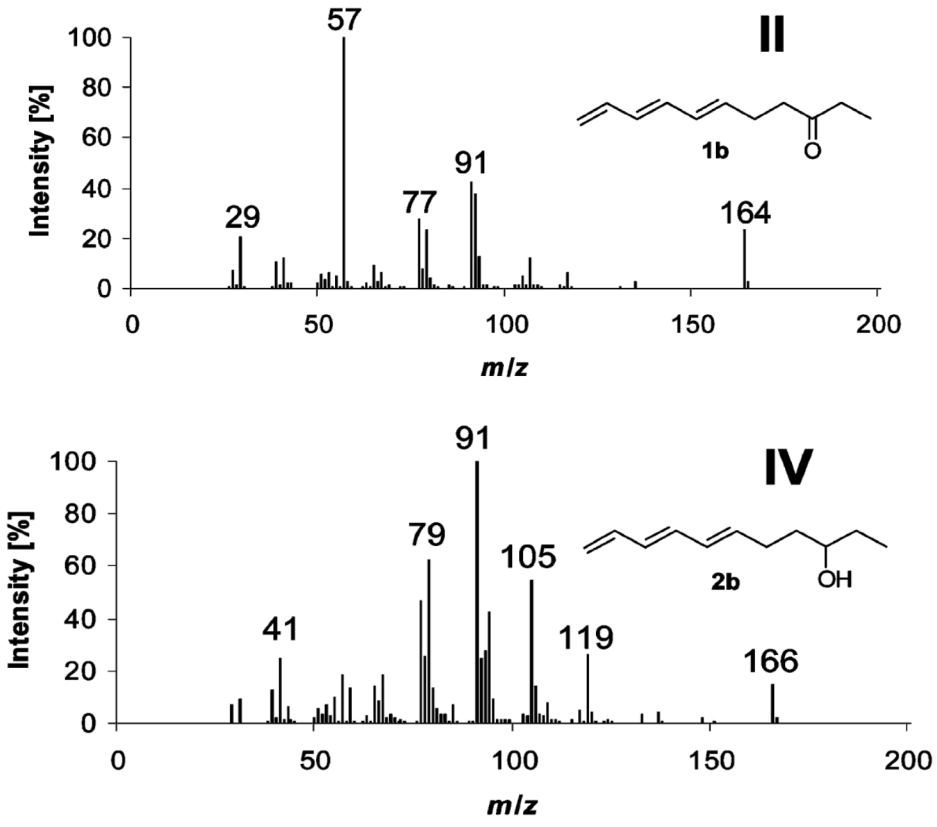

Fig. 19: Massenspektren von (6*E*/8*E*)-undeca-6,8,10-trien-3-on (II) und (6*E*/8*E*)-undeca-6,8,10-trien-3-ol (IV) nach *Miyazawa et al. [2009]*

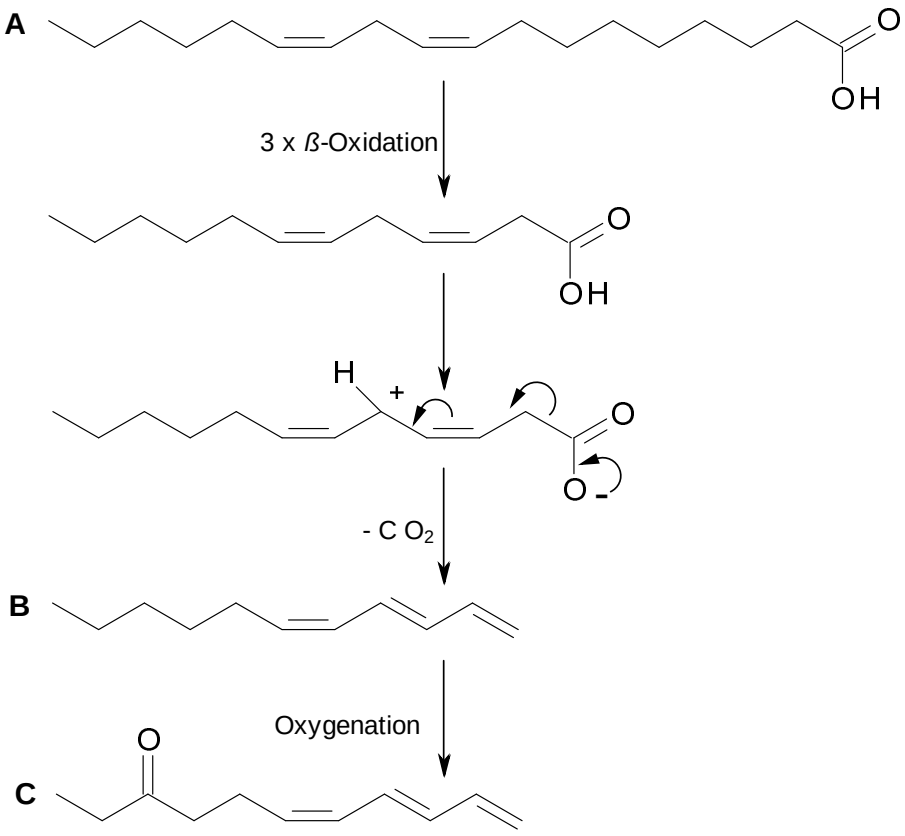

Fig. 20: Möglicher Biosyntheseweg von Yuzunon. A: Linolsäure, B: (3*E*, 5*Z*)-Undeca-1,3,5-trien, C: (6*Z*, 8*E*)-Undeca-1,3,5-trien-3-on (Yuzunon)

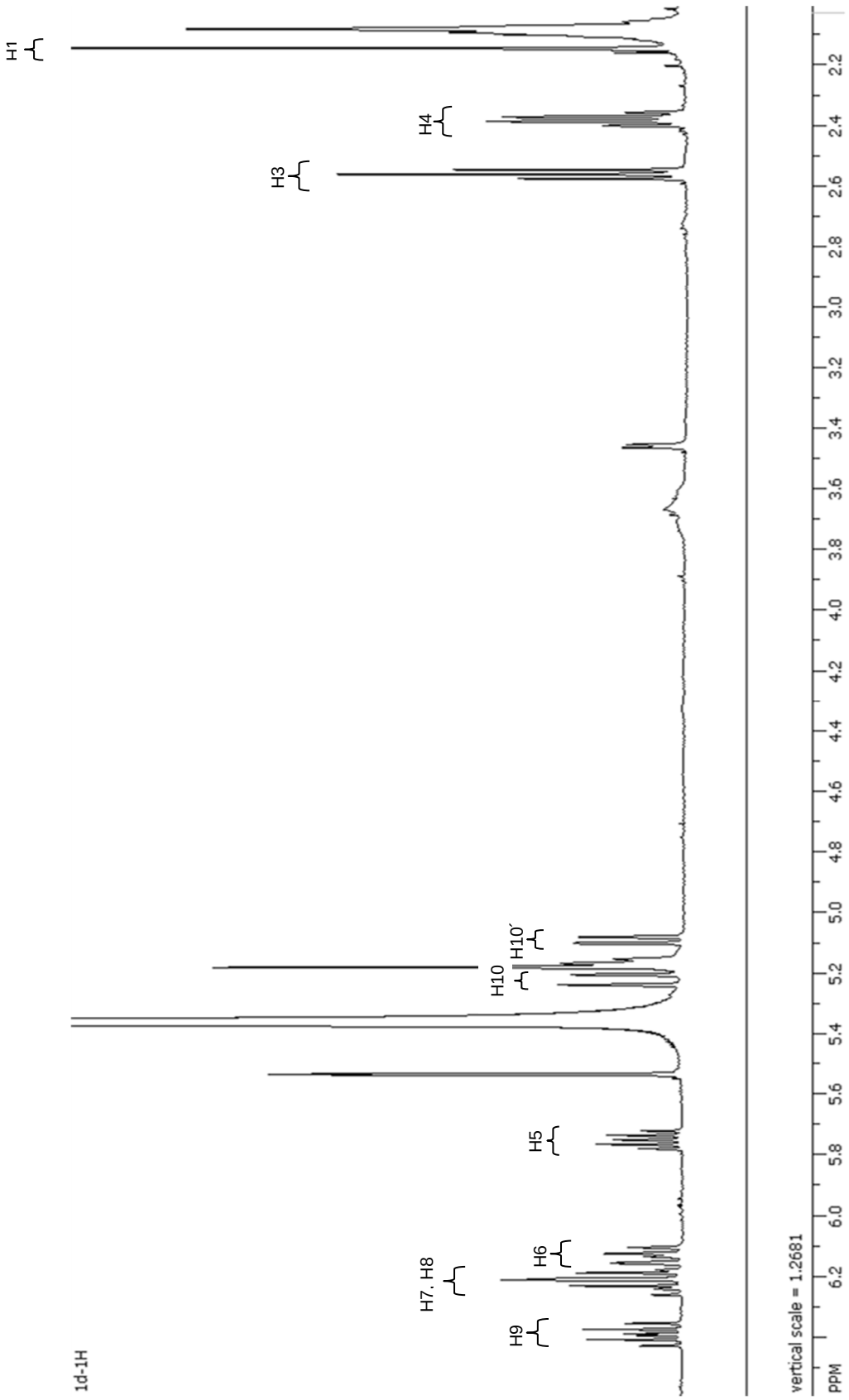

Fig. 21: ^{1}H-NMR Spektrum von (5*E*,7*E*/*Z*)-Deca-5,7,9-trien-2-on [G]

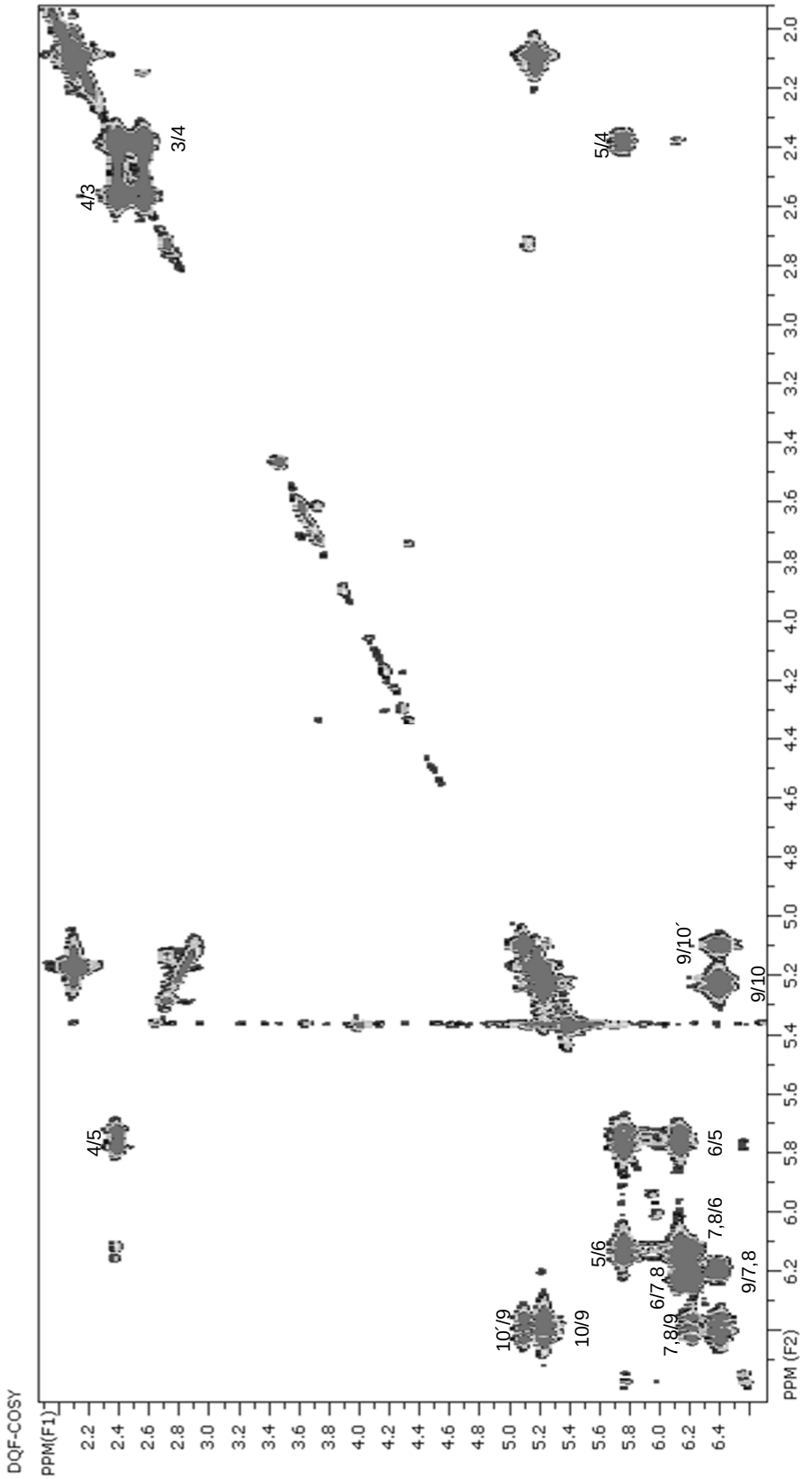

Fig. 22: DQF-COSY Spektrum von (5*E*,7*E*/*Z*)-Deca-5,7,9-trien-2-on [G]

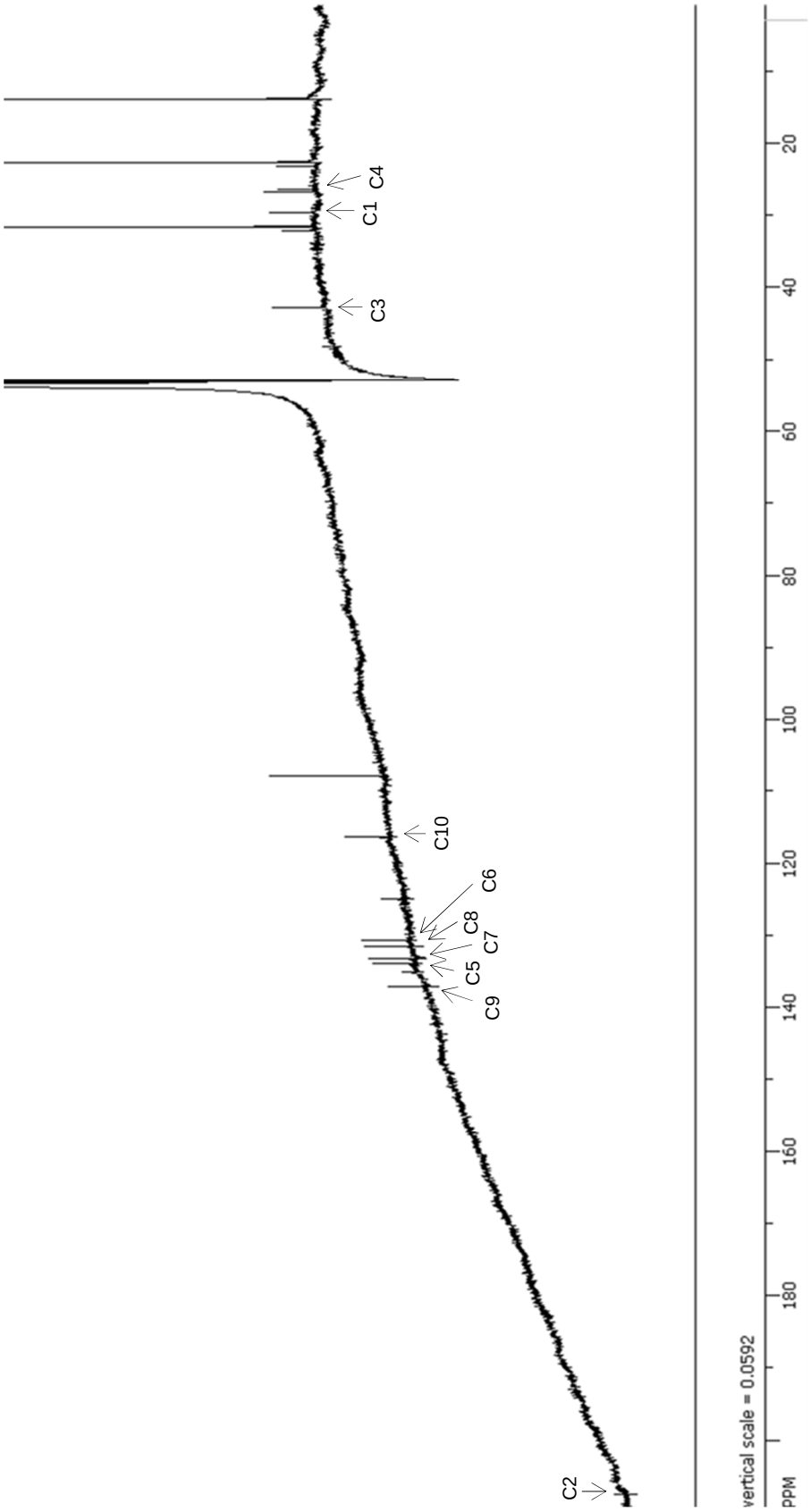

Fig. 23: ^{13}C-NMR Spektrum von (5*E*,7*E*/*Z*)-Deca-5,7,9-trien-2-on [G]

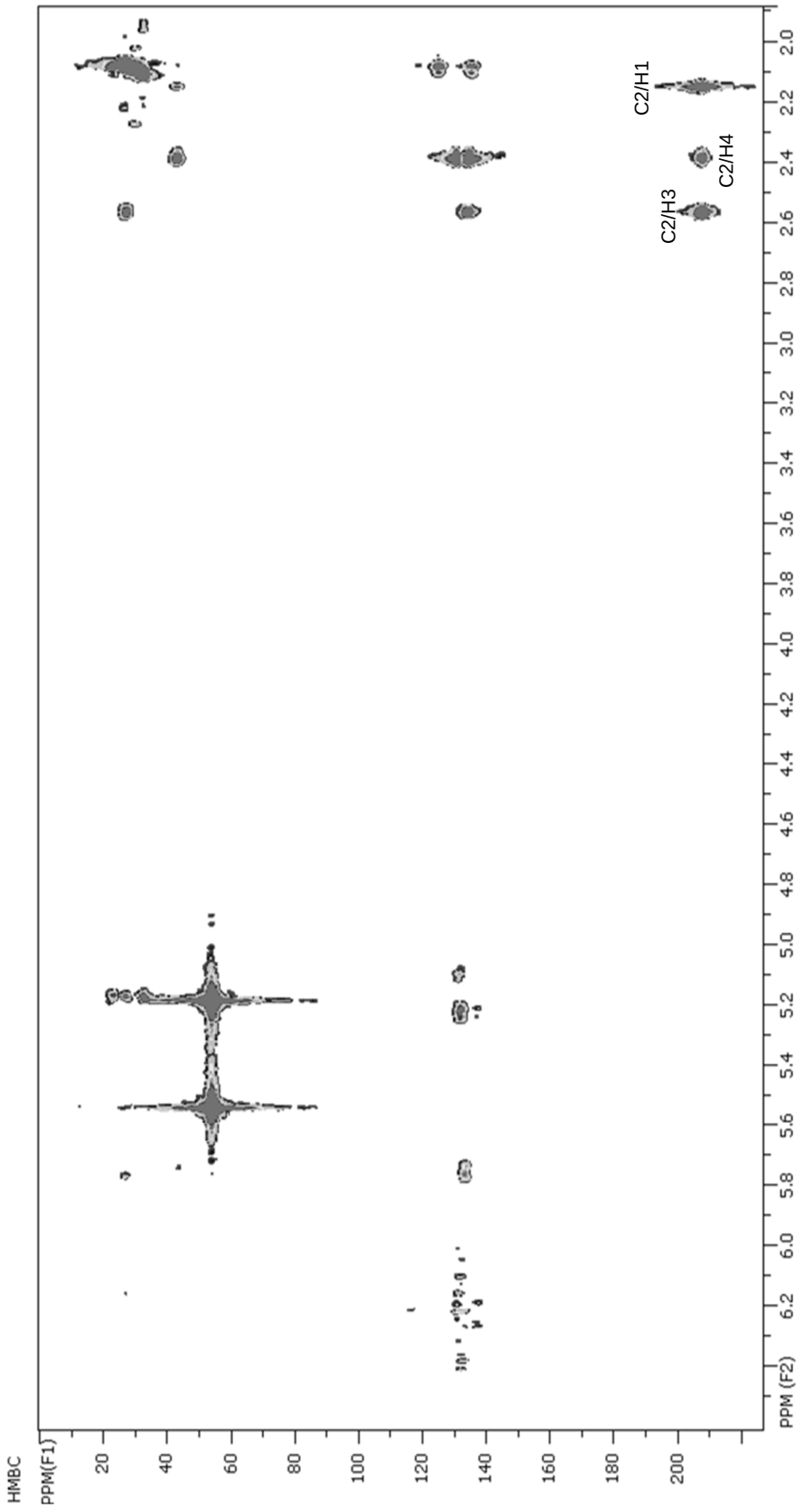

Fig. 24: HMBC Spektrum von (5*E*,7*E*/*Z*)-Deca-5,7,9-trien-2-on [G]

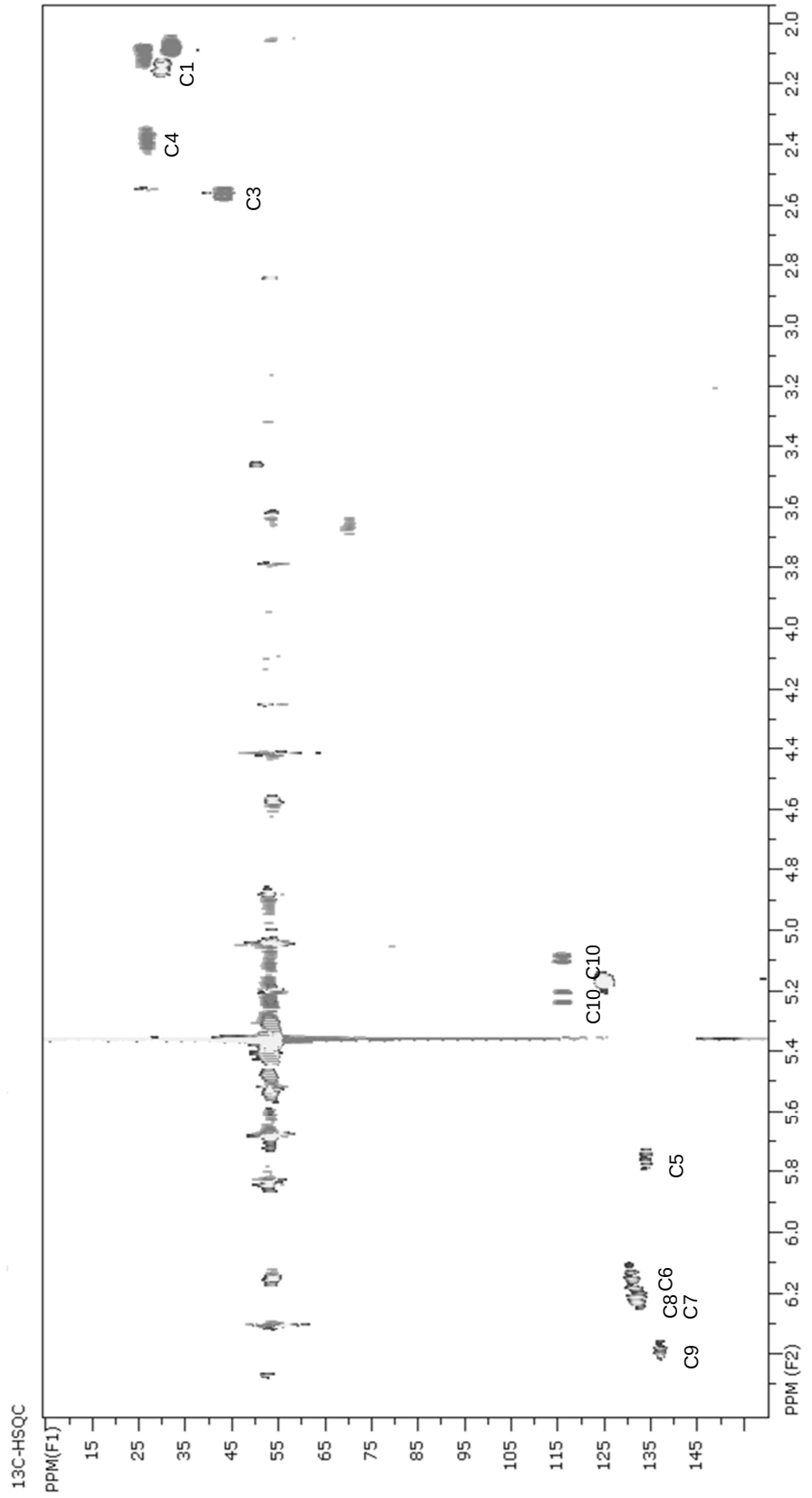

Fig. 25: 13C-HSQC Spektrum von (5*E*,7*E*/*Z*)-Deca-5,7,9-trien-2-on [G]. Hellgrau: positive Signale (CH, CH_3); Dunkelgrau: negative Signale (CH_2)

10. Lebenslauf

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