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Isolation, Separation and Bioactivity of Wood Extracts

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“You need to believe in things that aren't true. How else can they become”?

-Terry Pratchett, Hogfather

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Summary

In the course of evolution, plants have developed protective mechanisms and actions to ensure their survival. These strategies include the natural durability of wood, which has underlying functional mechanisms, which are not yet fully understood. In particular, tropical species harbor many effective tools, whose mode of actions are still to be revealed in detail. Soluble wood compounds, the so-called extractives, are known to contribute to the biological defense strategies to a considerable extent. These extractives consist of a great variety of different compounds and show a great inter-species diversity.
Many "lesser known" tropical species are traded on the world market today and are of special interest as some of them stand out due to attractive colouring and their high durability against wood-degrading fungi. To understand the variations in durability, the chemistry of the extractives needs to be further elucidated.

In the present thesis, eight tropical wood species, namely Bangkirai (*Shorea laevis*), Ipé Noir (*Handroanthus spp*.), Itaúba (*Mezilaurus itauba*), Jatoba (*Hymenea coubaril*), Massaranduba (*Manilkara bidentata*), Merbau (*Intsia spp*.), Muiracatiara (*Astronium graveolens*), and Pequia (*Caryocar villosum*) are examined. The methodological focus is on isolation and separation of their extractives as well as on testing the extractives for their bioactivity against two wood degrading fungi. The aim is to elucidate whether the principals behind the durability of the wood can be attributed to specific substances. However, analysis of bioactive key substances is time-consuming and often necessitates sophisticated laboratory equipment. Efficient, rapid and straightforward isolation methods of extractives have to be developed to enable a concentration of the analytical effort on the causative compounds.

To achieve this goal, two methodological approaches were adapted to wood for the first time. Initially, a specific isolation procedure, called foam fractionation, was applied to isolate, separate, and accumulate, in particular, soap-like compounds, the saponins. This method is based on the fact that surface-active molecules contain hydrophobic and hydrophilic parts in their structure. Therefore, they can be dissolved in aqueous solutions on the one hand and are able to adsorb at gas bubbles and emerge through the initial solution on the other. Together with the raising bubbles, the adsorbed molecules leave the solution and can be enriched in the formed foam.

The results proved the applicability of the method to solid wood analysis, and specified that specific experimental conditions have to be adjusted to the targeted extractives of the wood species. It was observed that the method is suitable to rapidly extract saponins, without the need to separate them from other extractives. However, compared to conventional water extraction, foam fractionation is not an alternative to a comprehensive analysis of water extractable compounds. At present, foam fractionation can be applied to wood species that contain sufficient amounts of surface-active substances making the wood „self-foaming “. However, for a more versatile application, the method needs to be further refined. Optimization of various process parameters, the application of surfactants other than tested, as well as techniques like the tweezing technique, offer potential to adapt the foaming process to the specific wood extract chemistry.

Secondly, an agar-overlay bioautography for basidiomycete fungi was successfully implemented using *Rhodonia placenta* and *Trametes versicolor*, which do not sporulate easily. Therefore, hyphae were incubated in a malt agar bouillon and afterwards homogenized and mixed thoroughly with a warm potato-dextrose agar. After adding a vitality stain to the agar, the mixture was then poured over a preparative thin-layer chromatography (TLC)-plate with the separated extractives on it. The prepared bioautography was allowed to incubate again until bioactivity zones could be detected. An additionally developed TLC-plate was used to isolate the respective compounds.

The homogenized hyphae recovered rapidly from the treatment and the added vital stain enhanced the visible assignment of the inhibition zones containing antifungal agents. The combination enables a more rapid method for the investigation of bioactive wood extracts, providing isolation and identification of possible key substances against microbial attack. This might accelerate the elucidation of potential molecular structures, for example for biocidal, pharmaceutical or chemical uses. However, the prime benefit of the method is its rapid overview on the bioactivity potential of extractive mixtures from lesser-known wood species.

Results from both methods unfolded possible contributions of the extractives to the durability of the tested wood species. In Massaranduba (*Manilkara bidentata*), ursolic acid, hederagenin and quillaic acid and in Pequia (*Caryocar villosum*), oleanolic acid and hederagenin were identified from foam fractionation. They are obviously present as saponins, which are known to be bioactive in many cases. For Muiracatiara (*Astronium graveolens)*, Merbau (*Intsia* spp.), Bangkirai (*Shorea laevis)* and Itauba (*Mezilaurus itauba*), in most of the bioactive zones of the bioautography, several known fungicidal compounds were identified. This can be rated as a

proof of principle, with prominent examples like gallic acid, catechin and resveratrol. Furthermore, the isoquinoline alkaloids bicuculline and norbicuculline were detected, which have not yet been described as extractive components of *Mezilaurus itauba*.

It was shown that both methods represent straightforward analysis tools to isolate and separate compounds even out of complex extractive mixtures. The hyphae agar bioautography additionally provides insights into the antifungal behavior of the extractives. Further studies need to optimize the presented separation techniques of extracts followed by the implementation on an enlarged preparative scale, to allow additional analytical methods for an in-depth identification of the substances responsible for the natural durability.

Zusammenfassung

Um ihren Fortbestand sicherzustellen haben Pflanzen im Laufe der Evolution eine Reihe von Verteidigungs- und Schutzstrategien entwickelt, welche der Mensch nutzbar machen könnte. Einen interessanten Aspekt stellt hierbei die natürliche Dauerhaftigkeit mancher Holzarten dar. Allerdings sind bis heute die Mechanismen, welche hinter der Widerstandsfähigkeit gegenüber mikrobiellem Abbau stehen, nicht vollumfassend verstanden. Speziell tropische Holzarten beinhalten Wirkmechanismen, welche zum Teil noch unbekannt sind. Ein nennenswerter Beitrag zur natürlichen Dauerhaftigkeit von Holz wird löslichen Holzinhaltsstoffen, den sogenannten Extraktstoffen zugeschrieben. Ihre chemische Zusammensetzung unterscheidet sich von Holzarten zu Holzart und ist häufig nur zum Teil bekannt, da sie aus einer Vielzahl verschiedenster Verbindungen bestehen.
Derzeit werden auf dem Weltmarkt sogenannte „weniger bekannte“ tropische Holzarten gehandelt, welche hinsichtlich ihrer ansprechenden Farben, vor allem aber aufgrund ihrer herausragenden natürlichen Dauerhaftigkeit gegenüber holzabbauenden Pilzen von speziellem Interesse sind. Eine vertiefte Analytik ihrer chemischen Inhaltsstoffe soll hierzu weitergehendes Verständnis erzeugen.

In der vorliegenden Arbeit wurden die acht Holzarten Bangkirai (*Shorea laevis*), Ipé Noir (*Handroanthus spp*.), Itaúba (*Mezilaurus itauba*), Jatoba (*Hymenea coubaril*), Massaranduba (*Manilkara bidentata*), Merbau (*Intsia spp*.), Muiracatiara (*Astronium graveolens*) und Piquia (*Caryocar villosum*) untersucht. Im Focus standen hierbei sowohl die Isolierung und Trennung der jeweiligen Extraktstoffe als auch deren Prüfung auf fungizide Eigenschaften gegenüber zwei holzabbauenden Pilzen. Ziel war es, die Prinzipien hinter der jeweiligen Dauerhaftigkeit einer Holzart zu beleuchten und zu ergründen, ob diese Prinzipien speziellen Verbindungen, sogenannten Leitverbindungen zugeschrieben werden können. Die Analytik solcher Leitsubstanzen ist jedoch häufig zeitaufwändig und bedarf einer hoch entwickelten sowie anspruchsvollen Laborausstattung. Daher soll die Entwicklung zeitsparender und einfach anzuwendender Methoden es gestatten, den analytischen Aufwand auf die für eine möglich vorhandene Bioaktivität ursächlichen Verbindungen zu konzentrieren.

Hierzu wurden zwei methodische Ansätze gewählt, welche zum ersten Mal in Kombination mit Holz aus Ausgangsmaterial verwendet wurden.
Mittels einer speziellen Isolationsmethode, der Zerschäumungsanalyse, sollten seifenähnliche Substanzen (Saponine) aus dem Holz isoliert, abgetrennt und angereichert werden. Diese

Methode macht sich zu Nutze, dass oberflächenaktive Verbindungen, welche hydrophile als auch hydrophobe Anteile im Molekül aufweisen, sich einerseits in wässrigen Umgebungen lösen und andererseits an Gasblasen adsorbieren können. Auf diese Weise können entsprechende Verbindungen zusammen mit der Gasblase aus der wässrigen Umgebung ausgetrieben und im gebildeten Schaum angereichert werden. Es konnte gezeigt werden, dass die Methode grundsätzlich auf Holz und seine wässrigen Extrakte anwendbar ist, wobei die Versuchsparameter den jeweiligen Inhaltsstoffen der vorliegenden Holzart angepasst werden müssen. Im Speziellen lassen sich so Saponine, ohne die Notwendigkeit sie dabei von den anderen Extraktstoffen abtrennen zu müssen, schnell und v.a. selektiv isolieren. Die Zerschäumungsanalyse stellt allerdings keine Alternative zur konventionellen Wasserextraktion bei Raumtemperatur dar, da nicht alle Inhaltsstoffe im selben Umfang extrahiert werden. Holzarten mit ausreichend oberflächenaktiven Inhaltsstoffen eignen sich für eine Zerschäumungsanalyse am besten, da ihr Holz nach der Zerkleinerung „selbstschäumend", das heißt ohne zusätzliche Hilfsmittel schäumbar ist.

Zur Erschließung breiterer Anwendungsmöglichkeiten muss die Methode allerdings noch weiter optimiert und verfeinert werden. Die Variation verschiedener Prozessparameter sowie die Anwendung von weiteren Techniken (weitere oberflächenaktive Stoffe sowie die Tweezing Technik), welche noch nicht im Rahmen dieser Arbeit getestet wurden, bieten hier Potentiale die Methode an die jeweilige Extraktstoffchemie der verschiedenen Hölzer anzupassen.

Des Weiteren wurde eine Agar-Overlay-Bioautographie erfolgreich entwickelt und an die beiden, unter Laborbedingungen, nur schwer sporulierenden Basidiomyceten *Rhodonia placenta* and *Trametes versicolor* angepasst. Hierzu wurden Hyphen beider Pilze jeweils in einer Malz-Agar Bouillon inkubiert, anschließend homogenisiert und in warmen Kartoffel-Dextrose Agar gemischt. Nach Zugabe eines Vitalitätsfarbstoffes wurde das Gemisch über eine Dünnschicht-Chromatographie Platte gegossen, auf welcher zuvor der zu analysierende Holzextrakt aufgetrennt worden war. Diese beschichtete Platte wurde in der Folge erneut inkubiert bis sichergestellt war, dass der Pilz reaktiviert und mit den Extraktbestandteilen in Kontakt gekommen war.

Die Hyphen erholten sich von der Stressbelastung durch die Homogenisierung nach nur kurzer Zeit und der zugegebene Farbstoff ermöglichte eine klare Abgrenzung der Aktivitätszonen, die durch die fungiziden Verbindungen erzeugt wurden. Die Kombination einer Substanztrennung mit gleichzeitigem Bioaktivitätstest auf derselben Chromatographie-Platte bietet eine gute Möglichkeit, um Einblick in das Wirkspektrum weniger bekannter Extraktstoffmischungen, zu

erlangen. Im selben Zug können die betreffenden Verbindungen und möglichen Leitsubstanzen isoliert und identifiziert werden.

Aus den Ergebnissen beider Methoden ließen sich einige mögliche Beiträge der Extraktstoffe im Hinblick auf die Dauerhaftigkeit der untersuchten Holzarten ableiten. In Massaranduba (*Manilkara bidentata*) konnten Ursolsäure, Hederagenin und Quiallajasäure und in Piquia (*Caryocar villosum*) Oleanolsäure und Hederagenin mit Hilfe der Zerschäumungsanalyse isoliert und anschließend identifiziert werden. Diese Verbindungen liegen als Saponine im Holz vor. Saponine sind häufig bekannt für ihre Bioaktivität und leisten somit einen möglichen Beitrag zu der erhöhten Dauerhaftigkeit beider Holzarten. In den meisten Aktivitätszonen der Holzarten Muiracatiara (*Astronium graveolens)*, Merbau (*Intsia* spp.), Bangkirai (*Shorea laevis)* und Itauba (*Mezilaurus itauba*) konnten etliche bereits bekannte fungizide Verbindungen wie zum Beispiel Gallussäure, Catechin und Resveratrol gefunden werden. Dies kann als ”Proof of Principle” für die Funktionalität für die Methode gewertet werden. Zusätzlich konnten die Isoquinolin-Alkaloide Bicucullin und Norbicucullin identifiziert werden, welche aktuell noch nicht im Holz von *Mezilaurus itauba* beschrieben sind.

Bei beiden Methoden handelt es sich um einfach anzuwendende Analysenmethoden, welche es ermöglichen, auch aus komplexen Mischungen Verbindungen zu isolieren und abzutrennen. Des Weiteren erlaubt die Hyphenagar-Bioautographie Einblicke in das jeweilige bioaktive Verhalten der entsprechenden Verbindungen. Weitere Untersuchungen sollten zum einen die Optimierung des Trennerfolges als auch einen größeren präparativen Maßstab zum Ziel haben. Eine tiefergehende Analytik zur Identifizierung der für die Dauerhaftigkeit verantwortlichen Verbindungen könnte so erreicht werden.

Preface

The thesis begins with an introduction (1) into the topic followed by the aims and objectives (2). In chapter 3 the state of the art is presented, starting with a deeper description of the extractives-classes and a portrayal of the wood species, which were examined in this thesis. It covers an overview over common separation techniques and delivers insights to the foam fractionation, which is one of the major topics in this thesis. Furthermore, the prevalent ways of identifying bioactivity are introduced, providing the main knowledge on which the development of the hyphae-agar overlay method is based. A brief description of the fungi, which were used in the presented study as well as a short definition of the term durability are included. The relation between wood extractives and decay-prevention complements this chapter to outline the recent scientific work.

Thereafter follows the materials and methods in chapter 5 and results and discussions in chapter 6. Both chapters are mainly a rendering of the two publications this thesis is built upon, put into the overall context of this thesis. Finally, in chapter 7 the conclusions and future prospectives are considered.

Publications on which this thesis is based

Publication 1: Wanschura R, Windeisen E, Richter K (2019) Application of Foam Fractionation to Wood. Wood Science and Technology, March 2019, Volume 53, Issue 2, pp 349–371

Publication 2: Wanschura R, Baumgartner M, Linder CU, Windeisen E, Benz JP, Richter K (2020) Direct bioautography for the screening of selected tropical wood extracts against basidiomycetes. Holzforschung, January 2020, Volume 74, Issue 8, pp 733–743

Other publications

Heigenmoser A, Fuchs R, Windeisen E, Wegener G (2012) Characterization of different wood samples using a new combined method of evolved gas analysis and pyrolysis-gas chromatography/mass spectrometry. Wood Science and Technology, 46, 637-642

Conference Proceedings

Wanschura R, Windeisen-Holzhauser E, Richter K (2017) Establishing a screening method for bioactive extractives of wood species against basidiomycetes. In Proceedings of IUFRO2017 Division 5 Conference, prof 402

Wanschura R, Windeisen-Holzhauser E, Richter K (2016) Screening of bioactive extracts for selected tropical hardwood species and identification of key substances. In: Proceedings of 14th European Workshop on Lignocellulosics and Pulp, 433-43

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Wanschura R, Windeisen E, Richter K (2014) Analysis of extractives of tropical hardwoods and benefits for the surface treatment. In: Proceedings of IAWS and Hardwood Conference, 71-72

Fuchs R, Windeisen E, Richter K (2012) Extractives of some commercially traded tropical timber. In: Proceedings of 12th European Workshop on Lignocellulosics and Pulp, Edita Prima Oy/Finland, August 2012, S. 248-251

Fuchs R, Windeisen E, Richter K (2012) New aspects in the analysis of extractives of some commercially traded tropical timbers. In: Proceedings of COST FP0901 Meeting, Tulln/Österreich, März 2012. University of Natural Resources and Life Sciences, Vienna, S. 40

Abbreviations

BAN	Bangkirai
IPN	Ipé Noir
ITA	Itaúba
JAT	Jatoba
MAS	Massaranduba
MER	Merbau
MUI	Muiracatiara
PEQ	Pequia

BSTFA/TMCS	N,O-Bis(trimethylsilyl)trifluoroacetamide / chlorotrimethylsilane
DAD	Diode array detector
DMF	Dimethylformamide
dH_2O	Water, demineralised
ELSD	Evaporative light scattering detector
FIA-ESI-IT-MS-MS	Flow injection analysis- electrospray ionization-ion trap-tandem mass spectrometry
FTIR	Fourier-transform infrared spectroscopy
GABA	4-Aminobutanoic acid
GC/MS	Gas chromatography / mass spectrometry
HPLC	High performance liquid chromatography
HPTLC	High performance thin-layer chromatography
HPLC/PDA–ESI/MS	HPLC - photodiode-array and electrospray ionization mass spectrometry
LC-NMR	Liquid chromatography- nuclear magnetic resonance
MALDI-ToF-MS	Matrix-assisted laser desorption/ionization time-of- flight mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
NAD(P)H	Nicotinamidadenindinucleotidphosphat
NH_3	Ammonia
Rf	Retention factor

RI	Refraction index
SDS	Sodium dodecyl sulfate
TLC	Thin layer chromatography
2D-TLC	Thin layer chromatography with two-dimensional development
Tween20	Polyethylene glycol sorbitan monolaurate
UV-VIS	Ultraviolet–visible spectroscopy
PDA	Potato-dextrose agar

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1 Introduction

Wood is a versatile resource with great significance for the development of mankind: primarily as easy to handle raw material and energy source. From the early days onwards, wood has been used to make furniture, tools and construction elements. In addition, fibres, paper and other products (e.g., flavouring agents or xylitol as sugar substitute in chewing gums) are produced from wood. In individual cases, wood compounds even served as paradigm for bioactive substances, which lead to pharmaceutical products, e.g., medicaments like Aspirin® or Taxol. As a renewable resource, produced by the world's forests, wood is part of a natural cycle, meaning that under given conditions, it is decomposed into its basic elements, becoming part of the forest-ecosystem again. Forests are multifunctional ecosystems, they help mitigate climate change, which has become more and more of an issue for the world at large. Measures to reverse climate change contain reforestation and sustainable forestry, as trees and plants in general use carbon dioxide in their energy metabolism. Thus, more forest areas and more use of wood in long-lived products means more stocking of carbon, leading to less carbon dioxide in the atmosphere and mitigation of global warming.

Approximately 40 million square kilometres of the planet are covered by forests today and the number of tree species known to science in 2017 reached 60.065 [1] worldwide and about 1700 [2] species are traded commercially. Many of the traditional tropical timber species, known to our markets for decades, suffer from overexploitation, which resulted in a drop of acceptance from customers in the past.

Due to globalization, today, tropical wood species are traded worldwide to a rising extent [3, 4]. A change of thinking in society towards achieving a more sustainable development has led to changed social policy objectives promoting the use of natural resources. More efficiently monitored trade standards like the Forest Law Enforcement Governance and Trade (FLEGT) action plan including the European Timber Regulation (EUTR) combats illegal logging and ensures that the timber origins from certified forests to restore consumers' confidence [5, 6].

Quite a number of lesser-known tropical species reach the European market each year, called substitutes. This leads to an enormous variety of species with divers properties, posing challenges to traders, manufacturers and consumers. In most cases, only their mechanical properties and their basic chemical characteristics are known. Many of these species are appreciated due to their intense colouring and anticipated superior natural durability.

Nevertheless, particularly detailed knowledge on the chemical composition (extractive chemistry) and the related durability is often missing and is subject matter of many recent studies as the underlying biological principals are far from being fully understood.

Besides cellulose, hemicelluloses and lignin, extractives are the fourth large category of wood components accounting for a share of 0.5-10% the dry matter. Especially in many tropical wood species, shares are particularly high [7].

These components can be extracted from the woody biomass by organic solvents or water. The state of knowledge indicated that the extractives play an essential part for the resistance of the wood against attack and decomposition by microorganisms. However, the group of extractives is extremely divers and often builds complex chemical mixtures in individual species. To select and identify the biologically active compounds in the extractive cocktail of a species is subject of numerous studies [8–10] and much progress has been made in developing powerful methods in analytical chemistry leading to large amounts of analytical data. For example, sophisticated hyphenation techniques like FIA-ESI-IT-MS-MS and MALDI-ToF-MS for the characterization of gallotannins [11], HPLC/PDA–ESI/MS for the identification of ingredients in different species of eatable berries [12] or LC-NMR for analysis of different natural products [13] were recently presented. However, this does not necessarily give more insight of practical relevance.

Due to complex matrices of plant extracts, isolation and analysis of bioactive key substances is often time-consuming and requires usually highly developed laboratory equipment. In the scope of this thesis, two selected techniques should provide rapid and straightforward methods to isolate and evaluate bioactive compounds as well as possibly causative key substances against wood destroying fungi. An acceleration of the isolation and detection process should allow for an enhanced concentration of the analytical efforts on the causal components,

Initially, as a specific isolation method, a method called foam fractionation was chosen to isolate, separate, and accumulate, in particular, saponins. It is known that saponins, as surface-active compounds, form colloidal, soap-like solutions in water [14]. They thus are able to generate stable foams [15]. They consist of triterpenes, steroids or spirostanols as aglycone and one or more sugars. Saponins are widely distributed in the plant kingdom, for instance in several wood species, and many of them show biological activity [16]. However, as they are mostly present in highly diluted concentrations, they need to be accumulated to obtain a sufficient amount for chemical analysis or various further test methods. Foam fractionation is described as a separation method with no need for sophisticated equipment and which can accumulate

especially highly diluted substances [17]. Thus, an enrichment of specific components out of the wood extractives via foam fractionation could be a valuable approach.

Secondly, a bioassay method to evaluate bioactive compounds in wood extracts was applied. Despite sophisticated on-line HPLC coupled bioassays, also straightforward bioautography methods could offer valuable information for target-guided isolation of the active compounds [18]. However, conventional bioautography methods to test (wood) extractives are mostly designed for spore-producing fungi. In this study the basidiomycetes *T. versicolor* and *R. placenta* were used. These fungi are known to pose difficulties while producing spores under laboratory conditions although the production of basidiospores *in vitro* was improved over the last years [19, 20, 21–23]. Using a hyphae-seeded agar medium were the hyphae is mixed into molten agar [24] and applying it directly onto a developed TLC plate [25] could overcome those difficulties. Therefore, the respective extract is separated on the TCL plate and its bioactive compounds are located in specific areas of the plate. Presumably, the mixed-in and poured-over hyphae should not be able to grow in these areas. Combining the two techniques should provide a possibility to benefit from the advantages of a direct bioautography (rapid, easy to implement) while testing wood extractives against commonly used basidiomycetes, which do not readily sporulate. Especially for less well-known tropical wood species the method is posing an opportunity to better understand the increased durability by evaluating their bioactive components.

2 Objectives and research questions

The overall objective of this thesis is to develop methodological approaches for the isolation, separation and testing of potentially bioactive substances of several tropical hardwood species.

<u>Objective 1:</u> Establishing foam fractionation as a promising isolation method for saponins out of wood and water extracts thereof

Attendant research questions:

1.1. Is the foam fractionation principally applicable to wood samples? Does it make a difference if milled wood or previously isolated water extracts are used as input material for the process?

1.2. Are added tensides to foam non surface-active extracts effective to accumulate specific components?

1.3. Which influence have sizes of bubble devices and the usage of either N_2 or CO_2 as performing gases on the effectiveness of the method/ yield?

1.4. Are there differences between the spumates and the water extract regarding the extract components, their concentration and the extract amount?

1.5. Does a possible accumulation of specific substances directly out of the wood meal may open up a possibility for time and cost reduction?

<u>Objective 2:</u> Establishing a direct bioautography for hardly sporulating basidiomycetes using a hyphae agar in order to employ it as a target-guided fractionation method for wood extractives

Attendant research questions:

2.1. Is the hyphae agar applicable to the TLC plate?

2.2. Do the homogenized cultures recover fast enough and does the usage of viability stain MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] provide a good basis/ an improvement to evaluate fungal inhibition?

2.3. Is it possible to determine the antifungal activity of the extract fractions via 2D-TLC agar overlay method and can the inhibiting compounds be assigned to the bioactive zones?

2.4. Does the presented technique provide a rapid method to evaluate bioactive components in complex extract mixtures?

3 State of knowledge

3.1 Organic compounds of wood

Wood as an organic material is chemically heterogenic, whereby three main substance classes can be differentiated, which are building up the cell wall. Cellulose is the most abundant polysaccharide in wood and its decomposition is especially important with respect to the carbon cycle. As a linear polymer, it consists of glucose monomers, which are linked by β-1, 4-glycosidic bonds. More precisely, the repeating unit of the chain is the dimer cellobiose, which is synthesized in the cell wall and accounts for a percentage of 40 % to 50 % of cellulose in wood. Hemicelluloses, also denominated as polyoses, are polymeric sugars and can be distinguished from the cellulose as they consist of many different sugar-monomers, shorter molecular chains and branching of the chain molecules. In hardwood, polyoses count for 25 – 30 %, and a little less in softwood for 20 – 25 % to the wood biomass. Lignin is the second most abundant natural polymeric carbon source on earth after cellulose. It is a three-dimensional network of phenyl propane units, with various types of linkages, which is still a matter of models [26]. Equal shares of coniferyl- and sinapyl alcohol compose hardwood, while softwood is constituted almost purely of coniferyl alcohol monomers. Lignin contributes to the cell wall mass with 20-40%.

The macromolecular components cellulose, hemicelluloses which constitute for the majority of the wood cell wall are supplemented by wood-specific accessory organic substances, which are low-molecular in mass and mostly extractable up to specific molecular masses by either water or organic solvents. The so-called extractives can be divided into primary and secondary extractives.

Primary extractives are part of the metabolic process of the plant and serve as nutrients and storage substances (e.g., lipids, sugars or amino acids). They are deposited mainly in the sapwood, which shows therefore never a natural durability.

The secondary wood extractives are located in the heartwood [27] and are biosynthesised in the transition zone between the sap- and the heartwood. They are mainly stored in the wood ray cells and in the axial parenchyma of the heartwood and can be found as coatings on the lumen-side of the cell walls (S3) [28–31]. The concentration of secondary extractives varies between individual trees of a species, and per tree across the transversal section as well as in stem-height [32] and ranges from 0.5-10 % for species from the northern hemisphere up to 15-20 % (e.g. in *Handroanthus spp*. or *Intsia spp*.) for some species growing in tropical areas [7, 33]. Even up

to 46 % were reported for Quebracho (*Schinopsis balansae)* whereby it should be noted that this measurement includes extractives obtained with a 1%- NaOH-solution [34]. Secondary extractives are assigned to have influence on the natural durability of wood [35–37]. They are often described to be bioactive against microorganisms due to their toxic nature or the ability to inhibit fungal enzymes [38]. However, the exact relationship between the substances and the respective fungus is in many cases still to revealed [7].
The many different substance classes amongst the group of extractives are described below. Examples for the respective substance classes are given in Table 1.

3.1.1 Starch and fats and waxes

Starch is a form to store carbohydrates. It consists of amylose and amylopectin. Fats and waxes are lipids that are mainly stored as triglycerides. More than 20 fatty acids like lignoceric- or cerotic acid are identified in different wood species [7]. Some of them are known to be highly effective against wood degrading fungi [39]. Due to the hydrophobicity of the compounds, a high contend of fats and waxes enhances the natural durability of the wood by reducing the liquid water penetration into the bulk wood, by retarding the moisture diffusion into the cell wall and thus making the entry of microorganisms more difficult. Furthermore it influences the equilibrium moisture content and the swelling and shrinkage of the wood [7, 40]. As lipids are a common nutrient for many organisms, wood-degrading fungi can probably metabolize many lipids in wood as well. However, they are not the main substrate [41, 42].

3.1.2 Alkaloids

Alkaloids are defined as nitrogen containing products of the secondary metabolism and are a very diverse group. Their nitrogen generally origins from amino acids, but can also be a product of transamination reactions. The nitrogen usually reacts as an alkaline. With over 15.000 described compounds [43], they are the second biggest group (after the phenolic substances) of secondary plant substances. Many alkaloids are found in molluscs, fishes, amphibians, ferns and microorganisms. However, the probably biggest source are angiosperms [43]. As they are usually toxic to vertebrates, they show a good effectiveness against insects. The most prominent alkaloid is probably caffeine, but alkaloids also take part in antiviral, antibacterial and antifungal mechanisms in many species [44]. A further example is quinine which is used as the bitter principle in tonic water, due to the bitter taste of a majority of alkaloids [45].

3.1.3 Flavonoids, Tannins

Flavonoids are composed of aromatic rings and can be separated into chalcones, flavan-derivates, aurones and isoflavones [43]. A consequent definition and classification does not always exist [46]. As very common extractives, flavonoids can be found in all higher plants as pigments and especially frequent in photosynthetically active cells. They were used as natural dyes in earlier days and are known to have many beneficial health effects, including antioxidative, antiallergic, anti-inflammatory, antidiabetic, gastroprotective and antiviral activities [47]. Another key factor concerning their biological potential is their ability to interact with cellular proteins. Polyphenols can function as activators or inhibitors of numerous cellular enzymes and therefore have potential for applications in nutraceuticals, nutracosmetics and skin care products [48]. They are known to influence the formation of the actin-chains that are essential for the speed of many cellular processes, were they have an either activating (epigallocatechin) or inhibiting (quercetin, 14, Table 7) effect [49]. With the ability to scavenge free radicals they can block enzymatic processes and stop fungal growth [48]. Additionally, they provide a metal chelation potential. A prominent plant metabolite having a fungicidal effect is catechin (15, Table 7) [32], which can be isolated from tropical wood species like e.g. Bangkirai *(Shorea laevis)* as well as from European wood species,.

Tannins are polyhydroxyphenols and are mostly recognized for the bitter taste of tea [50] or wine [51]. They are divided into hydrolysable tannins (gallotannins) and condensed tannins (catechintannins).

A simple example for a hydrolysable tannin is a 1-galloyl-ß-D-glucose. Gallotannins are known to have antimicrobial, anti-inflammatory as well as anti-tumoral effects [43]. In higher concentrations, they are also known to restrain fungal growth due to their complexing abilities of enzymes [52]. Ellagitannins are also hydrolysable tannins. The most abundant ellagitannins in oak wood are castalagin (9, Table 1) and vescalagin, which represent between 40% and 60% of the total extract content [53].

Condensed tannins represent a big group amongst extractives with either catechin/gallocatechin or epicatechin/epigallocatechin as basic components, having characteristic molar masses between 500 and 20000 amu [43]. They were already extracted from oak wood in Europe in ancient times and used for tanning hides into leather [54]. Today, a main source of condensed tannins is the bark of *Acacia mearnsii* and *Schinopsis balansae* wood. They are still used in leather production, but are also utilized as a component in adhesives. Furthermore, the use of

tannins is expanding into nutritional and pharmaceutical areas [55]. They can form complexes with secreted fungal enzymes and therefore protect the biomass from degradation. They are applied medically in the treatment of tonsillitis, pharyngitis and diarrhoea due to their styptic effect (contracting tissues or blood vessels) and are an antidote for glycosidic, metallic and alkaloidal poisons, as they make these substances insoluble under physiological conditions [52]. Their antitumor potential was also recently examined [56].

3.1.4 Lactones

Lactones are cyclic esters derived from lactic acid in which the functional group of the ester has become part of a ring structure [57]. Many simple examples occur in essential oils. The antibacterial effects of lactones have been well-known since macrolide antibiotics were found [58] and are traditionally thought to play an important role as mucolytics [57]. Lactones like bergapten and capralactam are known to be bioactive against certain insects and to have a very specific ability to inhibit the growth of some brown rot fungi [32]. Another prominent representative, coumarin, is e.g. responsible for a vanilla- and caramel-like odour [59] as well as it is responsible for the smell of new-mown hay [60] .

3.1.5 Phenols, Lignans, Stilbenes

Phenols are aromatic compounds consisting of a phenyl group and one or several hydroxyl group(s) [61] which as an “extractives-class” cannot be assigned to flavonoids, lignans or stilbenes. As dyes and tanning-agents, they are widely distributed in nature. Because of the occurrence of structural different phenols in all analysed plant extracts, it´s suggested that each plant has its own individual “phenol pattern” [16]. The solubility of the phenols depends strongly on the particular substituents (ranging from polar to non-polar) and due to their big amount and diversity, no generalized predictions on chemical and physical behaviour can be made. The antioxidant activity of phenolics is mainly due to their redox properties. This allows them to act as reducing agents, hydrogen donators and singlet oxygen quenchers [62]. One representative is vanillin (4-Hydroxy-3-methoxybenzaldehyd) (5, Table 1), which is gained as a by-product in the pulping process (besides a biotechnological production) and is the most produced aroma in the world [63]. Phenols are also known to be fungicidal.

Lignans are formed by a condensation of coniferylalcohol and sinapylalcohol and it is assumed that the biosynthesis pathway is similar to the one of lignin. They are crystalline compounds of low volatility [43]. A diet rich in lignans has been associated with a decrease of the risk of

cardiovascular diseases but also with an increase of the risk of developing breast cancer, due to their interaction with estrogenic receptors [64]. The lignan pinoresinol (6, Table 1) is known to have a fungicidal activity and can be found amongst others in *Pinus* species [36].

Stilbenes occur naturally in various families of plants. They consist of two benzene units joined with an alkene linker. In plants, stilbenes are produced by stilbene synthases, enzymes specifically catalysing the biosynthesis of resveratrol (7, Table 1), the basis of most stilbenes [65]. Grapes and related products are considered to be the most important dietary sources of these substances. Trans-resveratrol is postulated to function as a potential signalling pathway modulator and is demonstrated to affect a multitude of signal transduction pathways associated with tumorigenesis [66]. Some stilbenes show fairly well fungicidal activity while tested on agar plates. For example, extracts from *Pinus pseudostrobus* containing pinosylvin, pinosylvinmonomethylether and pinoysylvindimehtylether were found to have a distinct fungicidal effect against *Coniophera putenana, Chaetomium globosum* and *Trametes versicolor* [37] which doesn´t correspond to tests performed on wood [32]. These findings have not been completely understood yet. It´s suggested that the stilbenes are combined onto other wooden components, causing the suppression of the bioactivity [32].

3.1.6 Quinones

Quinones represent a class of quinoid compounds named after the parent system from which they are derived e.g. benzoquinones from benzene, naphthoquinones from naphthalene and anthraquinones from anthracene [67]. Due to their wide occurrence in nature along with their involvement in a number of essential biological and chemical processes, quinones correspond to a well-studied class of compounds [67]. The electrophilic nature of quinones is cited as the most likely explanation for their toxicity [68]. Thus, they have a very strong effect on biological systems and are able to trigger toxic responses in beings. Furthermore, in photosynthesis, plastoquinone acts as an electron acceptor in the light-dependent part of the reaction and is therefore essential to keep the photosynthesis running [67]. Tectoquinone, known as a biological active substance contributes to wood durability e.g., in particular in teak wood *(Tectona grandis)*. However, quinones show mainly insecticidal activity and their bioactivity against fungi is occasional [32].

3.1.7 Saponins

Saponins, as surface-active compounds, form colloidal, soap-like solutions [14] in water. Thus, they are able to generate stable foams [15]. Structural requirements for that property are the existence of a hydrophobic and a hydrophilic part of the respective compound. Therefore, they consist of triterpenes, steroids or spirostanols as aglycone (hydrophobic) and one or more sugars (hydrophilic). Saponins are widely distributed in the plant kingdom, for instance in several wood species e.g. *Quillaja spp* [69] or *Manilkara zapota* [7], and are often present in a complicated mixture of numerous and hardly separable compounds [7]. Many of them show biological activity and can be present in all plant organs in higher concentrations from 0.1 up to 30 % [43], especially in roots, barks and seeds [70]. Besides their role in defence mechanisms of plants against attack from microorganisms, fungi, insects and molluscs, saponins are of growing interest for drug research and provide valuable pharmacological properties [71]. They are part of medicine plants contributing to many traditional medicines all over the world having anti-inflammatory [72] and antibacterial effects [73–75]. The effective parts of ginseng (*Parax ginseng*) are mostly saponins though ginseng is one of the most important oriental medical plants and is distributed worldwide [76]. Another prominent example is hederin, which is obtained from common ivy *(Hedera helix)* and is also commercially used [77]. In wood, selected saponins are known to increase natural durability [32]. They are mainly able to inhibit insects and are mollucicidal. Their mode of action towards fungi is proposed to base on the formation of complexes with membrane sterols resulting in pore formation and loss of membrane integrity [70].

3.1.8 Steroids

Steroids play a major role in physiology as constituents of membranes and as hormones. Naturally, occurring steroids are considered to be derivatives of a hydrocarbon, which contains three cyclohexane rings and one cyclopentane ring. They have various substituents (alkyl, hydroxyl, aldehyde, ketone, carboxylic acid) attached to the steroid nucleus [78]. Some of them are cardio active like cardenolide and bufadienolide [43]. In many wood species like silver fir *(Abies alba)* or Scots pine *(Pinus sylvestris)* sterines were identified [79].

3.1.9 Terpenes and terpenoids

Terpenes and terpenoids are a structurally diverse and widely distributed family containing over 25.000 well-defined compounds isolated from all biological kingdoms [80, 81]. This diversity

can be attributed to enzymes known as terpene synthases, which convert acyclic prenyl-diphosphates and squalene into acyclic and cyclic forms. Additionally, some terpene synthases produce multiple products from a single substrate [82]. The nomenclature of terpenes is based on the number of isoprene units that they contain. Accordingly, these compounds are classified as monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), and so on [81, 83]. Based on the number of cyclic rings in the structure, terpenes can also be classified as acyclic, monocyclic, and bicyclic [82]. They are pure hydrocarbons, whereas terpenoids or isoprenoids are derived from isoprene but contain additional functional groups. Monoterpenes and sesquiterpenes are often very volatile and belong to the major components of essential oils [82, 84]. They are of special interest as they contribute to the VOC (volatile organic compounds) emissions [84] from wood in indoor air which can influence human well-being and health [85].

Monoterpenes are endowed with various pharmacological properties including antifungal, antibacterial, antioxidant and anticancer activities. Their antimicrobial properties are exploited for numerous uses, including medicinal and food applications [86, 87]. Strong antibacterial activity against bacteria has been observed for e.g. (–)-linalool [88]. It´s mode of action is based on disruption of the lipid membrane structure in these microorganisms, increasing the permeability of the cell membranes [89]. Citral was found to exhibit strong fungicidal properties by being able to form a charge-transfer complex with an electron donor of fungal cells, resulting in fungal death [87].

Sesquiterpenes are, with more than 11.000 compounds the most extensive group among the terpenes and are widely distributed in the *Asteraceae* family, where they can be found almost ubiquitously. They can be divided according to their functional groups: sesquiterpene hydrocarbons, sesquiterpene alcohols and sesquiterpene lactones [43]. Sesquiterpenes and, in particular, sesquiterpene lactones make up a group that show a wide variety of biological properties including plant growth regulating, antibacterial, cytotoxic and mollucicidal functions. Therefore, they play an important role on protection of plants against pathogens, herbivorous insects, mammals and function as allopathic agents [90].

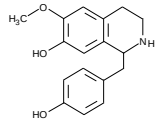
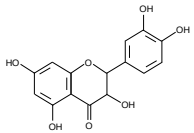
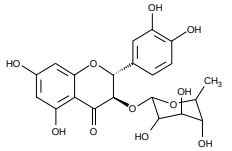
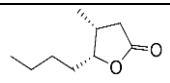
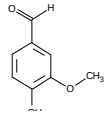
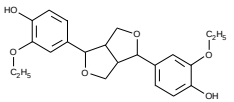
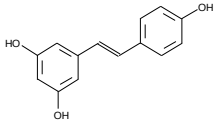
Resin acids are diterpenes and the major component of natural resins, which are mainly found in conifers. They consist of two structural stereo chemical groups: the abietanes and the pimaranes. In seeds from *Picea sp.* abietic acid dominates while pimaric acid is typical for *Abies sp.* seeds. Abietic acid is well known for its fungicidal properties. Furthermore, resin acids can cause red blood cell hemolysis, hepatocellular damage, affect epithel cells geno-toxicity and have anti-inflammatory properties. Because of their toxicity, they are used as parent compounds for pharmaceutical substances with a broad bioactivity spectrum [91].

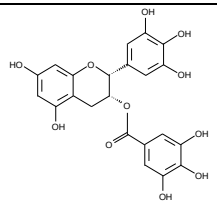
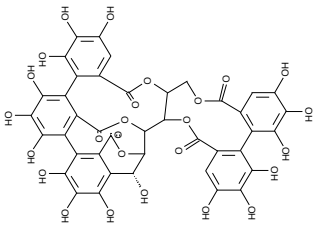
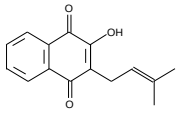
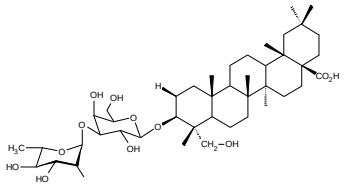
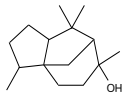
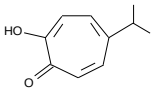
Triterpenes are complex molecules that are, for the most part, beyond the reach of chemical synthesis. Simple triterpenes are components of surface waxes, whereas complex glycosylated triterpenes (saponins) provide protection against pathogens and pests. Simple and conjugated triterpenes have a wide range of applications in the food, health, and industrial biotechnology sectors [92]. There is an ongoing growing interest in the last few decades in their potential biological and pharmacological activities, described in several reviews on their anti-inflammatory, antiviral, antitumor, anti-HIV and insecticidal activities and for treatment of metabolic and vascular diseases [93–95]. For many triterpenes fungicidal characteristics are documented as well [43], e.g. ß-amyrin has been found to exhibit antifungal and antimicrobial activity against various microorganisms [96].

3.1.10 Tropolones

Tropolones are a very small group among extractives. Their basic structure is an aromatic ring of seven carbon atoms with a hydroxyl function and a carbonyl function [97]. They can be divided into three groups: isopropyltropolones, hydroxytropolonecarboxylic acids and the colchicine group. Thujaplicin (13, Table 1) is a well-known example of a tropolone with a very potent antimitotic and antifungal bioactivity, which can be obtained from e.g. *Thuja plicata or Thujopsis dolobra* [60, 98]. Because of its universal effect, it is often used as a control agent in bioactivity tests. An antimitotic effect has also been proven for colchicine. In medicine, tropolones have been applied against several diseases, such as tuberculous fistulae, tuberculous pyothorax or pulmonary abscesses. The effect probably attributes to the inhibitory effect of tropolones on the oxidation of inositol [97].

Table 1: Overview over the extractive groups found in wood, a respective example, some well know representatives and species known to be a source. Numbers in brackets are included to create a reference from the text.

Group	example	compounds	Wood species or other crops
Alkaloids	 (1)	coclaurine (1) liriodendrine	*Mezilaurus synandra* [99] *Michelia formosana* [100, 01]
Flavonoides	 (2)	catechin pinocembrin taxifolin (2)	*Shorea laevis* [32] *Pinus sylvestris* [102] *Pseudotsuga taxifolia* [32]
Flavanone-glycoside	 (3)	neoastilbin (3), astilbin, isoastilbin glycosides of naringin gylcosides of isorhamnetin	*Dicorynia guianensis* [9] *Betula pubescence* [100] *Eucalyptus cumaldulensis* [103]
Lactones	 (4)	*trans*-oak lactone (4)	*Quercus spp*. [104]
Phenols	 (5)	vanillin (5)	*Vanilla planifolia* [63] and derived from lignin metabolism in coniferous trees [7]
Lignans	 (6)	pinoresinol (6) dimethylpinoresinol, lariciresinol, matairesinol,	Pinus spp. [36] *Picea abies, Pinus sylvestris* [102, 105]
Stilbenes	 (7)	resveratrol (7) pinosylvin, pinosylvinmonomethylether, pinosylvindimethyether,	*Picea abies* [54] *Pinus sylvestris* [102] *Picea abies* [106]

Tannins condensed	 (8)	epigallocatechingallate (8)	Astronium *balansae* [107]
Tannins hydrolyzable	 (9)	castalagin (9), vescalagin, roburin (ellagitannins) 1-galloyl-ß-D-glucose (gallotannin)	*Castanea sativa* [108] *Quercus spp.* [53] *Acer rubrum*
Quinones	 (10)	lapachol (10) juglone, tectoquinone, tectol, anthraquinone	*Tabebuia avellanedae* [100] *Juglans regia* [83] *Tectona grandis* [197]
Saponins	 (11)	caryocaroside II-22 (11) quiallic acid saponin	*Caryocar villosum* [109] *Quillaja saponaria* [69]
Terpenes/ Terpenoides	 (12)	cadinol, cedrol (12), muurolol betulinol dehydroabietic acid, borneol, terpineol	*Cunninghamia konishii* [110] *Betula pendula* [54] *Pinus sylvestris* [102]
Tropolones	 (13)	thujaplicin (13)	*Thuja plicata* [98]

3.2 Wood species

Many tropical wood species are known to have outstanding durability properties. In this chapter, the species chosen for this thesis are described more in detail. They are all traded timber species and are available commercially on the European market. As the scope of the research focuses on the extractives of the wood, the utilization of the timber is only addressed briefly. Most of the already identified compounds mentioned in the following chapter do not refer to the wood itself but mostly to the bark of the species. For some species, still only little of the wood extractives is known today.

3.2.1 *Hymenea coubaril* (Jatoba)

Figure 1: Macroscopic photo (1:1) of a longitudinal section of Hymenea *coubaril*

Jatoba (*Hymenaea courbaril* L.—family Fabaceae), which is also known as Brazilian Cherry, occurs in South and Central America and is one of the world's most demanded tropical hardwoods. This is due to its exceptional hardness and appealing colour (Figure 1). It is used for flooring, decking, furniture and stair components. Its colour can range from orange and red to darker shades of purple and brown. [111, 112] The trees exude a resin named copal, which is used to make varnish. The bark and leaves contain terpenes, tannins, and glycosides, which permits its usage as a fungicide and the bark is also used in a syrup recommended for the treatment of bronchitis and an infusion of the bark is used for stomach troubles [113]. Known bark extractives are several diterpenes like different labdan-acids [113], various clerodane-type diterpenes [114] and halimadien-acids [8]. Furthermore, procyanidin oligomers with different degrees of polymerization (up to nonamers) were purified [115], (−)-fisetinidol, (+)-*trans*-taxifolin (2, Table 1) [116], and fustin are also found in the bark [117].

3.2.2 *Manilkara bidentata* (Massaranduba)

Manilkara bidentata is a member of the family of Sapotaceae, and native to the Caribbean, as well as to Central and South America. It is also known by the tradenames Bulletwood, Balata or Massaranduba. The wood is often used for load bearing construction, decking, flooring,

boats, bent parts or turned objects, has a high density and is difficult to glue due to its high content of lipids [118]. The heartwood is of reddish-brown colour (Figure 2), which tends to darken and its yellow sapwood is clearly differentiated from the heartwood. Allergic reactions to *Manilkara bidentata* wood or its dust can cause skin irritation [119]. It is used as replacement for tropical wood species like Bangkirai or Bongossi [118]. *Manilkara bidentata* is known to be rich in triterpenes, though it has no characteristic odour. Examples are oleanolic acid (34), bassic acid and β-amyrin [120]. The two former compounds have antibacterial bioactivity [121] and oleanolic acid also seems to inhibit cancer cells [122]. β-Amyrin has been shown to be fungicidal [123]. Thus, the wood is very durable against microorganisms and has a high resistance to most insects. Furthermore, balataresinol, a triterpene, the isoprenoid cyclolaudenol, and α-spinosterol, a phytosterol, were found in *Manilkara bidentata* [120]. Moreover, cycloartenol, 24-methyl-enecycloartanol, lupeol and squalene could be detected in extracts of plant latex from *Manilkara bidentata* [124].

Figure 2: Macroscopic photo (1:1) of a longitudinal section of *Manilkara bidentata*

3.2.3 *Tabebuia spp. /Handroanthus spp.* (Ipé noir)

Handroanthus spp. is better known by its common name Ipé and is a member of the family of the *Bignoniaceae* [125]. Due to gene studies, the species formerly known as *Tabebuia spp.* with a high content of lapachol (10, Table 1) and with a heavy, red-brown to black-brown wood (Figure 3) were reorganized to the genus of *Handroanthus*, whereas species with hardly any to no lapachol in their wood stayed in the genus of *Tabebuia* [126]. The wood dust can cause allergic reactions [119]. An extract of *Handroanthus spp.* has been shown to be a very active phytotherapeutic agent against skin lesions [127]. The wood contains several naphtoquinones, which are discussed to show antibiotic activity against bacteria and resistance against antibiotics. α-lapachone, a natural

Figure 3: Macroscopic photo (1:1) of a longitudinal section of *Handroanthus spp.* (Ipé noir)

product known to have antivascular activity in tumours, was also found in extracts of *Handroanthus spp.* [128, 129].

3.2.4 Mezilaurus itauba (Itauba)

Figure 4: Macroscopic photo (1:1) of a longitudinal section of *Mezilaurus itauba*

Also known by the common name Itauba, *Mezilaurus itauba* is a hard and durable tropical wood species. It is part of the family of the *Lauraceae* and grows in Latin America [112]. The heartwood of *Mezilaurus itauba* is yellowish to orange brown (Figure 4) and its sapwood is beige white and clearly differentiated from the heartwood [130]. *Mezilaurus itauba* is traded only in small quantities and is often used for boat building, bridge construction, exterior uses, flooring, furniture, etc. The wood contains silica, which affects its workability. With its hardness and very high natural resistance against decay and termites, *Mezilaurus itauba* is promoted to be a potential substitute of teak. *Mezilaurus itauba* is not very well known yet. However, some extractives have already been found: the wood contains several neolignans [131] and alkaloids such as oxaporphines, pavine or morphinandienones as well as gamma-lactones [132]. More recent works by Acosta et al. yielded several compounds, e.g. naphtalenes, the sesquiterpenes α-cadinol, cadinene and valencene [133].

3.2.5 Shorea laevis (Bangkirai)

Figure 5: Macroscopic photo (1:1) of a longitudinal section of *Shorea laevis*

Bangkirai is a member of the family *Dipterocarpaceae* and grows mainly in Borneo, Sumatra, Malaysia, Singapore and Myanmar and is also called Balau [134]. The wood has a high density and the heartwood is of yellowish-brown colour (Figure 5), which becomes dark brown over time.

It´s mainly used for construction works and high performance furniture [134]. *Shorea laevis* is

known to be of high durability against wood destroying microorganisms and contains catechin (15) [32], gallic acid (20), ellagic acid (22), dilactons from flavogallonic- (23) and valoneaic-acid (24) [135], hopeaphenol (25), a tetramer of resveratrol [136] and the stilbenes laevifonol (26) und laevifosid (27), (Table 7) [137].

3.2.6 *Intsia spp.* (Merbau)

Merbau is a trade name for the species *Intsia bijuga* und *Intsia palembanica*, and origins from south-east Asia and Madagascar and is also cultivated in managed forests [138]. It has a reddish-brown colour (Figure 6) and is mainly used for outside constructions but also for furniture [138]. Known extractives are vanillic acid (5), gallic acid (20) and various fatty acids as well as stigmasterol (19), (Table 7) [40].

Figure 6: Macroscopic photo (1:1) of a longitudinal section of *Intsia spp.*

3.2.7 Astronium graveolens (Muiracatiara)

Muiracatiara is the trade name for *Astronium* graveolens und *Astronium urundeuva* species, which belongs to the Anacardiaceae family. Other tradenames are tigerwood, goncalo alves or urunday [112]. The name tigerwood is referring to the remarkable pronounced red and black pattern of the wood (Figure 7). Both species mainly occur in South America [112]. It is known for its resistance against the attack of fungi or insects and is mainly used in ground contact applications like railway sleepers or fence posts, but also for furniture and sliced veneer [112] due to the decorative colour. In both species gallic- (20) and ellagic acid (22, Table 7) as well as some of their derivates and glycosides and the flavonoid quercetin (14, Table 1) can be found [116].

Figure 7: Macroscopic photo (1:1) of a longitudinal section of *Astronium graveolens*

3.2.8 *Caryocar villosum* (Pequia)

Pequia (*Caryocar villosum)* is spread all over the Amazonian [112] and belongs to the family of Caryocaraceae. It´s wood has a bright yellow to brown-greyish colour (Figure 88) and sap- and heartwood are not separated sharply. The latter is mainly used for indoor applications like floors and stairs as well as for flooring for bridges and as construction wood. The wood dust is known to cause dermatitis [112] and especially in its bark different saponins like e.g. hederagenin (33, Table 7) and more triterpenoid saponins were already identified [109].

Figure 8: Macroscopic photo (1:1) of a longitudinal section *of Caryocar villosum*

3.3 Methods for evaluating antimicrobial activity

3.3.1 Separating natural extracts

Plant extracts usually occur as a combination of various types of compounds with different polarities and their separation remains a big challenge for identification and characterization of bioactive compounds. Classical isolation methods, including solvent or supercritical CO_2 extraction, precipitation, crystallization, (steam-) distillation and salting-out are still commonly used. However, more “modern” techniques such as thin layer chromatography (TLC), column chromatography, high performance liquid chromatography (HPLC), flash chromatography and ultrafiltration, where the molecules are separated based on their size, shape and charge play an important role in the separation of phytochemicals today [139]. On the scope of this thesis, mainly TLC and HPLC were chosen from these techniques to separate the obtained wood extractives. As their principles are commonly known, only a brief description should be given. A lesser-known technique, the foam fractionation, which stands at the core of this thesis is described further in the oncoming paragraphs.

In context of classical chromatographic techniques like TLC and HPLC the extent or degree of separation is mostly determined by the choice of the stationary phase and the mobile phase by utilizing the fact that certain compounds have different migration rates under given particular phases.

TLC is a versatile, simple and inexpensive technique that permits the analysis of complex samples with minimal sample pre-treatment steps. The procedure gives a quick answer to the number of components contained in a mixture. It can also be used to identify a compound by comparing the retention factor (Rf) to the one of known compounds. Compounds with a large set of polarities can be analysed and many samples can be run on a single TLC plate simultaneously. The resolution can be managed either in only one direction or as a 2D-TLC [140] were the plate is turned for 90° and is developed for a second time with an eluent of a different polarity. Favre-Godal et al. (2013) presented a comprehensive review concerning TLC techniques with special emphasis on assessing antifungal activity [141]. They mention approximately 100 antifungal compounds of natural origin.

In addition to TLC, HPLC is often used to profile crude extracts with high resolution [12, 142]. It has been used to analyse a broad variety of sample matrixes [143] and affords the possibility to be hyphenated with different spectroscopic detectors [141]. It is ideally suited for rapidly processing multi component samples either on an analytical as well as on a preparative scale. Modern HPLC mainly uses non-polar solid phases and a polar liquid phase, e.g., a mixture of water and another solvent. It offers the possibility of micro fractionation by connecting the instrument to a fraction collector. The micro fractionation can be realized in tubes or microplates and in some cases, the biological studies can be directly performed after solvent removal [141].

3.3.2 Foam Fractionation

Foam fractionation is described as a separation method with no need for sophisticated equipment which can accumulate especially highly diluted substances [17]. It is part of the Adsorptive Bubble Separation Methods and is differentiated from foam flotation methods, were a separation of dispersed parts takes place and particulate material is removed by foaming [144, 145]. The compounds are enriched by introducing gas to water or other solvents. These methods are subdivided into methods using the foamability of certain solutions and methods providing separations without foams (Figure9). In non-foaming bubble separations, bubbles raise through a solution with entraining particles. They are therefore enriched either in an upper phase (bubble fractionation) or in a different overlying solvent (solvent sublation) [144] whereas in foam fractionation a separation of dissolved compounds can be achieved [146].

Forming of foam

Basically two kinds of foam can be distinguished: spherical and polyhedral foam [147]. Spherical foams are unstable, wet and only slightly coordinated. Polyhedral foams have a longer lifetime, become dryer through drainage whilst rising and persist long enough to establish lamellas in-between each other with almost uniform thickness.

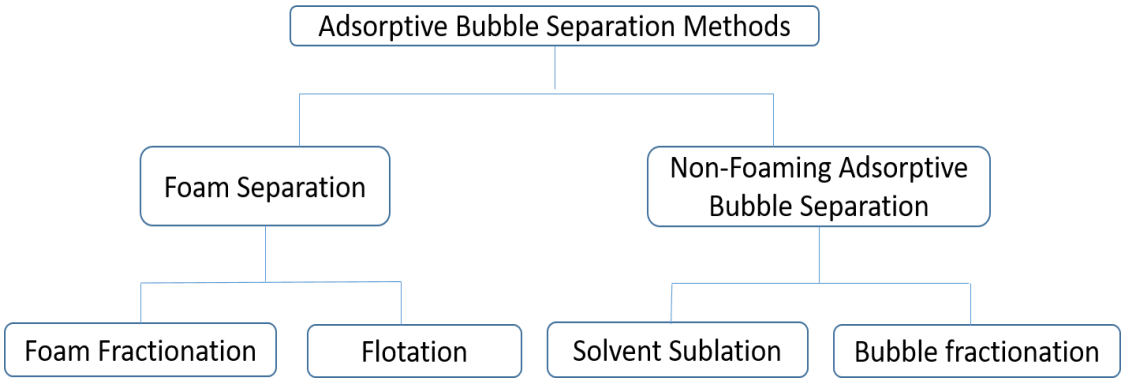


Figure 9: Schematic representation of Adsorptive Bubble Separation methods [230]

Thus, the formed bubbles create an emerging foam column above the liquid level. These foams are generated by gas injected into, and flowing through a solution. Both foams can persist side by side. Forming polyhedral foam is based on the tendency of surface active molecules (surfactants, tensides) (Figure 0) present in highly diluted aqueous solutions to selectively adsorb at the gas-liquid interface of foams [148]. These surface active molecules have amphiphilic characteristics (hydrophilic and hydrophobic) [149] and promote foaming due to the decrease of the surface tension at the interface [148].

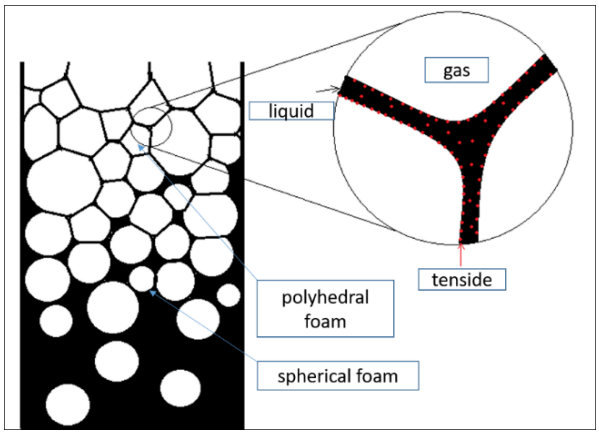


Figure 10: Foam formation; modified based on [156]

The term drainage is referring to the effluent flow of the initial solution that was entrained with the bubbles back into the flask, causing the foam to become dyer while rising. This is caused (1) by gravity and (2) by a suction effect from the plateau borders (in which a lower pressure

exists than in the bubble) [150]. The plateau borders are the channels formed in the capillary-liquid film in-between the bubbles. Consequently, the bubbles collapse or fuse (coalescence). Coalescence leads to the intended accumulation [150] of the adsorbed molecules.

Description of the procedure

For the enrichment of surface-active substances via this method, inert gases (like N_2 or CO_2) are introduced into dispersed (ground) organic solids in water, substances dissolved in water (like extractives from plants or waste waters) or plant saps. Surface-active molecules contain a hydrophobic and a hydrophilic part and in case of dispersed organic solids, they first are dissolved in the surrounding water and then become adsorbed at the surface of the gas bubbles (Figure 81). Hereby the hydrophobic part of the molecule is located in the gas phase of the bubble [147] because it tends to avoid the aqueous surrounding. Together with the emerging bubbles, the adsorbed molecules leave the initial solution and can therefore be enriched in the formed foam, the so-called spumate [151]. It is distinguished between the initial solution (containing the organic matter in solution), the spumate (liquid obtained after disintegration of the formed foam) [144] and the residue (solution in the flask after performing the foaming) [152].

Influencing factors

Some important factors have been identified in literature to influence the method efficiency, like the elementary presence of a sufficient concentration of surface-active substances. Low concentrations are proven to be more suitable to achieve effective separations [153, 154]. The forming of micelles in higher concentrations, which has a negative effect, is found to be the reason therefore [155].

PH-value is reported to be essential to remove or separate molecules like proteins from diluted aqueous solutions as it influences the sign and magnitude of the charge of these molecules [156–158]. Adjusting the pH-value to their specific isoelectric point, their solubility can be lowered to a minimum. It is proven, that the enrichment for proteins is higher at their corresponding isoelectric point [153, 154].

Length and diameter of the used column are found to be important parameters for the efficiency of the separation [159–161]. A longer column improves the drainage and the dwell time in the column [162]. However, the length of the column is the limiting factor due to the disintegration

of the foam, which means that at a specific height of the foam in the column, it gets labile and degrades before it can leave the column [150, 161].

A higher surface area should achieve higher recovery rates. Therefore, smaller bubbles, obtained by using frites with smaller pores should increase the amount of adsorbed substances [145]. As higher gas-flow rates also provide more bubbles it should also lead to a higher recovery rate. However, it was found that a low flow rate is in general beneficial for a higher enrichment. At low flow rates, less initial solution is entrained with the bubbles. Additionally the dwelling time in the column is higher, providing a better draining effect [163] and enhanced coalescence [164] resulting in bigger and dryer bubbles. Nevertheless, it was found that high flow rates can lead to higher separation rates of surface-active substances [165]. The optimum flow rate of the process has to be determined depending on the stability of the foam and the concentration of the surfactant [166, 167]. Using inert gases at room temperature reduces oxidative alterations at the extractive components [166].

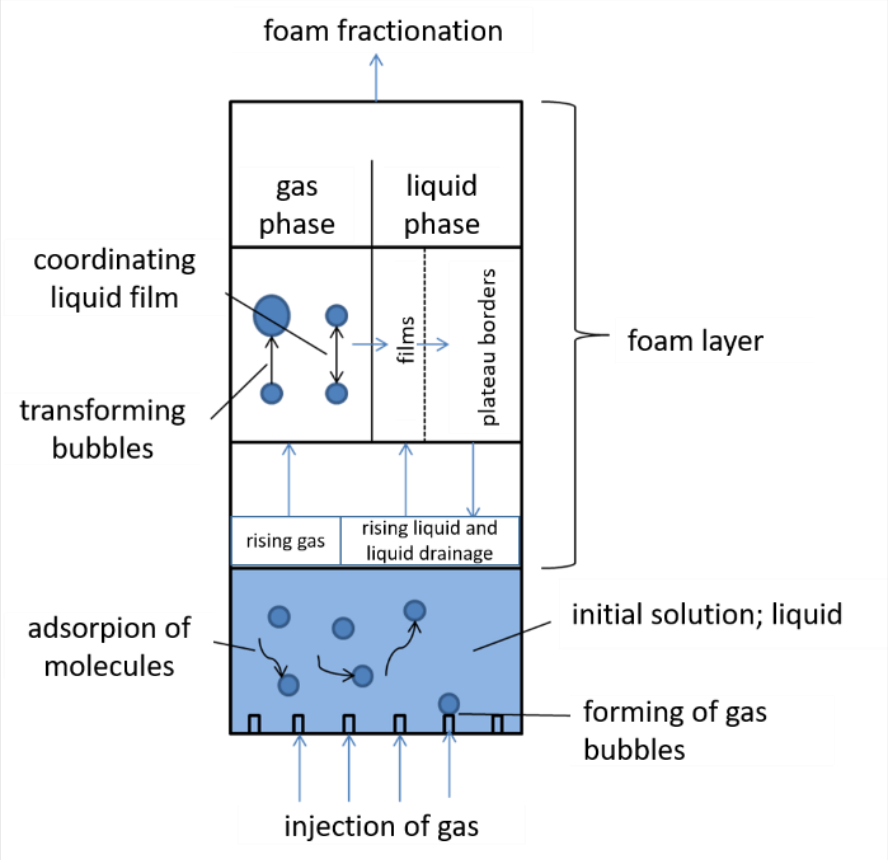


Figure 8: Schematic overview over the foaming process; adapted from [209]

Additives

Preliminary investigations have shown that not all plant extractives foam sufficiently. For a better performance tensides can be added [168]. Therefore, the anionic sodium dodecyl sulphate (SDS) and polyethylene glycol sorbitan monolaurate (Tween20) were chosen. The monolaurate Tween 20 (Figure 92) has no ionic parts but has hydrophilic and hydrophobic parts as well [166]. As an approximate value a concentration between 10^{-3} und 10^{-7} mol/l is suggested [155]. Good results were achieved analysing non-foaming saps from plants [169]. To remove the tenside, SDS can be precipitated with pottassiumchloride (KCl) as potassium dodecyl sulphate which is water insoluble [170] or separated with an amphoteric resin [171].

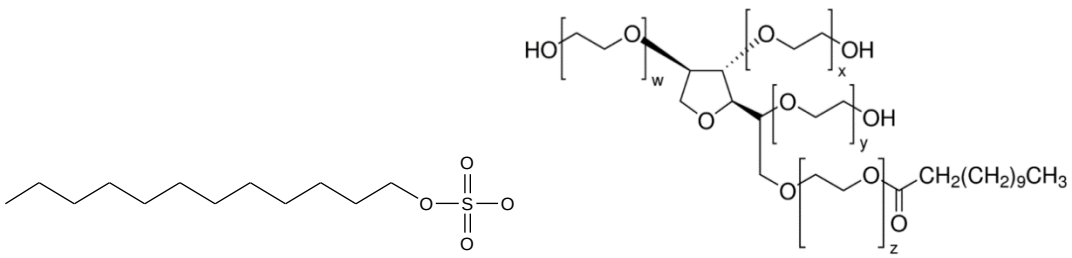


Figure 9: Sodiumdodecylsulphate and Tween20 (w + x + y + z = 16)

Applications of foam fractionation

The method was first mentioned in experiments that tried to separate protein from potato-water or sugar syrup [151], to accumulate hops-bitters from beer or for the purification and accumulation of different enzymes [152].

Foam fractionation is applied in different fields of plant science and has already been used for extraction, separation and accumulation of surface-active compounds. A comprehensive review regarding foam fractionation set-ups and applications was provided by Burghoff [145]. It is focused mainly on biotechnological applications and on protein separations. Some of the therein-mentioned examples as well as some more recent work are described in the following paragraph.

For example, oils and polyphenols were isolated out of selected spice plants and fruits by Parlar et al. [168] and Backleh [144, 156]. Humulones were separated from Pilsner beer [156] and an almost 100% recovery of glycol-alkaloids from potato-juice were achieved [169]. Separations

of catechol and phenol [172], recovery of *trans*-resveratrol from muscat grape pomace [173] or alkali metal ions from anionic surfactant solution [174] were accomplished. Application of adsorptive bubble separation techniques for metal extraction from diluted solutions like wastewaters is a well-tested technique [147, 175, 176] . For example, Jones and Robinson [177] removed 95% Ni(II) and Wu et al. [178] 97.2% copper ions from an aqueous solution via foam fractionation. Furthermore, a wide application field for foam fractionation is protein separation. It was shown that recovery, separation and enrichment of proteins like casein [179, 180] or bromelain [180] from highly diluted solutions, or laccase from basidiomycete strains are possible [181, 182].

3.3.3 Detecting bioactivity

The progress of modern methods in analytical chemistry sometimes conceals the problem that the ever-enlarging amount of analytical data does not necessarily give more insight of practical relevance [139]. Therefore, and for the target-oriented application of sophisticated as well as time- and cost consuming instrumental methods, bioactivity-based assays can deliver valuable information.

Typical bioassays of heartwood extracts on wood degrading fungi have been carried out since a considerable number of years [36, 183, 184]. Several different bioassays are well known and commonly used while others are not widely known. The most used assays are agar dilution, disk diffusion, and bioautography [141]. Comprehensive reviews regarding antimicrobial screening were assembled by Favre-Godal et al. [141, 185] and Balouiri et al. [185] which summarize the today applied methods:

(1) Different diffusion methods are used in many laboratories like the disk diffusion method, the antimicrobial gradient method also known as the E-test (bioMérieux AB BIODISK), the agar well diffusion method, the agar plug diffusion method, the cross-streak method or the poisoned food method. In these methods, the test compounds can be placed on the agar by impregnated filter disks or strips, directly into the agar or onto its surface (e.g., as a liquid) as well as via an incubated agar cylinder. Depending on the method, the agar is inoculated either prior or after the application of the antimicrobial substance. All methods have in common that they depend on the ability of the compounds to diffuse into the agar medium to detect them by the appearance of an inhibition zone around the inoculum. Some of them are designed to highlight antagonisms between microorganisms while others are mostly used to evaluate antifungal effects. Few studies already showed the possibility to apply diffusion

methods directly to solid wood [186, 187]. Munir et al. [188] additionally used the well diffusion method to test sawdust of some species from temperate climate.

(2) Dilution methods offer the possibility to estimate the concentration of the tested antimicrobial agent in agar (agar dilution) or broth medium (broth dilution). They are standardized. The most recognized standards are provided by the Clinical and Laboratory Standards Institute (CLSI) [189] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [190]. Both methods known to be suitable for the determination of MIC values, with the MIC being lowest concentration of an antimicrobial agent that completely inhibits growth of the organism as detected by the unaided eye [6]. The test compounds are either diluted in tubes or 96-well micro titration plates, or incorporated in molten agar and poured in agar plates (agar dilution) in the desired concentrations. Inoculation is done by adding a prepared suspension to the tubes/wells or a defined inoculum onto the agar plate surface. These methods are often combined with colorimetric methods based on the use of dye reagents.

Diffusion and dilution methods of full extractives or partly separated extracts have the major drawback that the biological effect cannot be clearly assigned to specific compounds and therefore a cause/effect relationship cannot be established. Furthermore, they require a relatively large amount of extract. However, these assays, as well as bioautography, are simple to perform and do not require complex instrumentation.

(3) Bioautography is a useful technique to determine bioactive compounds from plant extract having antimicrobial activity. Planar chromatography bioautographic methods combine chromatographic separation and microbial determination facilitating the localization and target-directed isolation of active constituents in a mixture [18]. Traditionally, bioautographic technique has used the growth inhibition of microorganisms to detect anti-microbial components of extracts chromatographed on a TLC layer. This methodology has been considered as the most efficacious assay for the detection of anti-microbial compounds [191]. Mainly three concepts of bioassays are practically applied today [192]:

a. The direct bioautography is the most applied method among these three methods. The developed TLC plate is dipped into or sprayed with amicrobial suspension and the microorganisms are growing directly on the TLC-plate. After incubation, tetrazolium salts are frequently used for visualization of the microbial growth. These salts are sprayed onto the bioautogram, which is then further re-incubated. Direct bioautography may be utilized with either fungi or bacteria.

b. The contact bioautography, also known as the agar diffusion assay is the least-employed one of the techniques. The antimicrobial agent is transferred from the chromatogram to an agar plate previously inoculated with the microorganism by scratching off the TLC surface or by putting it upside down onto the agar plate. After a defined time to allow diffusion, the chromatogram is removed and the agar plate is incubated. The growth inhibition zones appear in the places where the antimicrobial compounds contact with the agar layer.
c. The agar overlay or immersion bioautography, where a seeded agar medium is applied onto the TLC plate is a hybrid of the both previous methods. The TLC plate is covered with a molten seeded agar medium and after incubation, staining can be made with e.g., a tetrazolium dye. Like direct bioautography, this method can be applied to all microorganisms such as *Candida albicans* and moulds.

   A major advantage of the bioautography methods is that small amounts of sample are sufficient compared to the normal disc diffusion method and it can be used for bioassay-guided isolation of compounds. Therefore, the technique simplifies the process of identification and isolation of the bioactive compounds since the crude extract is resolved into its different components [18].

However, bioautography has some limitations, which makes the standardization of the bioautographic methods difficult. Factors like the tested organisms, the medium composition, and the pH and solubility of the sample culture make improvements necessary to achieve a better reproducibility [192]. Furthermore, in cases where compounds act synergistically in extracts, a chromatographic separation could be a disadvantage. Despite the limitations of the assay, TLC-bioautography has been used successfully for the screening of natural extracts, and several antifungal compounds have been isolated in various bioactivity-guided isolation studies [18, 139, 193].

However, conventional bioautography methods to test (wood) extractives are mostly designed for spore-producing fungi [141, 194]. The spores are easily harvestable and can be inoculated on agar. However, these standard antifungal assays cannot be readily adapted to basidiomycetes, like, in particular *T. versicolor* and *R. placenta*. The production of basidiospores in vitro was subject of several studies in the past [19, 20]. However, even if it was improved over the last years [21–23], the work with hyphae is still standard in many laboratories [195]. Combining the bioautographic agar overlay method [18] with hyphae agar [24] should provide a possibility to benefit from the advantages of a direct bioautography (rapid,

easy to implement) while testing wood extractives against commonly used basidiomycetes, which do not readily sporulate. Separating the compounds thoroughly should be achieved by performing the thin layer chromatography as a two-dimensional development (2D-TLC) as suggested by Wedge and Nagele [140].

3.3.4 Application of vitality dyes

Applying tetrazolium salts are a common technique in microbiology to assess cell viability. One of the most widely used dye thereof is MTT [3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium Bromide] which was introduced for the detection of dehydrogenase activity by Pearse [196]. It has been utilized successfully to quantify the effects of antifungal agents on cell viability or as a method of colorimetric determination of fungal cell densities on a number of fugal species [197–201].

Based on the reduction of the yellow tetrazolium salt, actively growing cells produce a blue/purple, insoluble formazan product [199]. This tetrazolium dye reduction is generally assumed to be dependent on the coenzyme Nicotinamidadenindinucleotidphosphat (NAD(P)H) and glycolytic enzymes at the endoplasmic reticulum [202, 203] and should be rather viewed as a measure of the rate of glycolytic NAD(P)H production [201]. However, it should be kept in mind, that several reductants like many biomolecules can reduce MTT [204].

The vitality of the mixed hyphae should be determined by using the viability stain MTT. By indicating the vitality of the mixed hyphae through a stain, the judgment of whether a compound (-mixture) causes fungal inhibition or not should be improved.

3.3.5 Identification of bioactive compounds and hyphenated techniques

Structural studies are often difficult to carry out with classical chemical methods, not at least because of the small amounts present in the plant (especially when the amount is lower than <50 ng g^{-1} plant tissue [205]). Since the material consist of multi-component mixtures, practically most of them have to be purified and analysed by a combination of several various methods. Therefore, spectral analysis is mainly used to obtain structural information and afterwards a comprehensive analysis is carried out with the assistance of literature data.

To obtain a compound in the first place by e.g., chromatographic elution, the presence of a compound must be detected. Popular detectors for this purpose are UV-VIS, Diode Array

(DAD), Refraction Index (RI) or evaporative light scattering (ELSD) detectors because they either offer high sensitivity or are non-specific and therefore most suitable [139].

To achieve structural elucidation, Fourier-transform infrared spectroscopy (FTIR) is a valuable tool for the identification of functional groups and mass spectrometry is a powerful analytical technique for the identification of unknown compounds and quantification of known compounds by defining both, the molecular weight and a diagnostic fragment of the molecule concurrently.

Most likely, the most powerful method for structural analysis is Nuclear Magnetic Resonance Spectroscopy (NMR), which provides physical, chemical and biological properties of the compound. However, its necessity of higher substance amounts is a major drawback, as the accumulation of such amounts requires a lot of time and work force.

Towards one-step or online setups, varieties of hyphenated techniques were developed in the recent years. Generally, hyphenation covers the different approaches to combine spectrometers with chromatography systems. However, Morlock and Schwack [206] defined it as a method that "represents a logical, rapid and efficient strategy for obtaining the most possible (relevant) information out of a single separation". They also summarize the major problems accompanying these techniques: they are costly and difficult to run in routine due to the complexity of the instruments as well because of the large amounts of data they produce. Furthermore, single eluents being optimal for all detectors are difficult to choose.

Liquid Chromatography as well as high performance thin layer-chromatography (HPTLC) coupled with Mass Spectrometry (LC/MS) provide separation and abundant information for structural elucidation of the compounds. They facilitate rapid identification of chemical compounds, and are considered as the gold standard these days [207].

LC–NMR combines the advantage of the outstanding separation power of liquid chromatography (LC) and the superior structural elucidating capability of nuclear magnetic resonance. LC–NMR developmental trends and its application in natural products analysis were recently reviewed [13].

Besides chromatographic techniques, exploitation of bioluminescence or genetic tools can also be of interest. Direct antibacterial bioautography assays were successfully performed on the bacteria *Photobacterium phosphoreum* which is able to emit bioluminescence and by screening

compounds against various deletion mutants of the *Candida albicans* genome, the mechanism of action of the antifungals was studied [141].

3.4 Durability

The term natural durability refers to the natural resistance of wood against biological degradation such as fungal decay and insect attacks (EN 113, EN 350, 2016). The natural durability of wood strongly varies according to wood species, geographical origin, individual trees, and different zones within individual trees [36, 208] . An accepted protocol to determine the natural durability is described in the European standards EN 350-1: 'Durability of wood and wood-based products: Testing and classification of the durability to biological agents of wood and wood-based materials. With respect to four common European wood species groups Picea abies, Pinus sylvestris, Populus spp. (*Populus canescens, P. alba*, *P. nigra*), Quercus spp. *(Quercus robur, Q. petrea*) five different classes of durability and a standardized test for heartwood [37] is introduced therein: (1) very durable; (2) durable; (3) moderately durable; (4) slightly durable; and (5) not durable. The sapwood of all species of wood ranks in durability class 5, the least durable class of all. The test is performed using small blocks of wood of a defined size which are weighed and then incubated with wood-degrading fungi (basidiomycete and soft-rot fungi), or other microorganisms like beetles, termites and marine organisms capable of attacking dry wood and under standard conditions. After a defined time, the wood blocks are weighed again and the loss of mass is determined (EN 113, EN 350, 2016) [209]. The wood species analysed in this thesis are all classified as durability class 1. However, they can also be less durable (class 2), depending on their growth conditions.

As fungi are among the most important wood-degrading organisms, the scope of this work focuses on two of their representatives. Generally, fungi require a suitable combination of moisture, temperature (opt. from 22–30 °C) and oxygen to grow. Most decay fungi will become inactive if the wood moisture content drops below 20% for extended periods [210]. As their nutrition's are dissolved in water to be taken up into the fungi hyphae cells, this explains why they can hardly decompose dry wood.

3.5 Fungi

The kingdom of fungi refers to a group of eukaryotic organisms including microorganisms such as yeasts and moulds as well as the more familiar mushrooms. They are considered sufficiently distinct from plants, animals or bacteria. They possess cell walls and vacuoles, but totally lack

chloroplasts and therefore do not photosynthesize. As they are heterotrophic organisms, they require preformed organic compounds as energy sources. They reproduce by sexual and asexual means, and are spore producing through their fruiting bodies (Asci for Ascomycetes and Basidia for Basidiomycetes). Most fungi possess a generally undifferentiated mycelium, which is formed by hyphae. Hyphae are cylindrical, thread-like structures 0.5–100 μm in diameter, up to several centimetres in length. Many fungi are parasites on plants, animals (including humans), and other fungi. Other species of fungi are saprotrophic, or obtain nutrients in a symbiotic relationship with plant species [211].

Wood destroying fungi organisms are classified into three types: brown rot, white rot and soft rot. They gain their energy through decomposing the organic matter by secreting different enzymes through their hyphae. In this thesis, *Rhodonia placenta*, a brown-rot fungus and *Trametes versicolor*, a white rot fungus are used as test species. Both belong to the family of the *Basidiomycota* [32].

The wood decayed by brown rot fungi can typically be recognized by its brownish colour, lateral and longitudinal ruptures and characteristic cubical fractures. Brown rots usually decompose mainly the polysaccharides (cellulose and hemicelluloses) of the wood cell wall [212]. The fungi contain cellulases and use a system of water and iron for feeding on these parts of the cell wall. By generating a hydroxyl radical according to the Fenton reaction ($H_2O_2 + Fe^{2+} + H^+$ --- $H_2O + Fe^{3+} + OH^{\bullet}$) [213, 214] and by modifying the lignin, the matter turns into its characteristic, name giving colour [212].

White rot fungi are typically associated with hardwood decay giving the degraded wood a bleached appearance and a whitish colour. The wood suffers severe decrease of density and strength due to loss of cellulose and lignin. White rot fungi possess both cellulolytic and lignin degrading enzymes (lignin peroxidases and manganese peroxidases) and therefore have the potential to degrade the entire wood structure [213]. *Trametes versicolor* is known to produce the enzyme laccase [170] that is used in industry to generate quinones and polymers from phenols, naphtols and bisphenols from wastewaters [215].

3.6 Wood extractives as decay-preventing agents and their potential utilization for wood protection

Several theories have been proposed to explain the natural decay resistance of heartwood, but the primary reason is likely to be the presence of heartwood extractives.

Schwager und Lange [32] published their comprehensive literature study on biological wood protection, which focused on wood extractives and their biological activity in 1998. Since then innumerable studies on fungicidal properties of wood extractives, application and fixing of extractives mixtures on non-durable wood species and coating possibilities have been published as well as several review papers concerning these topics were published in the following years [216–218].

For a considerable number of years extractives of several natural durable timber species have been removed by solvent extraction methods. It has been shown that the wood becomes more susceptible to decay and that non-durable timber impregnated with extractives, showed enhanced biological resistance [35, 36, 219]. The antifungal properties of various extractives have also been demonstrated using simple bioassays [219–221] and the interest in the bioactivity potential of extractives mixtures is still ongoing. Recent published work examined the potential of i.e., teak heartwood (*Tectona grandis*) extracts against decay fungi as a natural alternative to conventional wood preservatives. While Brocco et al. [222] found their results promising for the use of teak heartwood residues, Hassan et al. [223] could not find protective effects at tested concentrations when applied to non-durable southern pine or cottonwood. Stirling and Kus [98] used the extractives of western red cedar with the focus on the potential of the extracts to alter the appearance, stability and durability of non-durable timbers. They also report mixed results as the durability of extractive-impregnated wood was usually equal to or lower than that of the heartwood from which the compounds were sourced. Furthermore, the biocidal and antioxidant potential of the extractives of four Malaysian timbers against white rot and brown rot fungi were examined [224, 225] and it was found that the hot water extract of one of the species (*Neobalanocarpus heimii*) showed good fungicidal activities against wood decaying fungi.

As it can be seen, the relationship between extractives and decay resistance is not always straightforward. Several experiments have also shown, that extraction does not always fully eliminate the decay resistance of durable heartwood [35, 219, 226] and that discrepancies can be found between the antifungal activity in bioassays and the ability of the extractives to provide

decay resistance when impregnated into non-durable timber. These findings indicate the involvement of factors other than extractives and have also led to the assumption that not all extractives are actually removable by solvent extraction. They are believed to be polymeric or associated with the fibres, cell wall components as well as lignin [28, 29, 219, 226]. Furthermore, some extractives seem not to be involved in fungicidal defence at all when tested, but it is proposed that they may serve in synergistic action with more bioactive extractives [23, 227].

The mechanisms by which extractives probably accomplish the inhibition of the degradative capabilities of rot fungi are also far from fully understood. However, several different strategies are suggested [38]. Metal- and free radical scavenging is the strategy known to be the mode of action from e.g. the prominent tropolone ß-thujaplicin [228], some lignans and flavonoids [102, 105]. Antioxidants can scavenge free radicals by reacting with them, or they can prevent their formation by chelating metals that can catalyse radical-producing reactions such as the Fenton reaction (see section 3.5) [229, 230]. By directly binding onto wood degradative enzymes, e.g. the isoflavonoid medicarpin, identified in a *Dalbergia* species, inhibits laccase in *Trametes versicolor* [231]. A disruption of the fungal cell wall and the plasma membrane is the reason for the antifungal activity against white rots and brown rots of e.g., cinnamaldehyde. It is known that this compound is able to alter the cell walls of S*accharomyces cerevisiae* by inhibiting cell wall synthesizing enzymes. Furthermore, the antifungal effect of dehydrodiconiferylalcohol-glucoside has been attributed to changes in the plasma membrane. Thus, resulting in osmolality changes and reduced cell size [38]. Carvacrol, a monoterpene isolated from yellow red cedar is known to be fungicidal against various species including *Trametes versicolor* and *Coniophera puteana.* It causes disturbance of ion homeostasis by a transitory influx of Ca^{2+} from the surrounding [38]. A disruption of non-covalent bonds (chaotropic activity) affects the macromolecule-water interaction. This can be caused by many compounds found within wood extractives like alcohols or phenolics, resulting in disordering macromolecular structures affecting e.g. membranes [38]. Furthermore, extractives can reduce the rate of fungal degradation by lowering the equilibrium moisture content of wood.

However, many commonly used wood species are susceptible to biological degradation, which remains a major challenge for their utilization. However, wood can be protected from the attack of decay fungi, harmful insects, or marine borers by applying chemical preservatives as surface

coatings or impregnation with added photo protectives like inorganic particles, UV absorbers, radical scavengers, reactive metal compounds as well as by chemical modification (e.g. acetylation, furfurylation) or heat treatment [232]. Commonly used fungicides are applied to control mould, sapstain, and decay by basidiomycetes and soft-rots. Insecticides are largely used to control termites, but may also be used to protect wood against other insects such as carpenter ants and wood-boring beetles. Formulations used to control insects may have repellent and/or insecticidal activity. Repellents are typically volatile compounds that insects sense and avoid [233]. Most of the traditional wood protection methods employ chemicals that are considered toxic and can negatively affect human health (humatoxicity) and the environment (ecotoxicity) as they are leached into the environment, especially into ground water, waterways and soil [234]. They may then pose a potential risk for the soil organisms and thus for the soil ecosystem in general. These problems have raised serious concerns over the use of such chemicals and current practice of disposing treated wood products [216].

With this in mind, it becomes clear why protecting wood with synthetic chemical preservatives has come under increasing concern in public and is regarded suspiciously by the consumer today. It is true, that many of them are surely hazardous to water and some are corrosive to iron, steel or glass, flammable or can give off harmful odours. Therefore, chemical wood protection is known to be functional, but has major drawbacks. Nevertheless, a certain effective protection of wood against biodegradation is necessary under application in wet or moist environments, as many wood species are vulnerable without any protective measures. Thus, less toxic preservatives or wood modification techniques are desirable and wood extractives are considered a way to biological wood protection.

Therefore, effort is put in developing wood protection agents based on a natural basis. Plant oils and waxes are containing high portions of fatty acids which are known to be highly effective [39, 223]. They are widely used for wood protection [217] since ancient times. Additionally, different wood components are in focus as wood preservatives since the last decades and the interest is still ongoing. For example, different bark extracts from mimosa (*Acacia mollissima*), quebracho (*Schinopsis lorentzii*) and pine (*Pinus brutia*) known for their high condensed tannin amounts were examined. It was suggested that commercial mimosa and quebracho extracts can be utilized as alternative wood preservative chemicals against common wood decay fungi in indoor applications [235]. Tannin extract-based wood preservatives have also been applied and tested in different formulations in order to overcome leaching problems e.g. in complex with metal ions like boron and copper [236]. Hardeners like formaldehyde or hexamine [217] were

employed as well and suggest that tannin can be considered as a potential natural preservative product. Fernandez-Costas et al. [237] presented a different approach by applying an enzyme-mediated reaction to fix the compounds onto the exposed wood surfaces by forming covalent bonds between the bioactive compounds and the wood itself. Furthermore, biologically synthesized copperoxid nanoparticle formulations were also suggested as environmentally friendly wood protectants against decay fungi and termites. The metal nanoparticles were synthesized using plant extracts that are known to have wood preservative properties [238] without toxic by-products.

It can be seen, regarding the many studies in the recent years, that most of the researchers focus predominantly on the application of extract mixtures. However, some work is done on elucidating single compounds from wood with bioactive properties. Some of the very recent studies shall be mentioned here to outline the ongoing research.

Working on the phytochemical analysis of some classes of secondary metabolites present in *Croton Urucurana*, *Pelthoforum dubium*, *Jacaranda cuspidifolia* as well as on *Hymenaea courbaril*, Ribeo dos Santos et al. [239] detected the presence of alkaloids, phenolic compounds and triterpenoid. They suggested possible pharmacological actions of the species studied. Ahouhe et al. [9] examined the role of heartwood extractives of *Dicorynia guianensis* on antifungal properties against two white rot fungi using bio-guided fractionation. Catechin, epicatechin, neoastilbin, astilbin and isoastilbin were characterized and an obtained alkaloid fraction exhibited fungistatic activity against e.g., *Trametes versicolor*. Conde et al. [10] recovered bioactive compounds from *Pinus pinaster*, namely simple phenolics, phenolic stilbenes, flavonoids, lignans, juvabiones, resin and fatty acids, steryl esters and triglycerides. Minor amounts of epijuvabione, a sesquiterpenes were identified as well. Epijuvabione is said to play a role in plant defense. Francezon et al. [240] extracted spruce bark in hot water, which contained bioactive polyphenols as well as the stilbene glucoside isorhapontin, astringin, resveratrol, isorhapontigenin and the resveratrol glucoside piceid.

4 Overview of publications

4.1. Publication 1: Application of Foam Fractionation to Wood

Regina Wanschura, Elisabeth Windeisen, Klaus Richter
Wood Science and Technology 2019, 53, 2, 349–371

Abstract

Foam fractionation is a part of the adsorptive bubble separation methods and is a simple, gentle and rapid method for the extraction and accumulation of surface-active substances out of biomass. To assess whether the method is applicable to wood, two different device sizes were compared and two gases (N_2 and CO_2) were dispersed into the wood meal/water suspensions of several tropical wood species or into their respective water extracts. Furthermore, two tensides (Tween20 and SDS) were tested as supporting foaming agents. The compounds accumulated in the so-called spumates of these samples were compared to conventional water extracts of the respective woods and analysed by FTIR, sugar analysis and GC/MS to examine whether an accumulation of extractives was achieved. Results show that the method is applicable to wood meal and water extracts from wood, but the conditions have to be adjusted to the targeted extractives of the wood species. Furthermore, it was observed that it is suitable for extraction of saponins without the need to separate them from other extractives, but it is not an alternative to a comprehensive analysis of water extractable compounds. The presented method can be applied using wood species that contain sufficient amounts of surface-active substances to be "self-foaming", but for a more versatile application, it needs to be further refined. However, if the application of additives is required in order to achieve sufficient foaming, it could be shown that the use of Tween20 was not successful, whereas SDS allowed several experiments. Nevertheless, a more effective way for complete separation and recovery of the tenside has to be developed, in particular, when the targeted extractives shall be used in subsequent biorefinery processes.

Contribution

Regina Wanschura developed the research questions, designed the methodological approach, modelled the systems, conducted the analysis of the results and wrote the manuscript. Elisabeth Windeisen-Holzhauser and Klaus Richter supported the development of the study concept, the research questions and critically reviewed the manuscript.

4.2. Publication 2: Direct bioautography for the screening of selected tropical wood extracts against basidiomycetes

Regina Wanschura, Matthias Baumgartner, Claudia U. Linder, Elisabeth Windeisen,
J. Philipp Benz, Klaus Richter

Holzforschung 2020, 74, 8, 733–743

Abstract

To understand the reasons for the high durability of tropical wood species, the chemistry of the extractives needs to be elucidated. As these extractives consist of a great variety of components differing in quantity and composition, the analysis is often time-consuming. To focus on the key bioactive substances, bioassay-guided fractionation is helpful, but the established bioassay methods cannot be readily adapted to basidiomycete fungi that are commonly used for the respective durability tests, because they do not sporulate easily in laboratory settings. The research therefore aims at developing a direct bioautography using homogenized hyphae from basidiomycetes, to overcome this restriction. Extracts from four tropical wood species were analysed regarding their potential bioactivity on two selected basidiomycete fungi. To this end, the chemically complex mixtures and extract constituents were resolved by a two-dimensional planar chromatography and the metabolites were located in characteristic zones of fungal growth inhibition, which was accentuated by a colour reaction. The bioactive fractions were analysed by gas chromatography/ mass spectrometry (GC/MS). Potentially responsible compounds could be identified, such as the alkaloid bicuculline from *Mezilaurus itauba*, which has not been described in this species yet. The presented bioassay method can be used as a rapid screening method for bioactive components from wood.

Contributions

Regina Wanschura developed the research questions, designed the methodological approach, modelled the systems and achieved data, conducted the analysis of the results and wrote the manuscript. Design of methodical approach was supported by supervised master theses from Matthias Baumgartner, Claudia U. Linder. Elisabeth Windeisen, J. Philipp Benz and Klaus Richter, supported the development of the study, participated in scientific discussions and critically reviewed the manuscript.

5 Materials and Methods

5.1 Wood samples, test organism and sample preparation

The tropical wood species (Table 1) used in this research origin from South and Central America. The heartwood is classified in durability class 1 according to EN 350-2, and the raw density ranges between 0.65 -1.16 g / cm^3. The boards used to cut the specimens were purchased from local lumberyards. Wood blocks of one cm^3 were sawn out of the heartwood subsequently milled in a cross-hammer mill (Retsch) together with dry ice and sieved with a mesh size from 0.315 mm to 0.05 mm. The sample moisture content was determined via oven drying at 105°C as double determination. The sample weight is referred to as absolutely dry. Constant mass according to this test is reached, when the mass decrease after re-drying is less than 0.5 %.

Two fungi, the white rot fungus *Trametes versicolor* BAM 116 (CTB 863 A) and the brown rot fungus *Rhodonia placenta* BAM 113 (FPRL 280) were chosen as test organisms (according to EN 113), in order to detect a possible bioactivity of the wood extractives in the species.

Table 2: Wood species used in the experiments

Wood species		
Trade Name	Systematic Name	Abbrev.
Bangkirai	*Shorea laevis*	BAN
Ipé Noir	*Tabebuia spp. = Handroanthus spp.*[a]	IPN
Itaúba	*Mezilaurus itaúba*	ITA
Jatoba	*Hymenea coubaril*	JAT
Massaranduba	*Manilkara bidentata*	MAS
Merbau	*Intsia spp.*	MER
Muiracatiara	*Astronium graveolens*	MUI
Pequia	*Caryocar villosum*	PEQ

[a] (Grose and Olmstead 2007)

5.2 Determination of water content by drying

Firstly, weighing bottles were constant-weighed. For this purpose, they were put into a drying closet (105 °C; Memmert, Schwabach, Germany) for about 1 h and were then left to cool in a desiccator for 30 min. They were weighed, using microscales (200g/0.1 mg, Sartorius, Göttingen, Germany) and the process was repeated, until a constant weight was reached. About 1 g of wood powder was weighed out in a constant-weighed weighing bottle and dried in a drying closet at 105 °C for about 1 h. Then the sample was left to cool in a desiccator for 30 min and was weighed by microscales and the process was repeated until a constant weight was reached.

5.3 Water extraction at room temperature

Water extracts from wood are made according to the Tappi Standard 207 cm-99 by digesting 2 g of air-dried wood meal in a beaker filled with 300 ml of demineralized water and left at room temperature for 48 h with incidental stirring. Extract contents are determined gravimetrically after drying the samples in a freeze dryer (Christ Alpha 1-2 LDplus) and are expressed as percentage of dry substance. Wood meal/water suspension and water extracts are subjected to foam fractionation resulting in lyophilized spumate samples for further investigation. Results from prior tests are used to adapt the amount to the volume of the device.

5.4 Organic solvent extraction

Determination of the extract content in organic solvents was done in a Soxhlet extractor using petroleum ether, acetone and methanol (all supplied by Merck, SupraSolv quality) successively for 6 h each as double determination with a relative deviation of less than 10 %. The Soxhlet extractor was filled with 5 g of wood flour and extracted with 170 ml of the respective organic solvent in a 250 ml round-bottomed flask. Extract contents were determined gravimetrically after drying and are given as percentage of dry substance.

Solvent extraction for the separation process started with an extraction of lipophilic compounds from the wood meal using petroleum ether. The extraction was done in a soxhlet extractor thoroughly for 18 h with the petroleum ether followed by an extraction with methanol for 27 h until no more compounds could be extracted from the wood. The soxhlet extractor filled with 35 g of wood meal and extracted with 300 ml of the respective organic solvent in a 1000 ml round-bottomed flask. The extraction is done as a double determination.

5.5 Foam fractionation

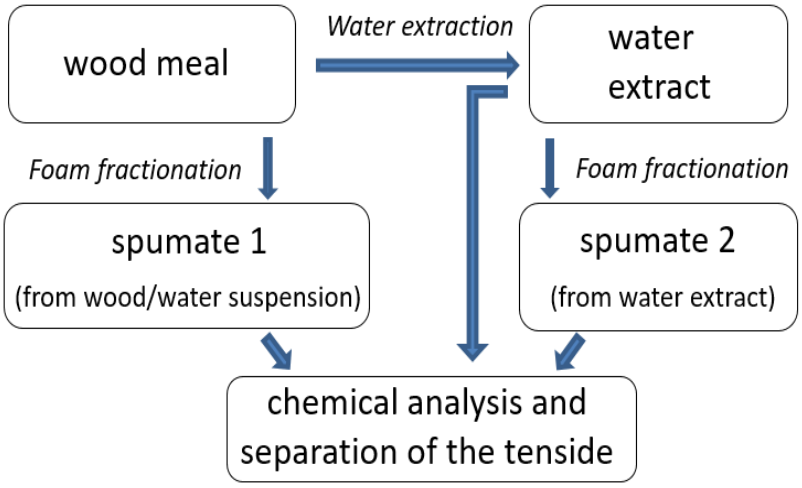


Figure 13: Flow chart of the experiments

A concentration of 1 g wood meal in 50 ml water for the wood/water suspension is chosen. Using the water extract, the initial flask is filled with 50 ml for device 1 and 300 ml for device 2. In the case of MAS, a reduced loading (0.1 g wood meal in 50 ml water or 25 ml water extract for device 1 and 0.3 g/ 300 ml and 150 ml for device 2) is necessary due to the strong foam formation. All spumates are compared to the corresponding conventional water extracts regarding the yield of extractives (expressed in percentage of dry substance) and the contained compounds.

Three types of isolation methods on the samples are analysed (Figure):

- Extraction in water
- Foam fractionation of a wood meal / water suspension → Spumate 1
- Foam fractionation of the water extract → Spumate 2

5.5.1 Foaming device

The foaming device is an open system, and consists of a glass chamber with a porous frit at the bottom side, a column and a receiving flask (Figure). Separations are performed on two different sizes of columns and at room temperature. The smaller one (device 1) has a length of 24 cm and diameter of 1.6 cm and the bigger one (device 2) a length of 60 cm and diameter of 3 cm. The sizes (volume) of the glass chambers are 100 ml for device 1 and 500 ml for device 2. N_2 or CO_2 gas is introduced into a water/wood meal suspension (resp. water extracts) via the porous frit. The samples are loaded directly into the flask via the neck before the glass column is put on to the flask. The emerging bubbles rise through the sample solution, surface-active components can adsorb to the bubble surface and therefore, enrich in the formed foam. After passing the column, the foam is collected in the receiving flask, which is a two-necked flask with a volume of 250 ml for device 1 and a beaker of 1000 ml for device 2. In the receiving flask, the foam is allowed to collapse and is then called the spumate [151]. Afterwards the mass of the spumate is determined gravimetrically and is expressed as percentage of dry substance (based on wood meal). The tensides used to foam non-active samples are sodium dodecyl sulphate (SDS; Sigma-Aldrich) and polyoxyethylene-(20)-sorbitanmonolaurate (Tween20; MERCK) at a concentration of 20 mg/l (according to [169]). Gas flow rate is controlled via a gas flowmeter and experiments are started with 100 ml/min. When no more bubbles emerge, the flow rate is increased in steps of 50 ml/min and is ended at a gas flow rate of 500 ml/min because higher gas flow rates lead to a “foam over”. This means that the initial solution is only pushed through the column into the receiving flask by the pressure of the introduced gas and no accumulation or separation is performed.

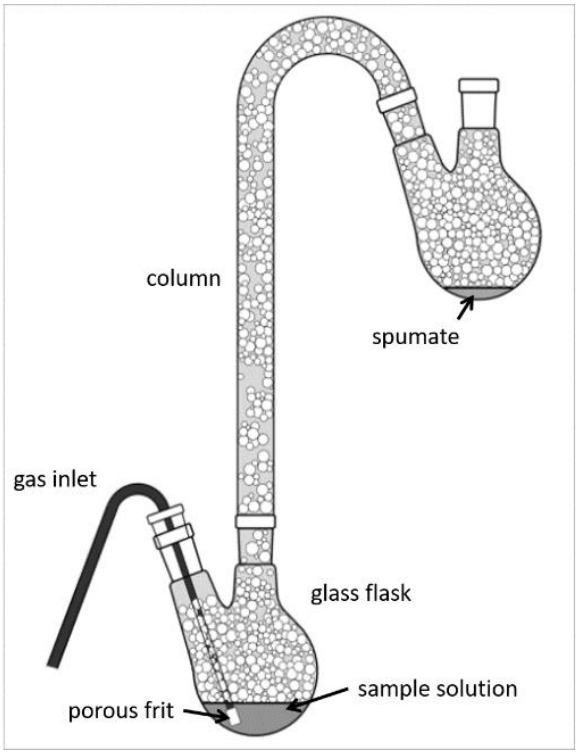


Figure 14: Scheme of a simple foaming device

The time of analysis is measured with an electronic time clock. The beginning of the experiment is defined at the time when the first bubbles reach the receiving flask and the end is defined at

the time when the bubbles disintegrate in the column and cannot reach the receiving flask anymore; latest at a gas flow rate of 500 ml / min (this is called the bubbling time). Therefore, the samples are not all treated for the same time (but until foam formation ends). All experiments are repeated 3 times if the percentage deviation from the average of the spumate amount is lower than 15 %. After performing the experiment, a rest of the sample solution remains in the glass chamber. This rest is called the residual solution [151].

5.5.2 Separation and detection of the tenside SDS

To quantify the extracted compounds, SDS needs to be separated from the spumates after the experiment. SDS can be precipitated by adding potassium chloride as potassium dodecyl sulphate or separated by using an ion-exchange resin. Focus was to assess the feasibility of the treatments to the samples. Because the amounts gained by each foam fractionation with SDS were small, the tests were performed only with the spumate from one wood sample per treatment.

Precipitation with KCl is performed according to Carro et al. (1994): 0.7 mg spumate from MUI wood is solved in 20 ml H_2O and adjusted to pH 1.5 with trichloro acetic acid (TCA). Afterwards 100 ml 2M KCl is added and after 15 min of cooling in an ice bath the mixture is centrifuged at 4 °C with 4000 rpm for 30 min.

Separation with an amphoteric resin: separation via an ion change resin based on [241] and [171]: 10 mg of BAN spumate is solved in 15 ml H_2O and 2 g Dowex retardion 11A8 50-100 is added. After 15 min, the resin is removed and the spumate is lyophilized.

Energy dispersive X-ray analysis: semi-quantitative determination of the sodium content in the sample and calculation of the SDS content therefrom are exemplarily applied to four MER spumate samples, which are subjected to the energy dispersive X-ray spectroscopy (EVO40 X-Flash 5010 by Bruker).

5.6 Thin layer chromatography (TLC)

Thin layer chromatography was performed as a two-dimensional chromatography (2D) on preparative glass plates (stationary phase: silica 60; 2 mm; 20x20 cm. Merck) without fluorescence indicator for bioautography and for GC/MS analysis. Contrary to the findings by Osmonova [25], who reported that the silica TLC plates from Merck were not suitable, because the coating was not stable when covered with warm agar, this kind of plate worked well under the tested conditions.

For a proper detection of the substance spots, the separation was also performed on analytical aluminium TLC plates (stationary phase: silica 60; 2 mm; 20x20 cm) as well. The plates were subjected to derivatization by dipping the plates for 10 seconds in a mixture of $(NH_4)_6Mo_7O_{24}$ (5%) and $Ce(SO_4)_2$ (0.2%) in a H_2SO_4 solution (5%). Afterwards they were dried with a heat gun.

For the bioassays, 30 µl of extracts- solution with a concentration of 100 g/l (resp. 3 mg) were applied onto the plates. The acetone extracts were solved in a mixture of 75 % acetone / 25 % methanol, and the methanol extracts were solved in a mixture of 75 % methanol / 25 % H_2O, respectively. The solvents used for the mobile phases are given in (Table 3). For the acetone and the methanol extracts, the same solvent mixtures were used. After performing chromatography, the plates were left to dry overnight in a vacuum oven at 60 °C. By doing so, the organic solvents, which cause inactivation of enzymes or death of living organisms, were completely removed prior to biological detection.

Bioassays were performed as duplicates. Therefore, 12 plates per wood species were prepared:

- two TLC plates with acetone extract and
- two TLC plates with the respective methanol extract per fungi for the following bioassay analysis.
- one TLC plate per fungi and wood species without extractives as a reference.

Furthermore, one TLC plate per extract and wood species was prepared for the following GC/MS analysis.

Table 3: Solvent mixtures used for the separation of the extract components

	1D	2D
Shorea laevis	butanone: methanol: dH_2O (20: 3: 4) + 10 drops of formic acid to 300 ml solvent mixture	butanone: propanol: dH_2O (6.5: 2.5: 1) + 10 drops of formic acid to 300 ml solvent mixture
Mezilaurus itaúba	cyclohexane: ethyl acetate: ethanol (1: 1: 19.5) + 10 drops of NH_3 to 300 ml solvent mixture	chloroform: methanol: dH_2O (35: 18: 1) + 15 drops of NH_3 to 300 ml solvent mixture
Intsia spp.	cyclohexane: acetone: ethanol (3: 6: 1) + 10 drops of formic acid to 300 ml solvent mixture	chloroform: acetone: methanol (1: 2: 2) + 10 drops of formic acid to 300 ml solvent mixture
Astronium graveolens		

5.7 Inoculation of fungal cultures (hyphae agar) and bioactivity assay

Hyphae agar: Homogenized hyphae solution was prepared according to [24] (Figure 10): For each experiment, 5.1 g malt extract (4 %) was mixed with 125 ml dH_2O using a stir bar until the solution was clear. Then, the bouillon was poured into Erlenmeyer flasks and autoclaved (sterilization temperature 121 °C for 20 min; autoclave: HMC Europe). Three inoculation blocks (0.5 x 0.5 cm) were cut from the fungal film of an agar plate of either *R. placenta* or *T. versicolor*. The inoculation blocks were put into the malt extract medium and the liquid cultures were incubated in an incubator (Memmert) for approximately two weeks at 22 °C, 70 % relative humidity. 250 ml of dH_2O were added to 9.75 g of potato dextrose agar, which was then autoclaved as above. The malt extract medium was decanted from the liquid cultures of *R. placenta* (19,000 rpm) or *T. versicolor* (10,750 rpm) and the hyphae were homogenized using a homogenizer (VWR). Then the suspension was centrifuged (4 min), the supernatant was discarded and the hyphae were washed with 10 ml of sterile 0.9 % sodium chloride solution. Afterwards the hyphae were suspended in 2.5 ml of 0.9 % sodium chloride. 5 ml of hyphae solution were added to 250 ml potato dextrose agar with a temperature of > 50°C (hyphae concentration of approx. 2 %). Instead of spraying an aqueous MTT solution on the already

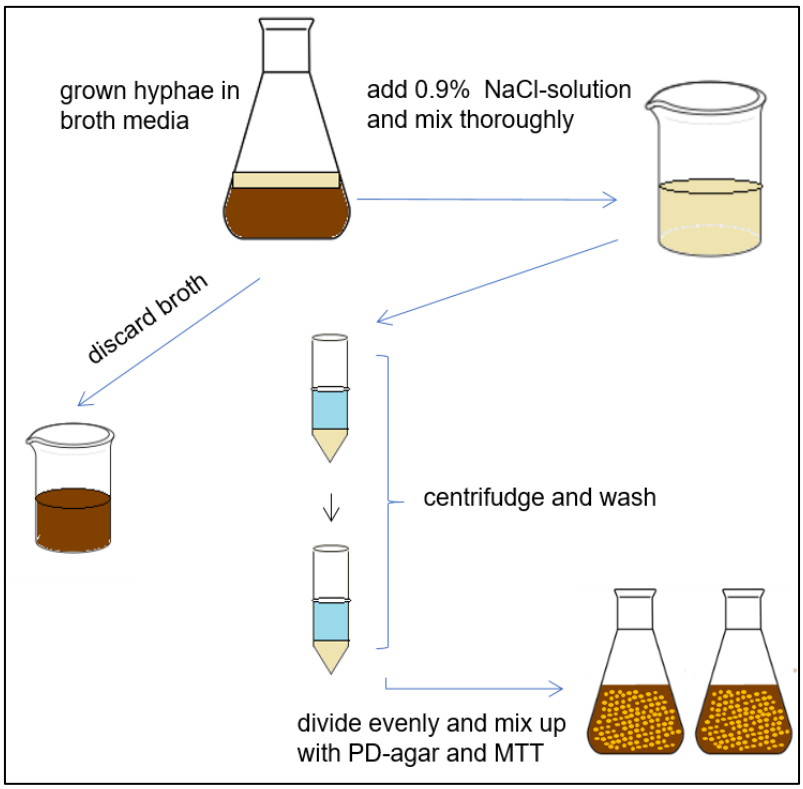


Figure 10: Flow chart of the production procedure of a hyphae agar

incubated TLC plates [18], 1 ml of the solution was mixed directly into the agar with a concentration of 10 mg/ml.

Bioassay: To generate a 1 mm thick agar layer on the TLC plate, the plate was locked into a suitable form and overlaid with 120 ml of the hot hyphae agar-MTT mixture (Figure 11). After drying (for 1-2 min), the plate was disassembled from the template form, stored in a bioassay dish (245×245×25 mm, Corning Life Science) and incubated in an incubator at 22 °C and 70 % relative humidity.

To determine the location of the compounds on the TLC designated for GC/MS-analysis, the bioassay plates were photographed after incubation. On each photo, the inhibition zones were marked. Together with the detection of the substance spots by UV, these pictures were used as an overlay to mark the corresponding spots on the "GC/MS-plate", which were then scratched from the plate. Furthermore, the TLC plates were compared with a respective chromatogram developed under identical conditions, which was derivatized. This compound visualization provided helpful information about their localization in the plate.

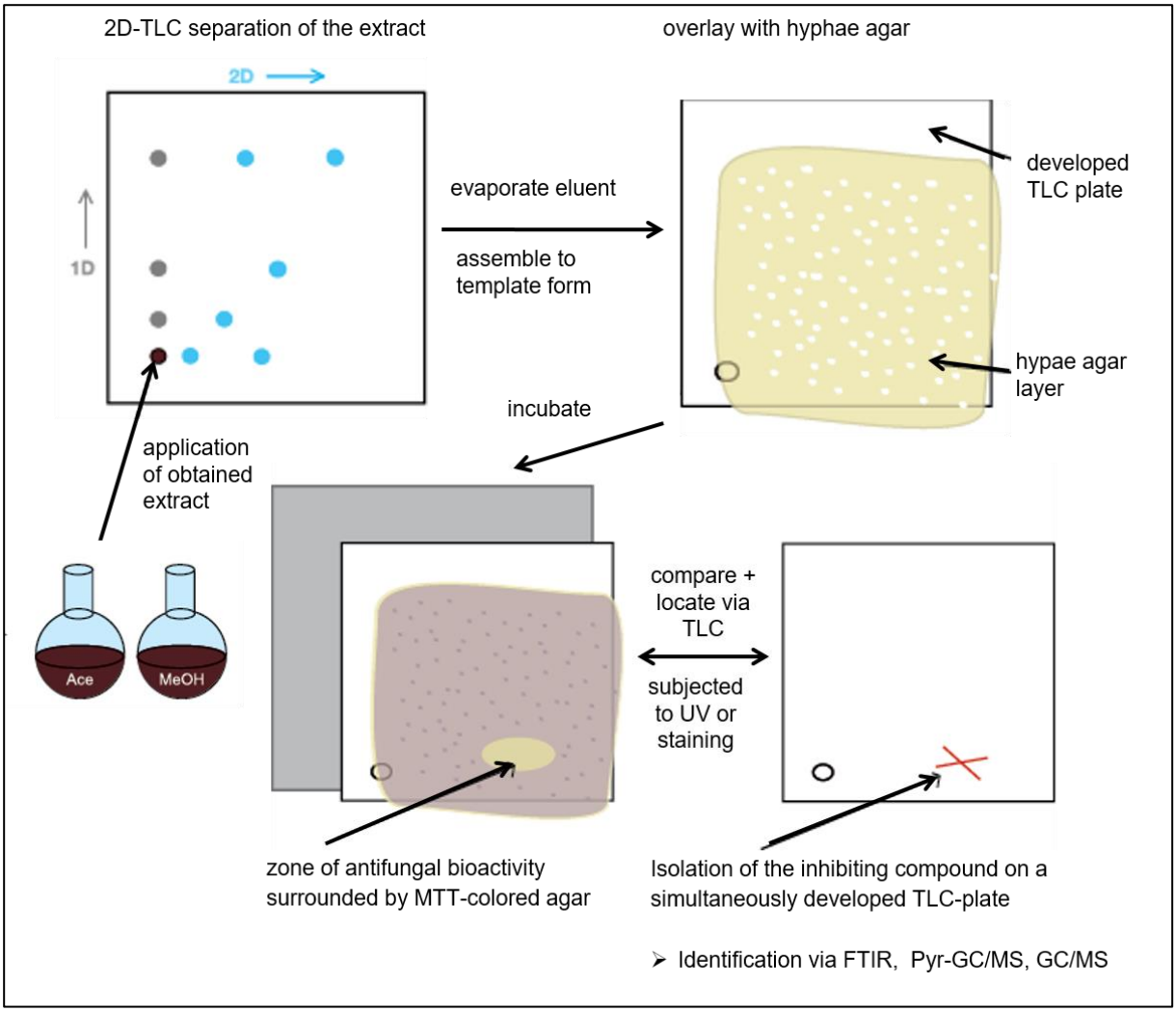


Figure 11: Schematic overview over the agar-overlay method

5.8 Fourier-transform infrared spectroscopy (FT-IR)

FTIR spectra are collected on a Nicolet is50 (Thermo Fisher) spectrometer with 16 scans/sample. The spectra are measured in extinction mode with a resolution of 0.09 cm^{-1} and recorded in a range from 4000 – 400 cm^{-1}. The dried samples of spumates or extracts (1 mg) are mixed with potassium bromide (KBr, 300 mg) using a vibrating mill (Perkin-Elmer, Waltham, PA, USA) and pressed to a pellet. Averaged spectra are baseline corrected (4-6 points) and normalized at 1045-1081 cm^{-1}.

5.9 Acidic hydrolysis and sugar analysis

Sugar analysis is done using a Biotronik LC 3000 to determine mono- and disaccharides directly as well as after hydrolysis with trifluoro acetic acid (TFA). Sugars are analysed in water extracts

and in selected spumate (foamed with N_2) thereof. 30 mg of extract or spumate are hydrolysed with 4.8 g TFA for 20 min at 102 °C in a thermoblock. Saccharides were detected via photometric detection (560 - 570 nm) of a bichinolat/Cu^+-complex, which is proportional to the amount of reducing sugars in the sample. Identification and quantification is done by external calibration (standard solutions consisting of cellobiose, rhamnose, mannose, arabinose, galactose, xylose and glucose).

5.10 Acidic hydrolysis and analysis of the aglycone

The spumates generated without a tenside (PEQ and MAS) are subjected to an acidic hydrolysis, followed by a liquid-liquid extraction with an organic solvent. For this purpose, 20 mg of the spumate are placed into a hydrolysis flask and diluted with 20 ml hydrochloric acid (HCl, 2M). The flask is placed into a thermoblock at 120 °C for 2 h and cooled to room temperature. The hydrolysate is extracted five times with 50 ml dichloromethane. After drying, the organic phase is subjected to GC/MS analysis.

5.11 Gas chromatography-mass spectrometry (GC/MS)

5.11.1 Liquid injection

Extracts and spumates are studied after silylation with BSTFA/TMCS (N,O-bis(trimethylsilyl)trifluoroacetamide/chlorotrimethylsilane; Sigma-Aldrich) by means of GC/MS (MSD 5975C by Agilent Technologies, Santa Clara, CA, USA). 1 mg sample is solved in 200 µl dimethylformamide (DMF) (4 mg/ml) and 50 µl BSTFA/TMCS are added. The sample is placed in a thermoblock at 80°C for 1 h. 1 µl of the sample is injected at an injector temperature of 300°C with a split of 30:1 (39 ml/min). GC is heated at 10 °C/min from 100 °C to 300 °C (held isothermally for 15 min). A HP5 separation column from SGE is used: 15 m x 0.25 mm, 0.25 µm film. Ionization: -70 eV. Detector temperature is set to 250°C, MS source to 230 °C. MS is recorded in a mass range from 40-800 amu.

Identification of compounds is done via comparison of spectra with a NIST08 database or measurement of reference compounds.

Quantification of a substance is done via the measured area of the peak in the chromatogram (area counts) using heneicosanic acid as an internal standard without considering different response factors. The mass of all detected and quantified compounds in the chromatogram in

relation to the mass of the sample used for analysis is expressed as percentage of “total quantified compounds”, reflecting the detectable (via GC/MS) parts of the measured sample.

The substance spots from the TLC plates were dissolved in 5 ml methanol and were separated from the silica via microfiltration. After evaporating the solvent, the residue was dissolved in 100 µl dimethylformamide (DMF) and 30 µl BSTFA/TMCS (N.O-bis(trimethylsilyl)trifluoroacetamide / chlorotrimethylsilane; Sigma-Aldrich).). A SGE-BPX 5 separation column was used: 15 m x 0.25 mm. 0.25 µm film. All other settings and further preparation are chosen as described above.

5.11.2 Pyrolysis - GC/MS

Pyrolysis temperature was 450°C for 0.2 min. The carrier gas was He. GC was heated at 7 °C/min from 40 °C to 320 °C (held isothermally for 15 min with a total runtime of 60 min.). A SGE-BPX 5 separation column was used: 15 m x 0.25 mm. 0.25 µm film. Ionization: -70 eV. Detector temperature was set to 150 °C. MS source to 230 °C and MS was recorded in a mass range from 40-800 amu.

6 Results and Discussion

The results presented in this chapter are a reproduction of the two publications this thesis is based upon.

6.1 Application of Foam Fractionation to Wood

Objective 1:

Providing a selective isolation method for potentially bioactive substances by enriching compounds (extractives) from wood meal as well as from wood water extracts using foam fractionation.

6.1.1 Is the foam fractionation principally applicable to wood samples? Does it make a difference, if milled wood or water extracts as a raw material is chosen for the initial solution?

The tested wood species behave quite different in the experiments, which is due to the type and amount of the present extractives. Two of the tested species show outstanding performance: Massaranduba (MAS) wood meal/water suspension and its water extract give stable and bulky foams, which are difficult to collapse while Pequia (PEQ) samples give moderate stable foams, which break down easier. One major difference between the wood meal/water suspension and water extract can be observed while foaming Ipé Noir (IPN) as only the water extract can be foamed successfully.

In contrast, the bubbles, which emerge out of all other wood species samples, collapse before they leave the flask with the initial solution. Furthermore, it is observed, that for wood meal samples (in this case PEQ) which are very hydrophobic the gas flow rates should be lowered, otherwise the wood particles tend to entrain with the bubbles.

Comparing the raw material, it is observed the yield of spumate 2 (origin: water extract) was higher than that of spumate1 (origin: wood meal). This can be due to the longer extraction time as the initial solution is already a conventional water extract. However, this is strictly speaking only observed while using CO_2 as a foaming gas and therefore pH-dependent components may also contribute.

The comparison of the yields based on the raw material in combination with the foaming gas N_2, tend to show the same behaviour but are more indecisive and unspecific.

The results showed a principal applicability of the method on wooden material and showed different results depending on whether wood meal or water extracts are chosen as raw material, in particular in the case of IPN foam fractionation became applicable only by using the water extracts as raw material.

6.1.2 Are added tensides to foam non-surface-active extracts effective to accumulate specific components and would they make the method suitable for a broader spectrum of (wood-) species and substance classes?

To enable the experiments to the samples, which do not foam for their selves, additional foaming agents (SDS and Tween20) are tested. By using SDS, spumates of Bangkirai (BAN), Merbau (MER) and Muiracatiara (MUI) as well as the spumate 2 of IPN can be successfully produced, too (Table 4). Tween20 is also tested, because the described enrichment factor is higher [168], but only IPN´s spumate 2 can be obtained, albeit barely. In the case of BAN, MER and MUI, no foam formation could be observed using Tween20. Spumate 1 of IPN cannot be produced at all under these conditions. This may be due to the contact time between the wood meal and the water, which is short, compared to the time of conventional water extraction (48 h) and is possibly not long enough to solve the compounds that interact with the additives.

Table 4: Foamability of the samples and used tenside

Wood species	Spumate1	Spumate2	used tenside
BAN	+	+	SDS
IPN	-	+	SDS
ITA	-	-	-
JAT	-	-	-
MAS	+++	+++	not necessary
MER	+	+	SDS
MUI	+	+	SDS
PEQ	++	++	not necessary

- no foam formation + low foam formation ++ moderate foam formation +++ very good foam formation

Under the tested conditions, with gas flow rates not higher than 500 ml/min, Jatoba (JAT) and Itaúba (ITA) do not form enough stable foam to ascend the column. They cannot be foamed at all even if the tested tensides were added.

However, as detected by GC/MS, an accumulation of some organic acids is achieved. In MUI, a clear accumulation especially of Gallic acid as well as (epi)catechin(-gallate) is observed. Thus, a significant effect of the foaming agent can be ascertained. It is known that catechins cannot be foamed without additives [167] because of their hydrophilic character. It was found by Parlar et al. [168] that catechins can be foamed very well adding Tween20 to the initial solution. In contrast, none of the wood species in this study, which contain catechins, can be foamed with Tween20 but by using SDS. Some of the extractives` components of these wood species must have inactivated Tween20, so it cannot produce a sufficient amount of foam.

For analytical purpose, the content of the additive is not a huge problem, even if it disturbs the gravimetric measurements. Semi-quantitative determination is achieved by means of energy dispersive X-ray spectroscopy that shows a content of SDS at about 4.8 % in MER wood spumate foamed with N_2. However, in case the components of the spumates should be further processed, the tenside should be removed as it probably interferes with following applications.

Different methods were tested to separate the tenside from the spumate. Precipitation with KCl as reported by Carro et al. [170] at low temperature and acidic pH-value is achieved but the results are not satisfying as a considerable amount of the extractives is precipitated as well. Furthermore, relatively high amounts of KCl are needed, making the method unsuitable to obtain SDS free extract largely. Using an amphoteric resin, good separation results are obtained but for instance, small amounts of extractives are removed from the solution as well and cannot be recovered by washing the resin sufficiently. Furthermore, it is not possible to regenerate the resin and it is therefore too expensive to provide a suitable method to purify the extractives at a larger scale.

In summary it can be concluded, that the addition of additives can help to apply the foam fractionation to wood species that do not foam sufficiently for their self, meaning that they do not contain enough surface-active substances. This provides the possibility to achieve separations and accumulations for other substance classes, using foam fractionation.

6.1.3 Which influence have sizes of bubble devices and the usage of either N_2 or CO_2 as performing gases on the effectiveness of the method/ yield?

If foam fractionation works, both sample types can be foamed in both devices and in combination with both gas types.

By comparing the size of the devices, it can be seen that the higher amounts of extractable components in spumate (Figure 12) can be found in device 1. This is possibly due to a disintegration of the foam in the longer column of device 2. Comparing the spumate´s origins (the raw material), it is observed that the yield of spumate (foamed with CO_2) is higher, independent of the device size. This can be due to the longer extraction time (initial solution is already a conventional water extract) and the above-described higher extract yield.

Differences in the performance of treatments carried out with N_2 or CO_2 can be summarized as follows:

- Lower yield of extractable compounds from wood using CO_2 (Figure 12).
- However, the yields of CO_2 foamed samples were more decisive and specific than those gained from foaming with N_2. In some cases, the deviation remained high, even if the determinations are repeated up to 8 times.
- Time of analysis is slightly increasing in all experiments (Table 6) using N_2.
- The spumate collected in the receiving flask produced with CO_2 disintegrates much more rapidly (about 10 min) than the one produced with N_2 (overnight in a cool environment).

A possible reason for the differences observed using CO_2 as foaming gas is shown by Backleh [144] by foaming beer: foam produced with N_2 is more finely pored and creamier than with CO_2. This has influence on the foam structure (higher non-polar surface due to more and smaller bubbles) and thus, on the produced foam amount and on the accumulation.

By performing the experiments with CO_2 a decline in pH-value in the spumate as well as in the residual solution can be observed. Starting with a pH-value of 7 in the wood meal/water suspension and 5 in the water extract, the pH-value declines to 4. Most likely, the dissolved CO_2 forms carbonic acid, which contributes to the pH decline. The adsorption of the molecules

at the gas liquid interface and the extent of their separation can be influenced by the pH-value [167] and can also be the reason for the smaller yield of extractable compounds using CO_2. Comparing all the spumates by means of FTIR, no obvious differences were detected neither between the two foaming gases nor between the sizes of the devices. It seems as both have an influence on the extract yield (higher content of extractable components in device 1) but not on accumulation.

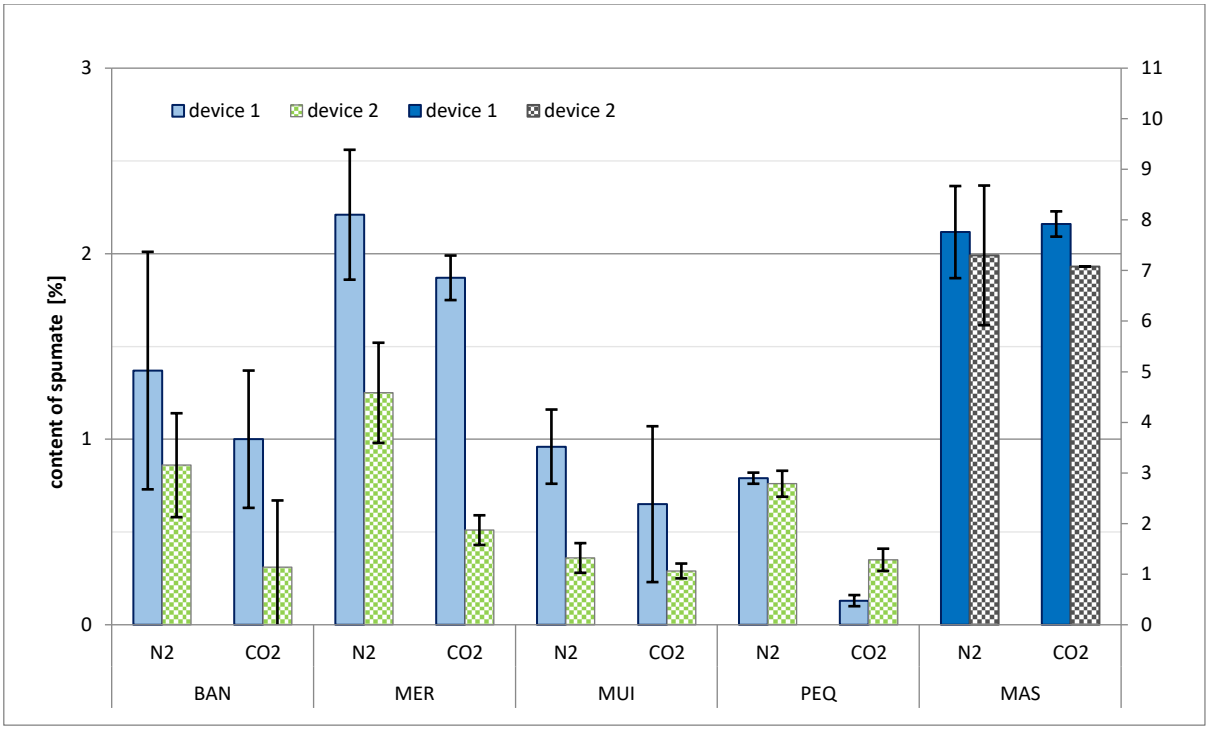


Figure 12: Content of spumate [in %] after treatment with CO_2 and N_2 in two different-sized devices. Content of MAS spumates refer to the second vertical axis.

Comparing device 1 and device 2, no differences could be detected via GC/MS (Table 5). In general, GC/MS quantification shows that the highest amounts are found in the spumates foamed with N_2 (water extract and wood meal) while the lowest contents are found in the wood spumate foamed with CO_2. Because spumate 2 of IPN cannot be compared to other spumates, the results are not mentioned in the table. However, slightly higher amounts of organic acids and triterpenes (e.g., $C_{30}H_{50}O_2$ like erythrodiol or derivates) are detected in the spumate.

6.1.4 Are there differences between the spumates and the water extract regarding the extract components, their concentration and the extract amount?

Massaranduba: Comparing the yield of spumates and water extracts, it was observed that the amount of extractable compounds from MAS in spumate 1 (7-8 %) is similar to that of conventional water extraction (9 %), meaning that nearly all water extractable compounds from MAS are foamable saponins. These findings are supported by the results of the sugar analysis. The small amount of mono- and disaccharides and the high amount of hydrolysed sugars found in the spumate indicates a high amount of oligosaccharides, which are very likely present as saponins. The sugars are attached at the aglycone, are therefore transferred to the spumate with the bubbles, and can be detected there after hydrolysation. Furthermore, MAS has a very consistent proportional composition of hydrolysed sugars between the water extract and the spumate, indicating that there is an accumulation of components but without a separation of different aglycones. Noticeable is the high proportion of Rhamnose after hydrolysation that only occurs in MAS to that extend in the study.

Furthermore, FTIR spectroscopy reveals only little differences between the water extract and the spumate regarding the dominating absorptions detected. A slight decrease of some band heights in the spumate can be observed indicating that there are different concentrations of some extractives between the spumate and the water extract. Hence, the compounds causing these absorptions show a lower surface activity and are therefore not extracted by foam fractionation to the same extent. MAS contains high amounts of saponins and consequently, absorbances that can be assigned to sugars and different aglycones like oleanolic or bassic acid are increased in spumate as accumulation happens.

The GC/MS spectra of MAS spumate compared to the spectra of the conventional water extract confirm the FTIR results. Both – the spumates and the water extract – contain the same compounds. As is known, MAS contains oleanolic acid (34, Table 7) [7] and bassic acid [120]. Based on the measured spectra, both compounds cannot be confirmed but ursolic acid (29, Table 7) and probably quillaic acid (30, Table 7) were identified instead. As Burnouf-Radosevich et al. [242] showed, oleanolic acid (34) and ursolic acid (29) could be distinguished from each other by the relative intensities of the two MS- fragments 73 and 203, which are found to be 90/100 for oleanolic acid (34) and 100/70 for ursolic acid (29) (Table 7). For further analysis, the MAS-spumate was subjected to acid hydrolysis, however the performance was insufficient.

Nevertheless, at least six triterpenes could be detected in the hydrolysate, which could not yet be identified beyond doubt by now.

It is observed that foam fractionation is successful for MAS as both compounds can be detected in the spumate as well. They seem to be accumulated in the spumate foamed with N_2 but not with CO_2.

Pequia:

On the contrary, PEQ samples show more obvious differences between the spumate and the water extracts. 3.9% extractives in conventional water extract cannot be gained by foam fractionation, which hardly reaches 1% extractives content. Alike MAS, the most remarkable difference regarding the sugar analysis is again the one between the small amount of mono- and disaccharides and the high amount of hydrolysed sugars in the spumate. Again, presence of saponins is plausible and is likely the reason for the good performance of the two wood samples during the experiment.

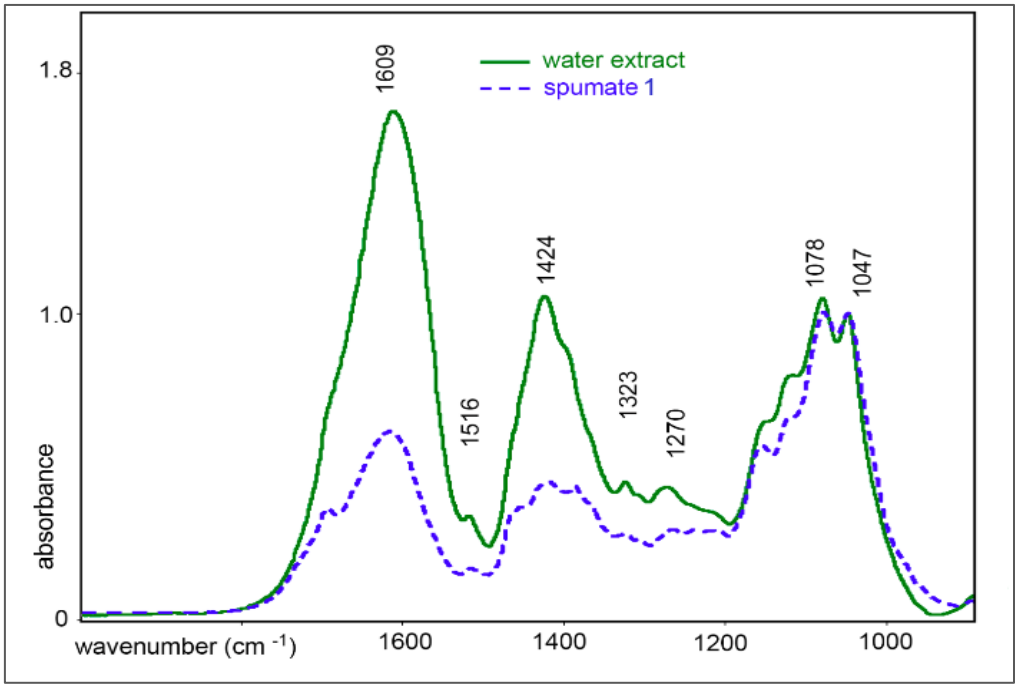


Figure 13: CO_2-spumate 1 of PEQ compared to the water extract; spectra normalized at 1047 cm^{-1}

The most noticeable differences in the FTIR spectra (Figure 13) origin from aromatic skeletal vibrations and from C-O stretch vibrations as well as C-H plane deformations. Gallic acid is one of the aglycones in PEQ (Table 5) and can therefore be the origin of the aromatic

absorbances. This would be consistent with a conformation like the suggested Hederagenin-galloyl-glc-ester [109]. Furthermore, FTIR implies a high content of hydrocarbons.

As GC/MS shows mainly sugars and a slight accumulation of gallic acid (Table 5) in the N_2 spumate of the water extract of PEQ, the spumate is also subjected to acid hydrolysis to elucidate the components. In the gained hydrolysate, hederagenin ((3β)-3,23-dihydroxyolean-12-en-28-oic acid) (31) could be identified by comparison with the measured spectra of a reference substance. It is known to be bioactive and is already used pharmaceutically. Additionally, GC/MS reveals gallic acid as aglycone in the spumate of PEQ wood. Hederagenin acylated with a galloyl group is known from the stem bark of PEQ: rha-gal-glc–hederagenin-galloyl-glc-ester [109]. GC/MS analysis as well as the behaviour of the PEQ water extract (hardly soluble, tends to precipitate easily) suggests a similar conformation of the aglycone from the spumate. Additionally, oleanolic acid and three more, not plainly identified triterpenes were found in the spumate.

All other wood samples have a distinctly lower yield after foam fractionation than after conventional water extraction showing that a bigger part of the compounds consists of water-soluble compounds without a hydrophobic part of the molecule being big enough to be adsorbed at the bubble surface and be entrained with the bubble in the foam.

Sugar analysis results for these samples provide a very species dependent picture regarding the kind and distribution either of the free as well as for the hydrolysed sugars, hardly indicating for any accumulation of wood components. Total amounts (referred to the sample taken) show that only a very small quantity of sugars can be found in the spumate. This is due to the generally little spumate amount gained by each experiment. Regarding the “total amount of quantified compounds”, the difference between the water extract and the spumates is mainly due to the lower content of sugars in these spumates.

This trend can also be seen via FTIR spectroscopy, even if additives are used to perform the experiments. As the absorbances of the additive strongly interfere with the absorbances from the sample, FTIR is not able to detect minor differences in these regions properly.

Regarding the quantified compounds via GC/MS, only minor differences between the spumates and the water extracts from BAN (triterpenes) and MER (flavonoids: i.e., Fisitinidol / Robinetinidol and isomers) are detected (Tab. 5). In IPN slightly higher amounts of organic

acids and triterpenes (e.g., $C_{30}H_{50}O_2$ like Erythrodiol or derivates) are detected in the spumate. Lapachone, which is detected in the water extract, cannot be found in the spumate.

Table 5 obviously shows that only a minor part of the compounds is detectable via GC/MS and the main peaks in the chromatograms are composed of carbohydrates. Although alternative derivatizations were performed, all other detected components were only found in traces and could not be identified beyond doubt by now. This is due to limitation of GC/MS when analysing very high molecular compounds.

Table 5: MS Fragments and assignment of identified and quantified compounds,

species	Spumate 1		Spumate 2		Water extract	MS Fragments *(of TMS-derivates)*	identified substances	substance class
foaming gas	**CO_2**	**N_2**	**CO_2**	**N_2**				
BAN								
Total quant. compounds [%]	12.95	17.59	7.60	13.27	22.86			
Quantified substances [%]	0.24	0.32	x	0.25	0.38	458(5) 433(18) 399(10) 355(12) 311(10) 281(69) 73(100)	*Gallic acid*	organic acids
	4.62	6.78	3.69	6.04	9.45	662(2) 647(10) 633(8) 207(23) 179(16) 73(100)		n.i.
						688(2) 635(8) 431(10) 255(10) 179(20) 193(31) 147(10) 73(100)		n.i.
MER								
Total quant. compounds [%]	7. 53	12.28	5.80	10.14	13.11			
Quantified substances [%]	0.22	0.14	0.32	x	x	355(10) 307(3) 265(5) 245(13) 233(24) 147(62) 73(100)	*butanedioic acid*	organic acids
	x	0.10	x	x	0.10	458(5) 433(18) 399(10) 355(12) 311(10) 281(69) 73(100)	*Gallic acid*	
	6.95	9.27	5.10	9.86	12.40	562(8) 368(100) 267(39) 73(60) 650(10) 560(5) 456(100) 368(5) 267(44) 73(75) 650(5) 560(2) 368(100) 355(28) 73(51) 647(5) 599 487 147	*fisetinidol or isomers* *robinetinidol or* isomers *(epi-)catechin* *quercetin*	flavonoids and flavonoid fragments
MUI								
Total quant. compounds [%]	4.74	31.03	0.82	24.64	12.28			
Quantified substances [%]	2.65	18.10	0.59	12.58	6.31	458(5) 433(18) 399(10) 355(12) 311(10) 281(69) 73(100)	*Gallic acid*	organic acids
	0.60	4.03	0.23	6.09	3.9	650(3) 560(2) 368(100) 355(28) 73 (51) 560(45) 471(25) 369 (70) 267(12) 207(40) 73(100)	*(epi-)catechin* *catechin (isomer)*	flavonoids
MAS								
Total quant. compounds [%]	21.40	29.89	18.30	35.80	42.69			
Quantified substances [%]	6.00	10.16	7.00	9.70	8.37	585[M-15] (8) 482(15) 393(25) 320(25) 203(50) 73(100) 673[M-15] (5) 570(15) 320(30) 203(60) 73(100) 687[M-15] 612 584 320 302 73 (100)	*Ursolic acid* *Hederagenin* *Quillaic acid*	triterpenes
PEQ								
Total quant. compounds [%]	20.90	31.54	16.71	30.50	32.08			
Quantified substances [%]	9.86	16.07	7.53	15.55	14.91	335(4) 245(11) 233(18) 147(54) 73(100) 465(3) 375(15) 363(18) 247(16) 273(76) 147(58) 73(100)	*malic acid* *carballylic acid*	organic acids
	0.95	1.07	0.88	1.30	1.07	458(5) 433(18) 399(10) 355(12) 311(10) 281(69) 73(100)	*Gallic acid*	

6.1.5 Does a possible accumulation of specific substances directly out of the wood meal may open up a possibility for time and cost reduction?

Apart from Massaranduba, foam fractionation has to be repeated several times to gain the same masses of extracts as from conventional water extraction. The higher extract content in water extracts can be due to the longer extraction time, which is method-specific. While performing the conventional water extraction where the wood meal is stirred in water for 48 h (according to Tappi Standard T207 cm-99, 1999), foam separations are performed within 30 min up to 5 h. It is observed that foam formation of MAS and PEQ continues for a longer time compared to the other wood species that are foamed using a tenside. Even if bubbling time of MAS and PEQ samples varies strongly, foam fractionation of BAN, IPN, MER and MUI using SDS is finished at the latest after 2-3 h (Table 6). Over all samples, the bubbling time in device 2 tends to be half an hour longer than in device 1, but this is within the variance of the experiments and has therefore no significance.

Table 6: Time of analysis of different foam fractionations

Wood species	**Bubbling time (h)**			
	Spumate 1		**Spumate 2**	
Foaming gas	CO_2	N_2	CO_2	N_2
BAN	0.5 - 1.5	1.5 - 2.0	1.5 - 2.0	2.0 - 3.0
IPN	-	-	1.0	-
MAS	2.5 - 6.5	3.5 - 5.5	3.0 - 5.0	3.5 - 5.0
MER	1.0 - 1.5	1.5 - 2.0	1.5 - 2.0	2.0 - 2.5
MUI	0.5 - 1.0	1.0 - 1.5	1.0 - 1.5	1.0 - 2.0
PEQ	0.5 - 3.0	0.5 - 3.5	1.0 - 3.0	2.0 - 4.0

Compared to conventional water extraction, foam fractionation is not an alternative to a comprehensive analysis of water extractable compounds. At present, foam fractionation can be applied to wood species that contain sufficient amounts of surface-active substances to be „self-foaming “. A principal answer concerning time and cost reduction cannot be given from the experiments carried out in this study.

6.2 Direct Bioautography for the Screening of Selected Tropical Wood Extracts against Basidiomycetes

Objective 2: Developing a target guided fractionation for wood extractives by establishing a direct bioautography method for hardly sporulating basidiomycetes using a hyphae agar.

6.2.1 Is the hyphae agar applicable to the TLC plate?

After placing in the TLC-plate into the metal template, the warm agar is distributed evenly by using a syringe or by directly pouring it out of the Erlenmeyer flask. After a few seconds of cooling the silica layer of the TLC plate and the poured agar result in a homogeneous and gelatinous layer on the glass plate (Figure). This provides a good starting situation for the extractives to diffuse into the agar and to get in contact with the homogenized hyphae.
Further improvement could be made by placing the template together with the TLC-plate on a heating plate to adjust the temperature before pouring the agar. This would enhance an even distribution because the hardening of the ager is temperature dependent and a very cold plate can lead to an undulated agar surface.

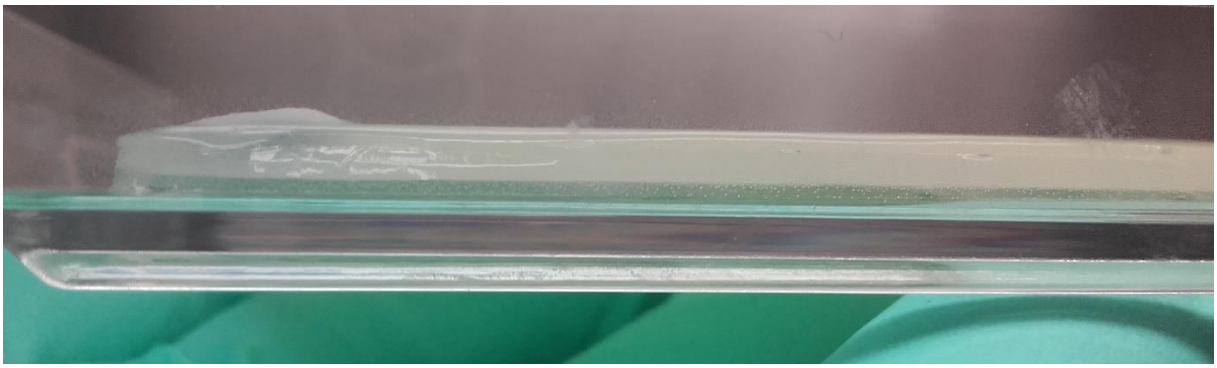


Figure 19: Gelatinous layer on the glass plate

6.2.2 Do the homogenized cultures recover fast enough and does the usage of viability stain MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] provide a good basis/ an improvement to evaluate fungal inhibition?

The best results concerning the homogenized hyphae are achieved using cultures that were incubated for 14 days in a liquid broth and adjusted to 2% hyphae in the PDA. Blue spots in the agar show that the hyphae are reactivating after the homoganisation process. It is observed that the blue staining on the *R. placeta* plate (Figure 14) is more homogenious than with *T. versicolor*. This probably results from the fact that *T. versicolor* (Figure 15) has a leather-kind matter that is more difficult to shred than *R. placenta*.

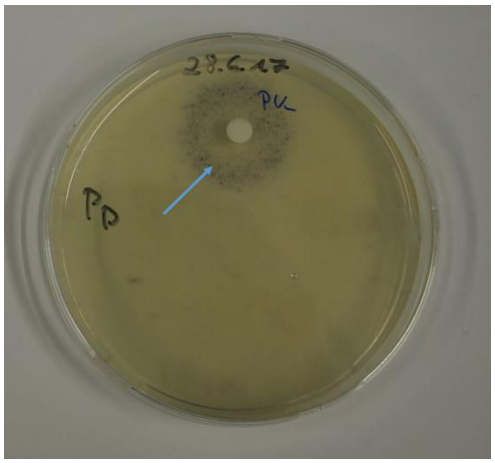


Figure 14: Hyphae agar of *R. placenta after* 2 days*:* blue stained flocks circled around the positive control shows reactivation of activity

Furthermore *R. placenta* reactivates faster than *T. versicolor* but is then growing slower afterwards, needing several weeks to overgrow the agarplate. *T. versicolor* needs a longer reactivation time instead, but is then overgrowing the agar plate in less than 5 days. The longer reactivation time is the result of an elevated stress level as a reaction to the harsh homogenisation process [243] which obviously affects *R. placenta* less than *T. versicolor*. The homogenized hyphae tend to sink to the bottom of the agar plate and are therefore located in an almost anaerobe environment. *R. placenta* seems to have more difficulties to overcome this situation and therefore needs more time to overgrow the agar surface.

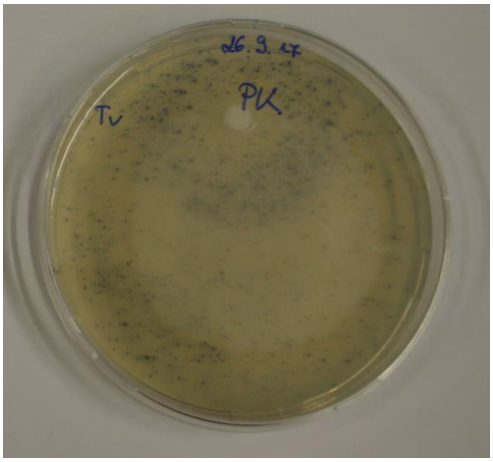


Figure 15: Hyphae agar of *T. versicolor* after 2 days, the hyphae agar is less homogeneous than the one from *R. placenta*, indicated by the visible blue different sizes flocks. The circle around the positive control is less pronounced.

The fungi activity tends to start around the positive control, which is indicated by the blue stained flocks circled around the positive control (Figure 14, Figure 15). The presence of the posive control (thujaplicin) which diffused into the agar around the cellulose disk is probably activating the defence mechanisms [244] of the fungi. Most likely, this is due to a secretion of laccase [245] which is more pronounced in the *R. placenta* than in the *T. versicolor* sample.

A difficult point during the production procedure of the hyphae agar is the temperature of the agar at the time were the hyphae are mixed into it. It has to be hot enough to be pourable onto the TLC-plate but cool enough not to kill the already stressed hyphae. The temperature of the agar was tested by placing the filled erlenmeyer flask onto the skin in the crook of the arm. As in some test the fungi didn´t reactivate on the plate, more attention must be paid to the temperature in the future. A possibilty could be measuring the temperature contact-free with e.g. a laser-thermometer.

To achieve the desired colour effect on one hand and not to perhaps falsify the results due to an excess of added dye on the other, different concentrations of the dye MTT are tested (Figure 16. It is possible to combine the homogenized hyphae from *T. versicolor* and *R. placenta* with MTT as an activity indicator, with a concentration of 40 µg ml^{-1} for the best optical results. Figure 22 shows the results of a diffusion test to evaluate the best conditions for the tests using β-thujaplicin, an established fungicide [98] at a concentration of 0.2 g l^{-1} that produced clear zones of fungal inhibition. Fungal growth inhibition could be clearly recognized by an absence of fungal growth and a bright beige colouring of the agar surrounded by the blue agar coloured by the MTT reaction.

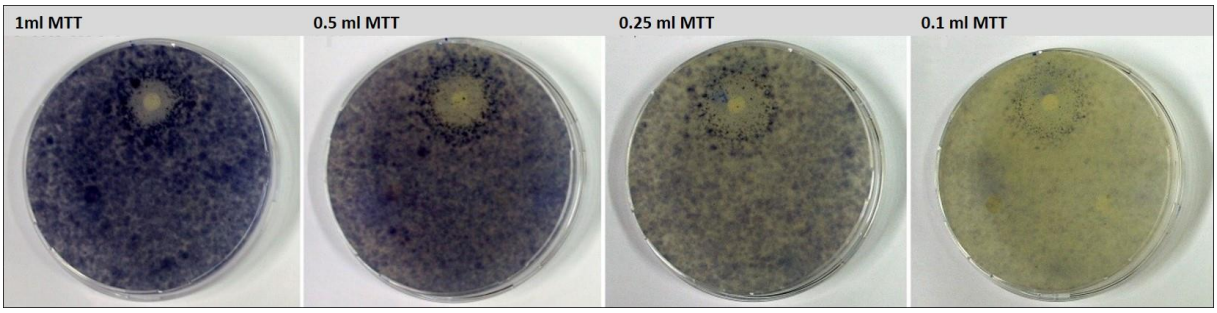


Figure 16**:** Different volumes (1 ml; 0.5 ml; 0.25 ml; 0.10 ml) of MTT in 250 ml of hyphae-PDA from *R. placenta,* after eight days. ß-Thujaplicin (conc.: 0.2 g/l) was used as a positive control.

The hyphae that are located close to the surface rapidly form white spots of hyphal growth on the agar surface. This takes more time for the hyphae located deeper in the agar. Consequently, parts of the plate are almost overgrown, while in other parts, less growth is visible. This may lead to misinterpretations of the bioactive zones (false positives). As a completely homogeneous dispersion of the hyphae is difficult, the optical determination of the bioactive zones is much more exact using the MTT vitality stain than an evaluation by judging the hyphal growth alone. Even though the intensity of the blue colouring varies, the optical determination of the bioactive zones is highly improved. The reaction time until the blue colouring of the MTT appears is

varying from 3 to 8 days, whereby the response time for *R. placenta* cultures is longer (concerning the whole plate). This was the case for all tested wood extracts (not all bioassay data shown).
An additional finding was that the hyphae solution (in 0.9% NaCl) could be stored in the refrigerator for up to 3 weeks. Activity tests showed that the hyphae were still vital after this period and showed activity in the hyphae agar. Therefore, it is also possible to prepare the hyphae solution prior to the preparation of the TLC plates.

6.2.3 Is it possible to determine the antifungal activity of the extract fractions via 2D-TLC agar overlay method and can the inhibiting compounds be assigned to the bioactive zones?

Figure 23 (I–IV) shows the bioassay results from *Astronium graveolens* acetone and methanol extracts tested against *T. versicolor* and *R. placenta*. The results of the method are presented for this species as an example. However, it was also carried out for Bangkirai, Itaúba and Merbau. Results from these tests are presented briefly without showing pictures.

For a better visualization of the bioactive zones on the photos shown in Figure 23 (I–IV), the zones a marked by a circle. Inside the circles, the partitioned antifungal constituents of the extract can be found. It could be observed that both fungi are not able to distinguish between compounds that are located very close together on the TLC. Instead, they formed inhibition zones with no or less fungal activity. On the one hand, this could be due to the fungal sensitivity, but more on the other hand, the substances very close together are more likely to be mixed during the diffusion process from the silica phase into the agar overlay, although the agar layer is very thin. Therefore, the detected fractions are aggregated to zones of fungal growth inhibition.

On the plates with the acetone extract, five zones with bioactivity against both fungi could be detected: D, E, F, G and H. The methanol extract shares the bioactive zones D, E, G and H, while K was only present in the methanol extract and zone A was only active against *R. placenta*.

In zone A, GC/MS revealed high amounts of a fatty acid, identified as caprylic acid. Fatty acids are known as fungicidal food additives [39] and several studies showed the fungicidal ability of fatty acid formulations like a caprylic acid-based formulation or a caprylicpelargonic- capric acid formulation against several different fungi [246]. A possible hypothesis for the mode of

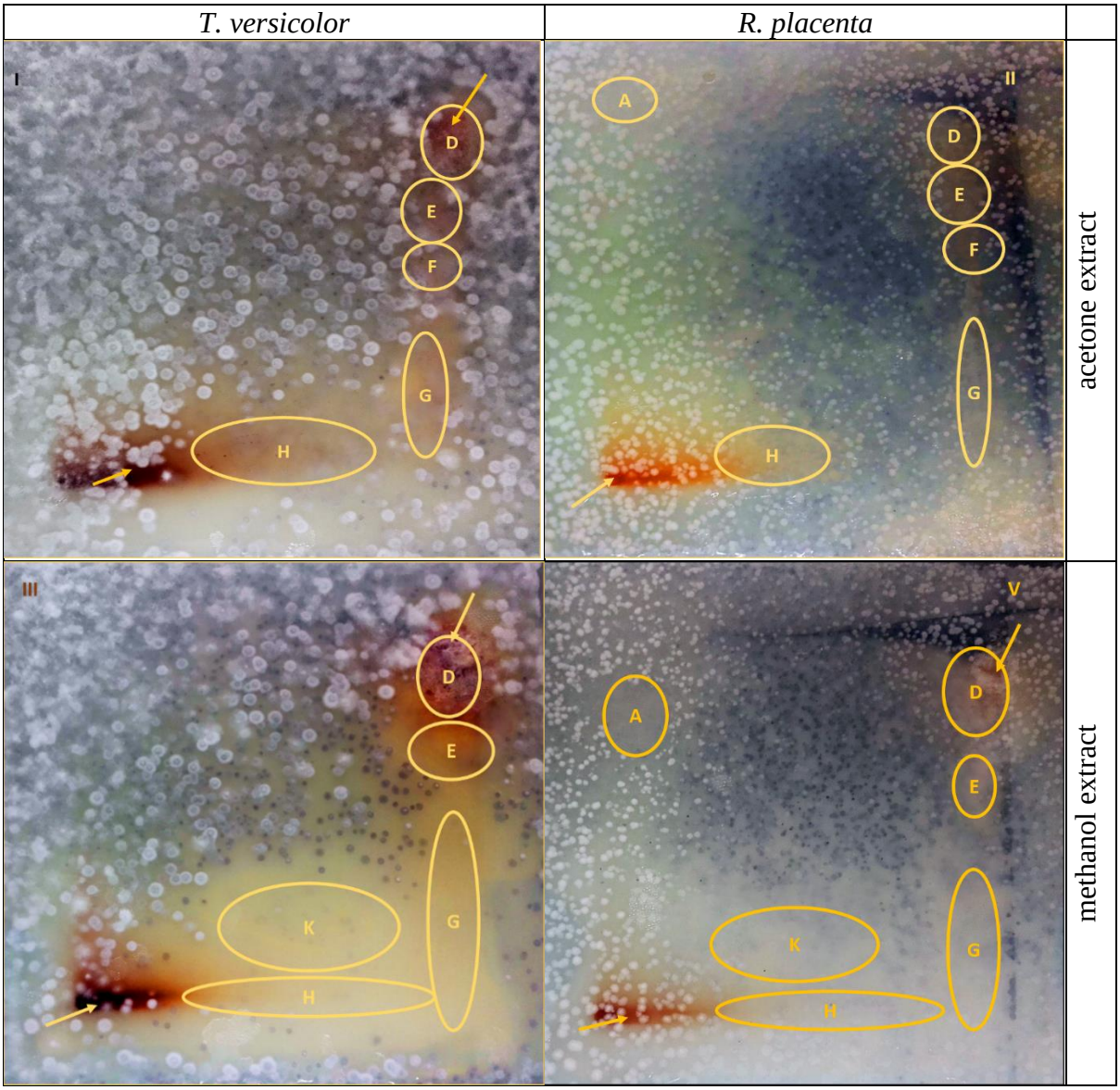


Figure 17: 2D-TLC direct bioautography of *Astronium graveolens* acetone (I/II) and methanol (III/IV) extract: bioactivity response from *T. versicolor* (l) and *R. placenta* (r) after 3 days. Marked and labelled zones are discussed in the text

action is that the fatty acids cause an increase in membrane fluidity, which will result in the leakage of the intracellular components and cell death [39, 246]. Therefore, an influence of caprylic acid on the growth of *R. placenta* hyphae may be also possible. Catechin (15) and an unidentified sterol were observed in zone D as well as two more compounds that are probably (epi-)gallocatechin gallates (4), which could only be detected in the methanol extract.

This is consistent with the fact that zone D on the methanol plates is more intense compared to the respective acetone plate (Figure 23 I/II). It is known that *Astronium* species contain different

hydrolysable tannins like gallotannins and ellagitannins together with different phenolic derivates [11].

In zone E and in the methanol zones H and K, an unidentified flavonoid and gallic acid are detected, as well as two triterpenes in methanol zone G. The bioactive potential of the acetone zones G and H cannot be explained sufficiently at this moment.

The response to the tested extracts is more pronounced in tests with *T. versicolor*. The inhibition zones are brighter, more differentiated and it took longer for the fungi to overgrow the inhibition zones. All zones of the separated extracts show clear inhibition zones and demonstrate that the method can be applied successfully to wood extracts.

Two compounds are detected, which have not been described in *Mezilaurus* species yet: norbicuculline and bicuculline (28), of which the latter is known as a GABA (4- Aminobutanoic acid) receptor antagonist [247], the chief inhibitory neurotransmitter in the central nervous system [248].

Furthermore, all spots, from locations with or without bioactivity in the bioassay, are subjected to GC/MS and measurements reveal that some compounds could be found in all wood samples: palmitic acid and stearic acid as well as different other fatty acids were detected in all zones. Together with varying contents of glycerol, it is supposed that they are present as triglycerides in the extracts, which are partly degraded during the sample preparation.

Most of the detected sugars are found in every examined zone: sometimes as a single component, sometimes associated with other compounds. To test whether the sugars are spread over the plate during the development, samples are taken from places with no substance spot. As these samples are negative to sugar compounds, it is suggested that many of the compounds in the extract are present as glycosides.

It is observed that the acetone and the methanol extracts of each wood sample have zones in common that are located on the same places on the plates. This localization is due to the use of the same solvent mixtures for the separation of the extracts. Furthermore, it seems as if the successive extraction with raising polarity of the solvents do not create the intended “pre-separation” effect and therefore many of the compounds were found in both extracts.

6.2.4 Does the presented technique provide a rapid method to evaluate bioactive components in complex extract mixtures?

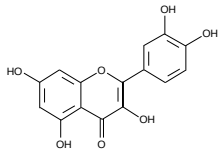
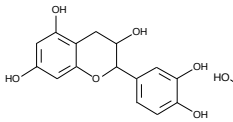
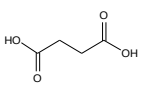
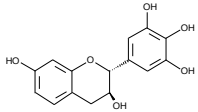
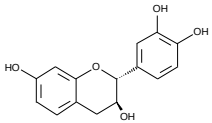
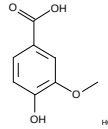
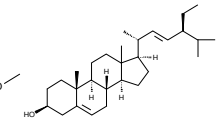
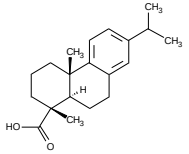
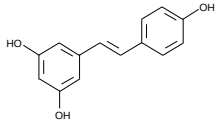
The results show that a part of the natural durability of the tested tropical wood species can certainly be attributed to the detected substances causing the inhibition zones on the TLC plates. Because this inhibition is also dependent on the substance concentrations, the minimal inhibitory concentrations should be determined in a next step. Furthermore, these concentrations must then be related to the concentrations that prevail in the wood. Some of the compounds may meet the requirements in potency and concentration to play an outstanding role for natural durability.

The relationship between durability and extractives contend as well as single compounds is addressed in several studies and both quantity and quality of extractives have a role to play. However, their relative contribution varies considerably from substrate to substrate and it should be kept in mind that there may be several defence strategies a species uses [9][35] . Therefore, consideration should also be given to the entire extractive mixture as well as micro-distribution of the extractives in the wood species [219]. Some of them play a role as an elicitor, regulator or water repellent and may have important influence on the natural durability.

As the main objective of this research was to elucidate whether the principals behind the durability of the tested wood species can be attributed to specific extractives, the following table (Table 7) summarizes the different compounds, which were detected in the examined species.

In both studies, varieties of compounds were detected. Some of them exhibit pronounced bioactivity and are therefore at least a part of the durability strategy the wood species have evolved (e.g., dehydroabietic acid in *Shorea laevis* as well as the alkaloids in *Mezilaurus itauba*).

Table 7: Identified compounds in the species examined in this study. Numbers in brackets are included to create a reference from the text.

<table>
<tr><td>Intsia spp</td><td colspan="2"></td><td></td></tr>
<tr><td></td><td colspan="2">found in spumate</td><td>found in spumate and in bioactivity zone</td><td>Additionally, known from literature</td></tr>
<tr><td colspan="2"> 
quercetin (14)</td><td> 
(epi-) catechin (15)</td><td> 
butanedioic acid (16)</td><td> 
robinetinidol (17)
 
fisetinidol (18)
gallic acid and further not identified flavonoids.</td><td> 
vanillic acid (5)
 
stigmasterol (19)
and various fatty acids [40]</td></tr>
<tr><td>Shorea laevis</td><td colspan="3"></td><td></td></tr>
<tr><td></td><td colspan="2">found in spumate</td><td>bioactivity zone</td><td>Additionally, known from literature</td></tr>
<tr><td colspan="2">gallic acid and some not identified compounds</td><td colspan="2"> 
dehydroabietic acid (21)
 
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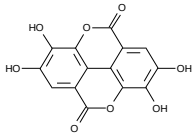
and some not identified triterpenes.</td><td> 
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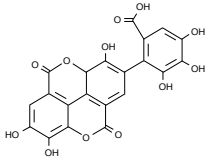
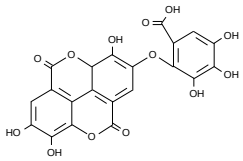
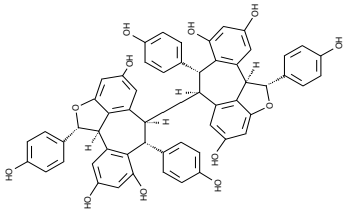
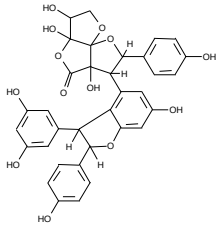
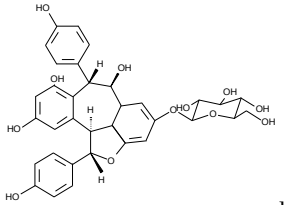
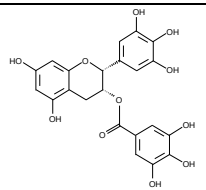
 
flavogallonic acid dilactone (23)
 
valoneic acid dilactone (24) [135]</td></tr>
<tr><td colspan="5">Additionally, from literature (Shorea laevis)</td></tr>
<tr><td colspan="5"> 
hopeaphenol (25) [136]
 
laevifonol (26) and
 
laevifosid (27) [137]</td></tr>
<tr><td colspan="5">Astronium graveolens</td></tr>
<tr><td colspan="2">Found in spumate</td><td colspan="2">Bioactivity zone</td><td>Additionally, from literature</td></tr>
<tr><td colspan="2">gallic acid, (epi-) catechin, not identified flavonoid</td><td colspan="2"> 
(epi-) gallocatechingallate (4) and two not identified triterpenes and an unidentified sterol.</td><td>quercetin</td></tr>
</table>

<table>
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<tr><td>bioactivity zone</td><td>Additionally, from literature</td></tr>
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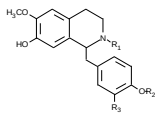
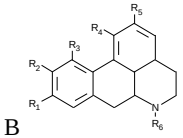
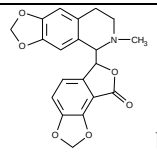

A

B

bicuculline (28)

A
R1; R2; R3 = H: coclaurine
R1, R2 =CH3; R3= OH: reticuline
R1= CH3; R2= H; R3=H: methylcoclaurine

B
R1, R5=OH; R2, R4=OCH3; R3=H; R6=CH3: boldine
R1, R4=OH; R2, R5= OCH3; R3=H; R6=CH3: isoboldine
R1=H; R2, R5= OCH3; R3, R4=OH; R6=CH3: corytuberin
R1=OH; R2, R4, R5 =OCH3; R3, R6 =H: lautrotetanine
R1=OH; R2, R4, R5, R6 =OCH3; R3= H: methyllaurotetanine
R1=H; R2, R4, R5; R6=OCH3; R3=OH: isocorydine
</td><td>neolignans, oxaporphines, pavine, morphinandienones
naphtalenes, α-cadinol, cadinene and valencene</td></tr>
<tr><td colspan="2">Manilkara bidentata</td></tr>
<tr><td>Spumate</td><td>Additionally, from literature</td></tr>
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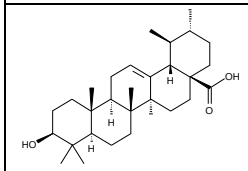
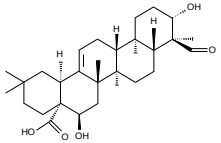
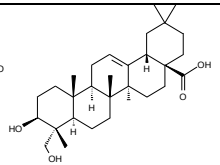
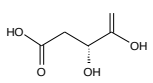
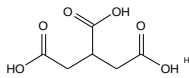
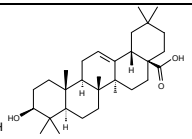




ursolic acid (29) quillaic acid (30) hederagenin (31)
and further not identified triterpenes.
</td><td>balataresinol, cyclolaudenol, α-spinosterol</td></tr>
<tr><td>Caryocar villosum</td><td></td></tr>
<tr><td colspan="2">Spumate</td></tr>
<tr><td></td><td>




malic acid (32) carballylic acid (33) oleanolic acid (34)

gallic acid, hederagenin, and further not identified triterpenes
</td></tr>
</table>

7 Conclusions and future perspectives

In the presented study, the nine tropical wood species Bangkirai (*Shorea laevis*), Ipé Noir (*Handroanthus spp*.), Itaúba (*Mezilaurus itauba*), Jatoba (*Hymenea coubaril*), Massaranduba (*Manilkara bidentata*), Merbau (*Intsia spp*.), Muiracatiara (*Astronium graveolens*), and Pequia (*Caryocar villosum*) were examined with specific emphasis on their extractive chemistry.

Attention was focused on isolation and separation of their extractives as well as on testing the extractives for their bioactivity against two wood degrading fungi. The objective was to elucidate whether the principals behind the durability of the wood can be attributed to specific extractives.

To achieve this goal, two methodological approaches were adapted to wood for the first time:

(1) by isolating, separating and enriching promising compounds in particular saponins in an efficient way by foam fractionation and
(2) by developing a bioassay guided fractionation method where wood extractives are directly applicable to wood decaying fungi, which are not readily sporulating.

The results of foam fractionation showed the principal applicability of the method to wooden materials, but made also clear that the conditions of the experiments have to be adjusted to the targeted extractives of the respective wood species, because the nature and composition of the extractives vary depending on the wood species. It was observed that the process is a suitable specific isolation method to extract selectively saponins in a fraction of time compared to conventional water extraction, without the need to separate them from other extractives. However, it is not an alternative to a comprehensive analysis of water extractable compounds. Due to the requirement that the method should be as simple as possible, it should be restricted to wood species with “self-foaming” extractives like the mentioned saponins. Nevertheless, poor foaming capacity and foam stability of the other wood species can be improved by adding suitable surfactants (tensides), but the subsequent separation of tensides was insufficient. Optimization of various process parameters, such as pH-value of the initial solution, gas flow rate, gas type and the size of the foaming device offer potentials to adapt the foaming process to the specific wood extractive chemistry. For a more versatile application, however, the method needs to be further refined.

The bioautographic agar overlay method and the hyphae agar method were combined. The combination was found to provide a possibility to benefit from the advantages of a direct bioautography (rapid, easy to implement), while testing wood extractives against commonly used basidiomycetes, which are not readily sporulating. The method was successfully employed using *Rhodonia placenta* and *Trametes versicolor* in media seeded with homogenized hyphae which recovered rapidly from the treatment. The added vital stain contributed to the visible assignment of the inhibition zones. The hyphae-agar bioassay provides a more rapid method for the investigation of bioactive wood extracts, enabling isolation and identification of possible key substances. This might accelerate the elucidation of potential structures, for example for biocidal, pharmaceutical or chemical uses. However, the method has to be seen as a possibility for a rapid insight into lesser-known extractives mixtures.

Results from both methods unfolded possible contributions of the extractives to the durability of some of the tested wood species. In Massaranduba *(Manilkara bidentata*) ursolic acid, hederagenin and quillaic acid and in Pequia (*Caryocar villosum*) oleanolic acid and hederagenin were identified from foam fractionation. They are obviously present as saponins, which are known to be bioactive in many cases. For Muiracatiara (*Astronium graveolens*), Merbau (*Intsia spp*.), Bangkirai (*Shorea laevis*) and Itauba (*Mezilaurus itauba*) in most of the bioactive zones of the bioautography several known fungicidal compounds were identified. This can be rated as a proof of principle, with prominent examples like gallic acid, catechin and resveratrol. Furthermore, the isoquinoline alkaloids bicuculline and norbicuculline were detected, which have not been described in Mezilaurus itauba yet.

In further experiments, purified compounds could also be applied in the bioassay to focus the analytical efforts on the possibly causative key compounds. The agar overlay technique can be combined with undeveloped TLC plates, spotted directly with extracts or substances, for example, with different concentrations. Several compounds in the TLC- fractions have not been yet identified. One reason might be the relatively low concentration of some compounds on the TLC, which could therefore not be isolated properly. For a more in-depth analysis, more suitable separation methods are needed as a prior step yielding higher concentrations. Preparative flash chromatography methods provide higher amounts of the pure compounds, so that compounds with weaker potency can be detected as well. Foam fractionation as a selective isolation and accumulation method for potentially bioactive substances may also allow for additional analysis methods, such as nuclear magnetic resonance (NMR). Therefore, different additives, the tweezing technique or the use of multi-stage foam fractionation [145] should be

tested to improve the separation and accumulation of wood extract components. A cost-efficient and applicable way to separate and recover added surfactants has to be developed. Enrichment factors of the separated components should be determined to be able to estimate the ecologic and economic benefit of the method when applied to lignocellulosic biomass.

The identification of bioactive compounds with valuable properties is a high priority research field. Several thousand counts of references in the web of knowledge (by January 2020 counting key words; references included since January 2000: "Bioassay" 45.599 references, "Biochemical detection" 13.593 references, "Bioassay-guided fractionation" 2.604 references, "Wood extractives" 2.623 references) show that studying phytochemicals from plants became popular. The only low increasing amount of registered plant based commercial products does not reflect this immense scientific effort. The success of modern methods in analytical chemistry sometimes masks the problem that the ever-increasing amount of analytical data does not necessarily give more insight of practical relevance [139]. The target should be not to build an ever-growing list of chemical compounds but to connect instrumental analysis with biological active compounds to create effect directed analysis methods. Analytical limitations and challenges in the elucidation of the multi-component mixtures are their extraction, identification and determination, which still create problems. The observed bioactivity effects often cannot be assigned to specific chemical compounds and most of them have to be purified by a combination of several chromatographic techniques and various other purification methods to isolate the bioactive compounds. Many of the bioactivity tests are too complex to be automated or miniaturized, and once extracted many bio components lack chemical stability. High costs of complex systems and reagents, insufficient method standardization and missing simple set-ups [139] are leading to an extensive development need. Poor acceptance could be the consequence in an interdisciplinary field, which is in need of broad knowledge and often suffers from poor interaction between non-related application fields. An interconnection of expert-knowledge from microbiology, chemistry and application orientated engineering sciences could possibly transform fundamental insights into practical benefits.

Although the current focus of wood preservation research is to develop wood protection based on natural products with little or no toxicity, there is still limited industrial uptake of the compounds. Certain limitations decelerate implementations, like occurring discrepancies between laboratory and field performance and variability in efficacy related to environmental conditions and fixation problems. As high and broad active levels are demanded in combination with small application rates with no or only small toxic side effects, especially legislation and

registration difficulties can pose major problems. For any new compound, regardless of its origin, a risk assessment is necessary to predict the effect on human health and environment prior to manufacturing or sale. These risk assessments involve exhaustive toxicity and exposure studies, which can require several years and are therefore not only very time consuming but also extremely expensive. These are considerable investment risks most companies in wood preservation area try to avoid and rely on approved synthetic agents [218].

For extractives mixtures, risk assessments are even more sophisticated as their composition would have to be standardized to meet a reproducibility of the action safety and it seems to be preferable to register a single compound, if available. Therefore, besides being necessary for analytical purposes, the compounds have to be to be extracted, separated, accumulated as well as purified. As a possible approach, to reduce time and effort in the first place, the adsorptive bubble separation was found to be an effective and potentially cost-efficient method to extract and accumulate extractives from wood. Furthermore, the combination of an agar overlay-assay with a hyphae agar to determine bioactive compounds in wood extracts also poses a possibility to benefit from a rapid and time saving method.

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9 Affidavit

Ich erkläre an Eides statt, dass ich die bei der promotionsführenden Einrichtung School of Life Sciences Weihenstephan der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Isolation, Separation and Bioactivity of Wood Extracts

am Lehrstuhl für Holzwissenschaft unter der Anleitung und Betreuung durch Univ.-Prof. Dr. rer. nat. Klaus Richter ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 6 und 7 Satz 2 angegebenen Hilfsmittel benutzt habe.

Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht, oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt. Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

Die öffentlich zugängliche Promotionsordnung der TUM ist mir bekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion) und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen.

Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

München, 22.04.2020 Regina Wanschura

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ORIGINAL

Application of foam fractionation to wood

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Abstract

Foam fractionation is a part of the adsorptive bubble separation methods and is a simple, gentle and rapid method for the extraction and accumulation of surface-active substances out of biomass. To assess whether the method is applicable to wood, two different device sizes were compared and two gases (N_2 and CO_2) were dispersed into the wood meal/water suspensions of several tropical wood species or into their respective water extracts. Furthermore, two tensides (Tween20 and SDS) were tested as supporting foaming agents. The compounds accumulated in the so-called spumates of these samples were compared to conventional water extracts of the respective woods and analyzed by FTIR, sugar analysis and GC/MS to examine whether an accumulation of extractives was achieved. Results show that the method is applicable to wood meal and water extracts from wood, but the conditions have to be adjusted to the targeted extractives of the wood species. Furthermore, it was observed that it is suitable for extraction of saponins without the need to separate them from other extractives, but it is not an alternative to a comprehensive analysis of water extractable compounds. The presented method can be applied using wood species that contain sufficient amounts of surface-active substances to be "self-foaming", but for a more versatile application, it needs to be further refined. However, if the application of additives is required in order to achieve sufficient foaming, it could be shown that the use of Tween20 was not successful, whereas SDS allowed several experiments. Nevertheless, a more effective way for complete separation and recovery of the tenside has to be developed, in particular, when the targeted extractives shall be used in subsequent biorefinery processes.

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Introduction

Extractives of tropical wood species consist of a great variety of components that differ in quantity and in composition (Hillis 1987; Xu et al. 2009), and there still is only limited data on their chemical composition available (Kilic and Niemz 2012). To understand variations in wood durability, the chemistry of the extractives needs to be further elucidated, and cost-efficient isolation methods of extractives have to be developed. It is known that saponins, as surface-active compounds, form colloidal, soap-like solutions (Neidhart and Häseler 2003) in water. Thus, they are able to generate stable foams (Ortega et al. 2010). They consist of triterpenes, steroids or spirostanols as aglycone and one or more sugars. Saponins are widely distributed in the plant kingdom, for instance in several wood species, and are often present in a complicated mixture of numerous and hardly separable compounds (Fengel and Wegener 1983). Many of them show biological activity and can be present in all plant organs in higher concentrations from 0.1 up to 30% (Hänsel and Sticher 2016), in particular in roots, barks and seeds (Osbourn et al. 1996). Besides their role in defense mechanisms of plants against attack from microorganisms or animals, saponins are of growing interest for drug research and provide valuable pharmacological properties (Augustin et al. 2011). However, as they are mostly present in highly diluted concentrations, they need to be accumulated to obtain a sufficient amount for chemical analysis or various other test methods.

Foam fractionation is described as a separation method with no need for sophisticated equipment and which can accumulate especially highly diluted substances (Uraizee and Narsimhan 1990). It is part of the adsorptive bubble separation methods and is different from foam flotation method, where a separation of dispersed parts takes place (Backleh 2001; Burghoff 2012).

The method was applied to other fields of plant science and has already been used for extraction, separation and accumulation of surface-active compounds. For example, oils and polyphenols were isolated from selected spice plants by Parlar et al. (2008), Backleh (2001) and Backleh-Sohrt et al. (2005). Separation of catechol and phenol (Huang et al. 2017), recovery of *trans*-resveratrol from muscat grape pomace (Wu et al. 2018) or alkali metal ions from anionic surfactant solution (Matsuoka et al. 2018) as well as heavy metals from wastewater (Peykar et al. 2013; Byambaa et al. 2018) were achieved. Furthermore, it was shown that recovery, separation and enrichment of proteins like casein (Shuai et al. 2018; Kamalanathan and Martin 2016) or bromelain (Li et al. 2016) from highly diluted solutions, or laccase from basidiomycete strains are possible (Blatkiewicz et al. 2017).

However, so far, no systematic attempts have been made to apply foam fractionation to biomass from trees. For the enrichment of surface-active substances via this method, inert gases are introduced into dispersed (ground) organic solids in water, substances dissolved in water (like extractives from plants or waste waters) or plant saps. Surface-active molecules contain a hydrophobic and a hydrophilic part and in case of dispersed organic solids, they first get dissolved in the surrounding water and then become adsorbed at the surface of the gas bubbles. Hereby, the hydrophobic part of the molecule is located in the gas phase of the bubble (Manegold 1953)

because it tends to avoid the aqueous surrounding. Together with the emerging bubbles, the adsorbed molecules leave the initial solution and can therefore be enriched in the formed foam, the so-called spumate (Ostwald and Siehr 1936). Some important factors have been identified in the literature, which influence the method efficiency, like the elementary presence of a sufficient concentration of surface-active substances. Preliminary investigations have shown that not all plant extractives foam sufficiently (Parlar et al. 2008). For a better performance, Parlar et al. (2008) added tensides like the anionic sodium dodecyl sulfate (SDS) or polyethylene glycol sorbitan monolaurate (Tween 20) that has no ionic parts but has hydrophilic and hydrophobic parts as well (Berner 2007). Good results were achieved analyzing non-foaming saps from plants (Parlar et al. 2008).

Furthermore, the efficiency of the method is found to be dependent on the pH value (Merz et al. 2011; Matouq 2008; Backleh et al. 2004; Liu et al. 1995) of the initial solution as well as on the length and diameter of the column used (Liu et al. 1995; Viehweg and Schügerl 1983; Banerjee et al. 1993). A longer column improves the drainage and dwell time in the column (Grieves 1975). However, the length of the column is a limiting factor due to the disintegration of the foam (Liu et al. 1995; Gerken 2005), which means that at a specific height of the foam in the column, it gets labile and degrades before it can leave the column. Additionally, higher gas flow rates can lead to a higher recovery rate (Brown et al. 1999) of surface-active substances, and using inert gases at room temperature reduces oxidative alterations at the extractive components (Berner 2007).

Thus, an enrichment of specific substance classes from the wood extractives via foam fractionation could be a valuable approach. In the presented research, it is applied to enrich compounds (extractives) from wood meal as well as water extracts with the objective of providing a selective isolation method for potentially bioactive substances. The spumates gained are compared to the conventional water extract regarding the respective amount of components and their concentration.

Materials and methods

Wood materials and sample preparation

All analyzed tropical wood samples (Table 1) originated from South and Central America. Their heartwood was classified into durability class 1 or 2 according to EN 113, and the raw density of all species ranged between 0.8 and 1.0 g/cm^3. The boards used to cut the specimens were purchased from local lumberyards. Wood blocks of 1 cm^3 were sawn out of the heartwood and subsequently milled in a cross-hammer mill (Retsch) together with dry ice and sieved with a mesh size ranging between 0.315 and 0.05 mm.

Three types of isolation methods were analyzed on the samples (Fig. 1):

(a) Extraction in water
(b) Foam fractionation of a wood meal/water suspension → Spumate 1

Table 1 Wood species used in the experiments

Wood species		
Trade name	Systematic name	Abbreviation
Bangkirai	*Shorea laevis*	BAN
Ipé Noir	*Tabebuja* spp. = *Handroanthus* spp.[a]	IPN
Itaúba	*Mezilaurus itaúba*	ITA
Jatoba	*Hymenea coubaril*	JAT
Massaranduba	*Manilkara bidentata*	MAS
Merbau	*Intsia* spp.	MER
Muiracatiara	*Astronium graveoles*	MUI
Pequia	*Caryocar villosum*	PEQ

[a]Grose and Olmstead (2007)

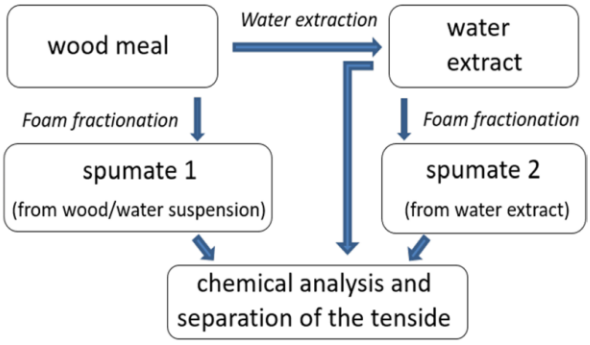

Fig. 1 Flowchart of the experiments

(c) Foam fractionation of the water extract → Spumate 2

Extraction in water and foam fractionation

Water extracts from wood were made according to the Tappi Standard T 207 cm-99 by digesting 2 g of air-dried wood meal in a beaker filled with 300 ml of demineralized water and left at room temperature for 48 h with occasional stirring. Extract contents were determined gravimetrically after drying the samples in a freeze dryer (Christ Alpha 1-2 LDplus) and were expressed as % of dry substance. Wood meal/water suspension and water extracts were subjected to foam fractionation resulting in lyophilized spumate samples for further investigation. Results from prior tests were used to adapt the amount to the volume of the device.

A concentration of 1 g wood meal in 50 ml water was chosen for the wood/water suspension. Using the water extract, the initial flask was filled with 50 ml for device 1 and 300 ml for device 2. In the case of MAS, a reduced loading (0.1 g wood meal in 50 ml water or 25 ml water extract for device 1 and 0.3 g/300 ml and 150 ml for device 2) was necessary due to the strong foam formation. All spumates were compared to the corresponding conventional water extracts regarding the yield of extractives (expressed in % of dry substance) and the contained compounds.

Foaming device

The foaming device was an open system and consisted of a glass chamber with a porous frit at the bottom side, a column and a receiving flask (Fig. 2). Separations were performed on two different sizes of columns and at room temperature. The smaller one (device 1) had a length of 24 cm and diameter of 1.6 cm and the bigger one (device 2) a length of 60 cm and diameter of 3 cm. The sizes (volume) of the glass chambers were 100 ml for device 1 and 500 ml for device 2. N_2 or CO_2 gas was introduced into a water/wood meal suspension (resp. water extracts) via the porous frit.

The samples were loaded directly into the flask via the neck before the glass column was put onto the flask. The emerging bubbles rose through the sample solution; surface-active components can adsorb to the bubble surface and therefore, enrich in the formed foam. After passing the column, the foam was collected in the receiving flask, which was a two-necked flask with a volume of 250 ml for device 1 and a beaker of 1000 ml for device 2. In the receiving flask, the foam was allowed to collapse and was then called the spumate (Ostwald and Siehr 1936). Afterward, the mass of the spumate was determined gravimetrically and expressed as % of dry substance (based on wood meal).

The tensides used to form non-active samples was sodium dodecyl sulfate (SDS; Sigma-Aldrich) and polyoxyethylene (20) sorbitan monolaurate (Tween20; MERCK) at a concentration of 20 mg/l (according to Backleh et al. 2004). Gas flow

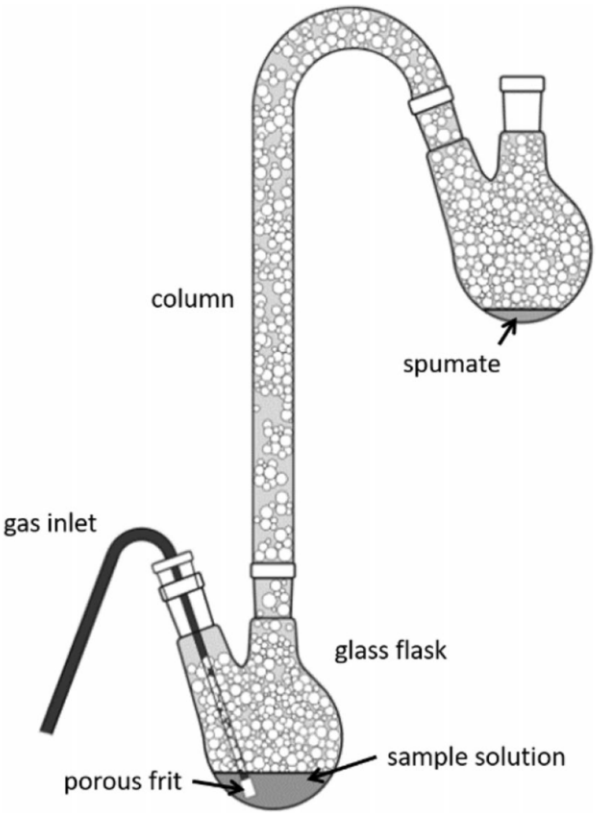

Fig. 2 Scheme of a simple foaming device

rate was controlled via a gas flowmeter and experiments were started with 100 ml/min. When no more bubbles emerged, the flow rate was increased in steps of 50 ml/min and ended at a gas flow rate of 500 ml/min because higher gas flow rates lead to a “foam over". This means that the initial solution was only pushed through the column into the receiving flask by the pressure of the introduced gas and no accumulation nor separation was performed.

The time of analysis was measured with an electronic time clock. The beginning of the experiment was defined as the time when the first bubbles reach the receiving flask and the end was defined as the time when the bubbles disintegrate in the column and cannot reach the receiving flask anymore; latest at a gas flow rate of 500 ml/min (this was called the bubbling time). Therefore, the samples were not all treated for the same time (but until foam formation ends). All experiments were repeated three times if the percentage deviation from the average of the spumate amount was lower than 15%. After performing the experiment, a rest of the sample solution remained in the glass chamber. This rest was called the residual solution (Ostwald and Siehr 1936).

FTIR

FTIR spectra were collected on a Nicolet is 50 (Thermo Fisher) spectrometer with 16 scans/sample. The spectra were measured in extinction mode with a resolution of 0.09 cm^{-1} and recorded in a range from 4000 to 400 cm^{-1}. The dried samples of spumates or extracts (1 mg) were mixed with potassium bromide (KBr, 300 mg) and pressed to a tablet. Averaged spectra were baseline corrected (4–6 points) and normalized at 1045–1081 cm^{-1}.

Acidic hydrolysis and sugar analysis

Sugar analysis was done using a Biotronik LC 3000 to determine mono- and disaccharides directly as well as after hydrolysis with trifluoro acetic acid (TFA). Sugars were analyzed in water extracts and in selected spumates (foamed with N_2) thereof. 30 mg of extract or spumate were hydrolyzed with 4.8 g TFA for 20 min at 102 °C in a thermoblock. Saccharides were detected via photometric detection (560–570 nm) of a bichinolat/Cu^+ complex, which is proportional to the amount of reducing sugars in the sample. Identification and quantification were done by external calibration (standard solutions consisting of cellobiose, rhamnose, mannose, arabinose, galactose, xylose and glucose).

Acidic hydrolysis and analysis of the aglycone

The spumates generated without a tenside (PEQ and MAS) were subjected to an acidic hydrolysis, followed by a liquid–liquid extraction with an organic solvent. For this purpose, 20 mg of the spumate were placed into a hydrolysis flask and diluted with 20 ml hydrochloric acid (HCl, 2M). The flask was placed into a thermoblock at

120 °C for 2 h and cooled to room temperature. The hydrolysate was extracted five times with 50 ml dichloromethane. After drying, the organic phase was subjected to GC/MS analysis.

GC/MS

Extracts and spumates were studied after silylation with BSTFA/TMCS (N,O-bis(trimethylsilyl)trifluoroacetamide/chlorotrimethylsilane; Sigma-Aldrich) by means of GC/MS (MSD 5975C by Agilent Technologies). 1 mg sample was solved in 200 µl dimethylformamide (DMF) (4 mg/ml), and 50 µl BSTFA/TMCS were added. The sample was placed in a thermoblock at 80 °C for 1 h. 1 µl of the sample was injected at an injector temperature of 300 °C with a split of 30:1 (39 ml/min). GC was heated at 10 °C/min from 100 to 300 °C (held isothermally for 15 min). A HP5 separation column from SGE was used: 15 m × 0.25 mm, 0.25 µm film. Ionization: − 70 eV. Detector temperature was set to 250 °C, and MS was recorded in a mass range from 40 to 800 amu. Identification of compounds was done via comparison of spectra with a NIST08 database or measurement of reference compounds.

Quantification of a substance was done via the measured area of the peak in the chromatogram (area counts) using heneicosanic acid as an internal standard without considering different response factors. The mass of all detected and quantified compounds in the chromatogram in relation to the mass of the sample used for analysis was expressed as % of "total quantified compounds", reflecting the detectable (via GC/MS) parts of the measured sample.

Separation and detection of the tenside SDS

To quantify the extracted compounds, SDS needs to be separated from the spumates after the experiment. SDS can be precipitated by adding potassium chloride (KCl) as potassium dodecyl sulfate (KDS) or separated by using an ion-exchange resin. The focus was to assess the feasibility of the treatments on the samples. Because the amounts gained by each foam fractionation with SDS were small, the tests were performed only with the spumate from one wood sample per treatment.

Precipitation with KCl was performed according to Carro et al. (1994): 0.7 mg spumate from MUI wood was solved in 20 ml H_2O and adjusted to pH 1.5 with trichloro acetic acid (TCA). Afterward, 100 ml 2 M KCl was added and after 15 min of cooling in an ice bath, the mixture was centrifuged at 4 °C with 4000 rpm for 30 min.

Separation with an amphoteric resin: separation via an ion change resin based on Henderson et al. (1979) and Bondareva et al. (2009): 10 mg of BAN spumate was solved in 15 ml H_2O, and 2 g Dowex retardion 11A8 50–100 was added. After 15 min, the resin was removed, and the spumate was lyophilized.

Energy-dispersive X-ray analysis: semi-quantitative determination of the sodium content in the sample and calculation of the SDS content therefrom were exemplarily

applied to 4 MER spumate samples, which were subjected to the energy-dispersive X-ray spectroscopy (EVO40 X-Flash 5010 by Bruker).

Results and discussion

Performance of foam fractionation concerning the wood species and water extracts

The wood species behaved quite different in the experiments. This is due to the type and amount of the present extractives. MAS wood meal/water suspension and its water extract gave stable and bulky foams, which were difficult to collapse, while PEQ samples gave moderately stable foams, which broke down easily. In contrast, the bubbles of all other wood species collapsed before they left the flask with the sample solution. If foam fractionation works, both sample types can be foamed in both devices and in combination with both gas types. The only exception is IPN, because the method could only be achieved successfully using the water extract and N_2 as foaming gas, and it is not applicable to JAT, ITA at all (Table 2).

In particular with PEQ, gas flow rates need to be low because of the hydrophobic character of the wood. Otherwise, the wood particles tend to entrain with the bubbles and thus need to be separated from the spumate afterward.

Performance of foam fractionation concerning an additional tenside

As already mentioned, MAS and PEQ obviously contain sufficient surface-active compounds, and therefore, all their samples are self-foaming and do not need any supporting tenside. To enable the experiments with all other samples, the additional foaming agents SDS and Tween20 were tested. By using SDS, spumate 1 and spumate 2 of BAN, MER and MUI as well as the spumate 2 of IPN could also be successfully produced (Table 2). Tween20 was also tested, because the described enrichment factor is higher (Parlar et al. 2008), but only IPN's spumate 2 could be obtained, albeit barely. In the case of BAN, MER and MUI, no foam formation

Table 2 Foamability of the samples and used tenside

Wood species	Spumate1	Spumate2	Used tenside
BAN	+	+	SDS
IPN	–	+	SDS
ITA	–	–	–
JAT	–	–	–
MAS	+++	+++	Not necessary
MER	+	+	SDS
MUI	+	+	SDS
PEQ	++	++	Not necessary

"–" No foam formation, "+" low foam formation, "++" moderate foam formation and "+++" very good foam formation

could be observed using Tween20. Therefore, Tween20 is not mentioned in Table 2. Spumate 1 of IPN could not be produced at all under these conditions. This may be due to the contact time between the wood meal and the water, which is short, compared to the time of conventional water extraction (48 h) and is possibly not long enough to solve the compounds that interact with the additives.

Under the tested conditions, with gas flow rates not higher than 500 ml/min, JAT and ITA do not form enough stable foam to ascend the column and cannot be foamed at all (neither the wood meal/water suspension nor the water extract) even if the tested tensides were added.

Analysis duration (bubbling time)

It was observed that foam formation of MAS and PEQ (spumate 1 and 2) continues for a longer time compared to the other wood species that were foamed using a tenside. Even if bubbling time of MAS and PEQ samples varied strongly, foam fractionation of BAN, IPN, MER and MUI using SDS was finished at the latest after 2–3 h (Table 3). Over all samples, the bubbling time in device 2 tends to be half an hour longer than in device 1, but this is within the variance of the experiments and has therefore no significance.

Influence of tested parameters on yield of foam fractionation

In Fig. 3, the yield of spumates 1 and 2 is presented. Influences regarding the two different foaming gases as well as from the two device sizes could be observed. The extract content of the conventional water extraction is presented in Fig. 4 for comparison. In Fig. 5, the influence of the raw material (if wood meal/water suspension or water extract is used for foam fractionation treatment) under CO_2 conditions on the yield of the experiments is presented. In some cases, the deviation remained high, even if the determinations were repeated up to 8 times. The influences of the tested parameters are described as follows:

Comparing the yield of spumates 1 (Fig. 3) and water extracts (Fig. 4), it was observed that the amount of extractable compounds from MAS in spumate 1 (7–8%)

Table 3 Time of analysis of different foam fractionations

Wood species	Bubbling time (h)			
	Spumate 1		Spumate 2	
Foaming gas	CO_2	N_2	CO_2	N_2
BAN	0.5–1.5	1.5–2.0	1.5–2.0	2.0–3.0
IPN	–	–	1.0	–
MAS	2.5–6.5	3.5–5.5	3.0–5.0	3.5–5.0
MER	1.0–1.5	1.5–2.0	1.5–2.0	2.0–2.5
MUI	0.5–1.0	1.0–1.5	1.0–1.5	1.0–2.0
PEQ	0.5–3.0	0.5–3.5	1.0–3.0	2.0–4.0

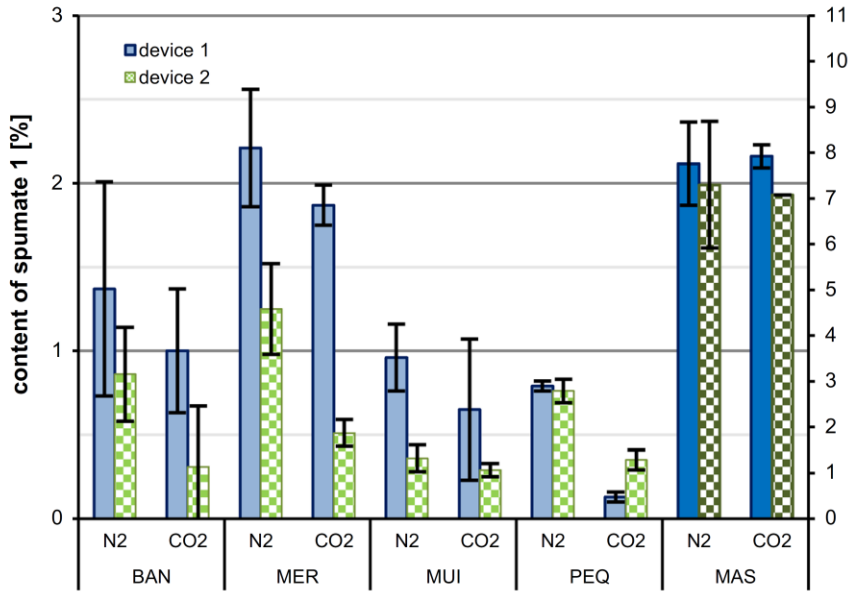

Fig. 3 Content of spumate 1 (in %) after treatment with CO_2 and N_2 in two different-sized devices. Content of MAS spumates refers to the second vertical axis

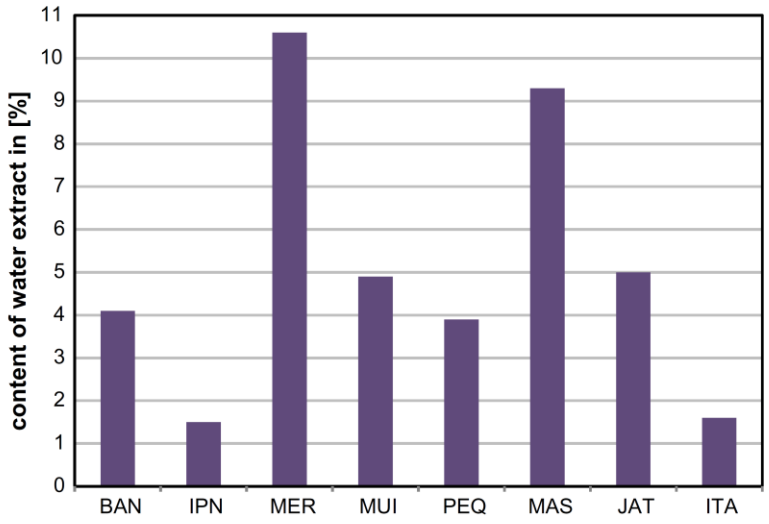

Fig. 4 Content of conventional water extracts (in %)

is similar to that of conventional water extraction (9%), meaning that nearly all water extractable compounds from MAS are foamable saponins.

Concerning the other wood species, including PEQ, the method had to be repeated several times to gain the same masses of extracts as from conventional water extraction. The higher extract content in water extracts can be due to the longer extraction time, which is method-specific. While performing the conventional water extraction where the wood meal is stirred in water for 48 h (according to Tappi

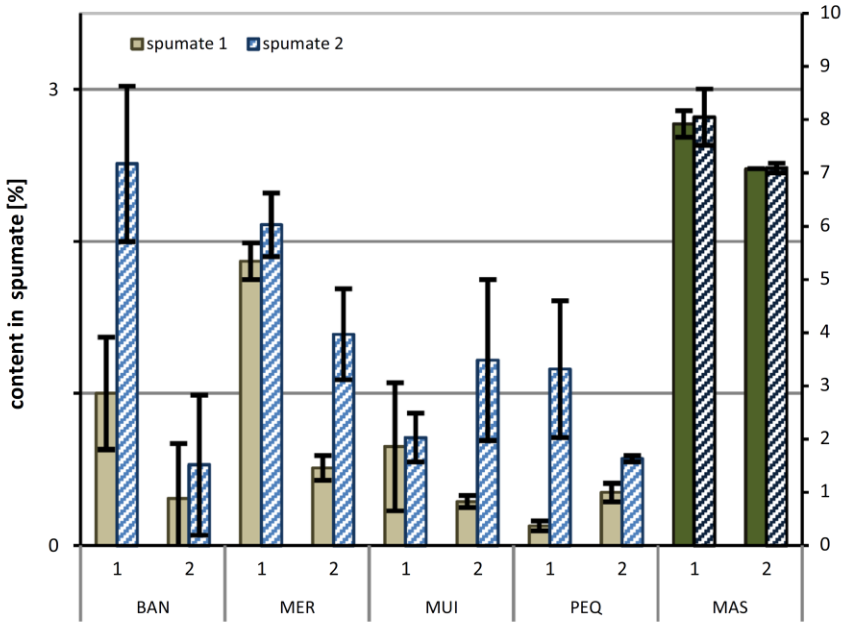

Fig. 5 Influence of raw material: spumate 1 and spumate 2 foamed with CO_2 in two different-sized devices. Contents of MAS refer to the second vertical axis

Standard T 207 cm-99, 1999), foam separations were performed within 30 min up to 5 h.

Influence of gas type

Differences in the performance of treatments carried out with N_2 or CO_2 could be observed:

- Lower yield of extractable compounds from wood using CO_2 (Fig. 2).
- Time of analysis is slightly increasing in all experiments (Table 3) using N_2.
- Furthermore, the spumate collected in the receiving flask produced with CO_2 disintegrates much more rapidly (about 10 min) than the one produced with N_2 (overnight in a cool environment).

A possible reason for the differences observed using CO_2 as foaming gas was shown by Backleh (2001) by foaming beer: foam produced with N_2 is more finely pored and creamier than with CO_2. This has influence on the foam structure (higher non-polar surface due to more and smaller bubbles) and thus, on the produced foam amount and on the accumulation.

Furthermore, by performing the experiments with CO_2, a decline in pH value in the spumate as well as in the residual solution can be observed. Starting with a pH value of 7 in the wood meal/water suspension and 5 in the water extract, the pH value declines to 4. Most likely, the dissolved CO_2 forms carbonic acid, which contributes

to the pH decline. The adsorption of the molecules at the gas liquid interface and the extent of their separation can be influenced by the pH value (Thompson 2004) and can also be the reason for the smaller yield of extractable compounds using CO_2.

Influence of device size and influence of raw material

Comparing the size of the devices, it can be seen that the higher amounts of extractable components in spumate 1 (Fig. 3) can be found in device 1. This is possibly due to a disintegration of the foam in the longer column of device 2. Comparing the spumate's origins (the raw material), it can be seen that the yield of spumate 2 (foamed with CO_2) is higher, independent of the device size (Fig. 5). This can be due to the longer extraction time (initial solution is already a conventional water extract) and the above described higher extract yield. The comparisons of the yields based on the raw material in combination with the foaming gas N_2 are indecisive and unspecific and therefore not presented here in detail.

Sugar analysis

Figure 6a and b shows the content of free mono- and disaccharides (Fig. 6a) and of hydrolyzed sugars (Fig. 6b) in water extract and in spumate 2 as well as the composition of the main sugar components. For better clarity, arabinose, galactose and mannose were summarized. The spumates 2 (from the water extracts) are presented, because for IPN, only this spumate exists.

The most remarkable difference regarding the sugar analysis is that between the small amount of mono- and disaccharides and the high amount of hydrolyzed sugars in the spumate 2 (after treatment) of the "self-foaming" species MAS and PEQ, indicating a high amount of oligosaccharides, which are very likely present as saponins. The sugars attached at the aglycone are therefore transferred to the spumate with the bubbles and can be detected there after hydrolyzation. This is the reason for the good performance of the two wood samples during the experiment. Furthermore,

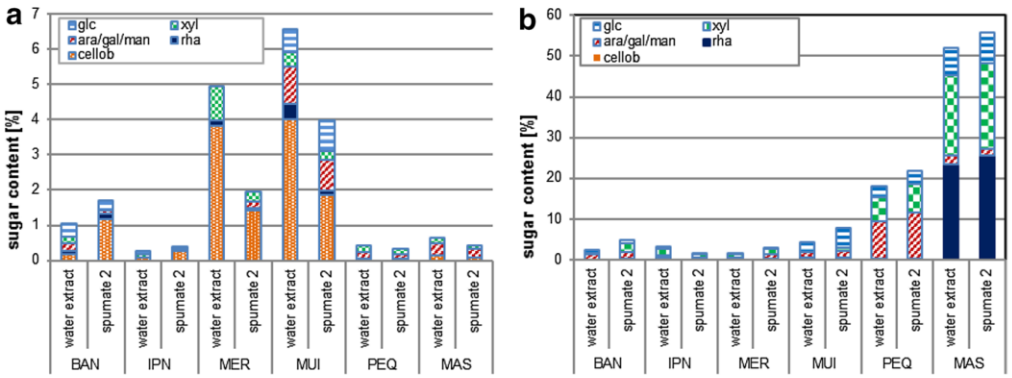

Fig. 6 **a** Sugar analysis of "free" mono- and disaccharides in extracts and in their spumate 2. **b** Sugar analysis after acidic hydrolysis in extracts and in their spumate 2

PEQ and MAS have a very consistent proportional composition of hydrolyzed sugars (6B) between the water extract and the spumate 2, indicating that there is an accumulation of components but without a separation of different aglycones.

In MER, no accumulation can be detected, whereas some accumulation of substances in MUI was observed (compare, Table 5), which may be due to the relatively high amounts of free sugars in the water extract. As is known from the addition of fructose to protein solutions, the addition leads to lower accumulation rates or can even inhibit an accumulation at all (Gschwendtner 2000).

BAN and IPN show a higher amount of free sugars in the spumate 2 than in the water extract (Fig. 6a). Since sugars are hydrophilic and, therefore, do not adsorb at the hydrophobic gas bubble surface, the determined sugars must be carried in the plateau borders and in the liquid film in-between the bubbles generated by SDS. This was verified by additional experiments and was found by Backleh (2001), too, who compared the distribution of glucose, fructose and saccharose between the initial solution, the spumate and the residual solution of orange juice. The sugars were distributed identically in all solutions and are therefore seen as not foam-active substances. Especially for IPN, the higher content of free sugars in the spumate 2 (6A) together with a lowered content of hydrolyzed sugars (6B) suggests a (auto-) hydrolyzation of sugars in the spumate 2 after foam fractionation.

The free mono- and disaccharides (Fig. 6a) consist mainly of cellobiose and xylose. In BAN, MER and MUI, rhamnose was detected as well. No free glucose was observed in MER, PEQ and MAS. The hydrolyzed sugars (Fig. 6b) of these species have high contents of glucose and xylose. Except for PEQ, rhamnose could be observed, too. Total amounts (referred to the sample taken) show that only a very small quantity of sugars can be found in the spumate. This is due to the generally little spumate amount (Figs. 3 and 5) gained by each experiment. Therefore, the presented percentages of sugars are referring to the respective extract or to the spumate and are not directly comparable. However, the results show clearly that in some cases an accumulation of sugar containing components by means of foam fractionation is successful.

FTIR spectroscopy

Comparing all the spumates by means of FTIR, no obvious differences were detected neither between the two foaming gases nor between the sizes of the devices. It seems as if both have an influence on the extract yield (higher content of extractable components in device 1, Fig. 3) but not on the included compounds. In contrast, the FTIR spectra of the water extracts and the spumates 1 show noticeable differences, which are illustrated exemplarily for MAS (Fig. 7a) and PEQ (Fig. 7b). The assignment of the detected absorbances in the FTIR spectra is given in Table 4.

In the case of MAS (Fig. 7a), only little differences can be detected between the water extract and the spumate 1. The spumate shows C–H and C–O absorptions at 2936 cm^{-1} and 1046 cm^{-1} besides a carbonyl absorption at 1733 cm^{-1}. Comparing the band heights in % (Table 4), it can be observed that there are some minor differences regarding the absorptions at 3405 cm^{-1}, 1633 cm^{-1}, 1516 cm^{-1}, 1451 cm^{-1}

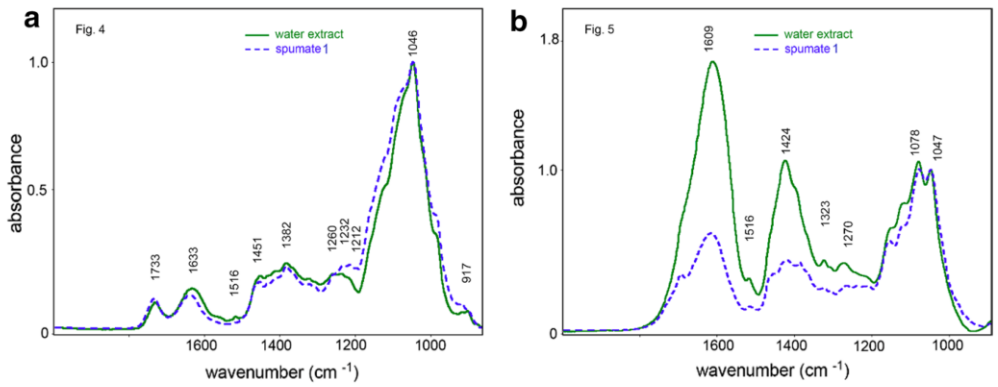

Fig. 7 **a** N_2-spumate 1 of MAS compared to the water extract; spectra normalized at 1046 cm^{-1}. **b** CO_2-spumate 1 of PEQ compared to the water extract; spectra normalized at 1047 cm^{-1}

Table 4 Band assignments in the infrared region of water extracts and spumates 1 of PEQ and MAS according to the literature

Band origin; comments	Maxima at cm^{-1}, % absorbance (%A) from water spectra		
		PEQ	MAS
	cm^{-1}	% A	% A
O–H stretch, hydrogen-bonded OH groups[d]	3405	55	87
C–H stretch in methyl and methylene groups[d]	2932	82	102
C=O stretch; unconjugated ketones; carbonyls; ester groups[b,d]	1733	x	103
C=O stretch; conjugated aldehydes and carboxylic aids[b]	1700	40	x
H_2O adsorbed; conjugated double bonds[d,e,f]	1633	x	69
C=C aromatic skeletal vibrations[d]	1609	36	x
C=C aromatic skeletal vibrations[d]	1516	46	39
C–H bending (asymmetric) from methoxyl[b], CH_2 of pyranring symmetric scissoring[f]	1451	x	73
C–H plane deformation[d]	1424	41	x
C–H bending[e]; –O–CO–CH_3[d]	1382	x	87
CH_2 rocking[f]	1323	60	75
C–O–C stretch; –O–H deformation[c]; C_{alkyl}–O–Aryl[a,c]	1260 1270	62	100
C_{alkyl}–O–Aryl; lactones[e]; C–C plus C–O plus C=O–stretch[b]	1232	x	106
OH plane deformation[f]	1212	x	110
C–O deformation in secondary alcohols and aliph. ethers (carbohydrates)[b,e]	1078	95	102
C_{alkyl}–O ether vibrations[c]	1046	100	100

Differences between the spumate and the water extract are presented as % absorbance (band height) from the spectra of the water extract

[x]no occurrence of the absorption in the spectra

[a]Fengel and Wegener (1983), [b]Faix (1992), [c]Collier et al. (1992), [d]Hesse et al. (1995), [e]Kotilainen et al. (2000), [f]Schwanninger et al. (2004)

Table 5 Results of GC/MS analysis of spumates 1 and 2 and of conventional water extracts

Species/foaming gas	Spumate 1		Spumate 2		Water extract	MS fragments (of TMS derivatives)	Substance class
	CO_2	N_2	CO_2	N_2			
BAN							
Total quant. compounds (%)	**12.95**	**17.59**	**7.60**	**13.27**	**22.86**		
Quantified substances (%)	0.24	0.32	x	0.25	0.38	**458** (5) 433 (18) 399 (10) 355 (12) 311 (10) 281 (69) 73 (100) *gallic acid*	Organic acids
	4.62	6.78	3.69	6.04	9.45	**662** (2) 647 (10) 633 (8) 207 (23) 179 (16) 73 (100)	n.i.
						688 (2) 635 (8) 431 (10) 255 (10) 179 (20) 193 (31) 147 (10) 73 (100)	n.i.
MER							
Total quant. compounds (%)	**7. 53**	**12.28**	**5.80**	**10.14**	**13.11**		
Quantified substances (%)	0.22	0.14	0.32	x	x	**355** (10) 307 (3) 265 (5) 245 (13) 233 (24) 147 (62) 73 (100) *butanedioic acid*	Organic acids
	x	0.10	x	x	0.10	**458** (5) 433 (18) 399 (10) 355 (12) 311 (10) 281 (69) 73 (100) *gallic acid*	
	6.95	9.27	5.10	9.86	12.40	**562** (8) 368 (100) 267 (39) 73 (60) *fisetinidol or isomers* **650** (10) 560 (5) 456 (100) 368 (5) 267 (44) 73 (75) *robinetinidol or isomers* **650** (5) 560 (2) 368 (100) 355 (28) 73 (51) *(epi-)catechin* **647** (5) 599 487 147 *quercetin*	Flavonoids and flavonoid fragments
MUI							
Total quant. compounds (%)	**4.74**	**31.03**	**0.82**	**24.64**	**12.28**		
Quantified substances (%)	2.65	**18.10**	0.59	**12.58**	6.31	**458** (5) 433 (18) 399 (10) 355 (12) 311 (10) 281 (69) 73 (100) *gallic acid*	Organic acids
	0.60	4.03	0.23	**6.09**	3.9	**650** (3) 560 (2) 368 (100) 355 (28) 73 (51) *(epi-) catechin* **560** (45) 471 (25) 369 (70) 267 (12) 207 (40) 73 (100) *catechin (isomer)*	Flavonoids
MAS							
Total quant. compounds (%)	**21.40**	**29.89**	**18.30**	**35.80**	**42.69**		
Quantified substances (%)	6.00	**10.16**	7.00	**9.70**	8.37	**585 [M-15]** (8) 482 (15) 393 (25) 320 (25) 203 (50) 73 (100) *ursolic acid* **673 [M-15]** (5) 570 (15) 320 (30) 203 (60) 73 (100) *hederagenin* **687 [M-15]** 612 584 320 302 73 (100) *quillaic acid*	Triterpenes

Table 5 (continued)

Species/foaming gas	Spumate 1		Spumate 2		Water extract	MS fragments (of TMS derivatives)	Substance class
	CO_2	N_2	CO_2	N_2			
PEQ							
Total quant. compounds (%)	**20.90**	**31.54**	**16.71**	**30.50**	**32.08**		
Quantified substances (%)	9.86	**16.07**	7.53	**15.55**	14.91	**335** (4) 245 (11) 233 (18) 147 (54) 73 (100) *malic acid* **465** (3) 375 (15) 363 (18) 247 (16) 273 (76) 147 (58) 73 (100) *carballylic acid*	Organic acids
	0.95	1.07	0.88	**1.30**	1.07	**458** (5) 433 (18) 399 (10) 355 (12) 311 (10) 281 (69) 73 (100) *gallic acid*	

Difference between quantification of detected target compounds the "total amount of quantified compounds" in [%] (reflecting the detectable parts of the measured sample) origins from sugar derivatives and minor components below quantification threshold. Contents with substantial increase which are due to a certain bubble gas, as well as absolute masses of detected compounds are given in bold

and 1382 cm^{-1}. A slight decrease in compounds with these groups (Table 4) can be observed in the spumate. Furthermore, no aromatic skeletal vibrations can be seen around 1515 cm^{-1} in the spumate, and only a very weak absorption in this region is shown in the water extract, indicating different concentrations of some extractives between the spumate and the water extract. Hence, the compounds causing these absorptions show a lower surface activity and are, therefore, not extracted by foam fractionation to the same extent.

Regarding the region from 917 to 1232 cm^{-1}, a variety of absorbances was found to increase in spumate compared to the water extract. Therefore, an accumulation of substances with these functional groups is indicated. As is known, MAS contains high amounts of saponins. Therefore, many of the absorbances should origin from sugars and different aglycones, like oleanolic or bassic acid. On the contrary, PEQ samples show more obvious differences between the spumate and the water extracts (Fig. 7b). Absorbances at 2920 cm^{-1} and 2849 cm^{-1} origin from CH_2– vibrations imply a high content of hydrocarbons. However, the most noticeable differences in the spectra can be seen at 1609 cm^{-1}, which origin from aromatic skeletal vibrations and 1424 cm^{-1} that derive from C–O stretch vibrations as well as C–H plane deformations. Gallic acid is one of the aglycones in PEQ (Table 5) and can therefore be the origin of the aromatic absorbances. This would be consistent with a conformation like the below suggested hederagenin-galloyl-glc-ester (compare GC/MS analysis; Alabdul Magid et al. 2006).

These differences can be explained by variations in the concentration of some extractives between the spumate 1 and the water extract. There probably is an accumulation of compounds by means of foam fractionation having strong absorptions at 1078 cm^{-1} and 1047 cm^{-1} usually (when analyzing polar wood extractives) deriving from sugars. This is supported by the fact that there also is a relatively high part of hydrolyzable sugars in the spumate (Fig. 5b).

This trend can also be seen in the spumates of the other wood species investigated, even if additives were needed to perform the experiments. Many absorptions indicating aromatic components were observed in the spectra of BAN (1613 cm^{-1}, 1516 cm^{-1}, 1455 cm^{-1}), MER (1620 cm^{-1}, 1509 cm^{-1}, 1455 cm^{-1}) and MUI (1616 cm^{-1}, 1518 cm^{-1}, 1455 cm^{-1}). Because SDS was needed to gain these spumates, mainly the additive caused important absorbances (2920 cm^{-1}, 2851 cm^{-1}, 1217 cm^{-1}, 1107 cm^{-1}) in the spectra. These absorbances interfere strongly with those from the samples, and therefore, in the case of SDS-gained spumates, FTIR is not able to properly detect minor differences in these regions.

GC/MS analysis

The GC/MS spectra of MAS spumate 1 compared to the spectra of the conventional water extract confirm the FTIR results. Both—the spumate 1 and the water extract—contain the same compounds. As is known, MAS contains oleanolic acid (Fengel and Wegener 1983 and literature cited therein) and bassic acid (Cocker and Shaw 1963). Based on the measured spectra, both compounds could not be confirmed, but ursolic acid and probably quillaic acid were identified instead. As

Bournouf-Radosevic et al. (1984) showed, oleanolic acid and urosolic acid can be distinguished from each other by the relative intensities of the two MS fragments 73 and 203 which were found to be 90/100 for oleanolic acid and 100/70 for urosolic acid (Table 6).

It was observed that foam fractionation is successful as both compounds can be detected in the spumate as well. They seem to be accumulated in the spumate foamed with N_2 but not with CO_2 (Table 5). As GC/MS shows mainly sugars and only a slight accumulation of gallic acid in the N_2 spumate of the water extract of PEQ, the spumate was subjected to acid hydrolysis to further elucidate the components (Table 6). In the gained hydrolysate, at $r_t = 34.7$, hederagenin ((3β)-3,23-dihydroxyolean-12-en-28-oic acid) (Fig. 8) could be identified by comparison with the measured spectra of a reference substance. Hederagenin is known to be bioactive and is already used pharmaceutically (Li 2000). Additionally, GC/MS revealed gallic acid as aglycone in the spumate of PEQ wood. Hederagenin acylated with a galloyl group is known from the stem bark of PEQ: Rha-Gal-Glc–hederagenin-galloyl-Glc-ester (Alabdul Magid et al. 2006). GC/MS analysis as well as the behavior of the PEQ water extract (hardly soluble, tends to precipitate easily) suggested a similar conformation of the aglycone from spumate 2. Additionally, oleanolic acid and three more, not plainly identified triterpenes, were found in the spumate. The spumate of the second "self-foaming" wood species MAS was also subjected to acid hydrolysis, but the performance was insufficient. Nevertheless, at least six triterpenes could be detected in the hydrolysate, which could not yet be identified beyond doubt by now (Table 6).

Furthermore, an accumulation of some organic acids was achieved. In MUI, a clear accumulation (with N_2 as the foaming gas), in particular of gallic acid as well as (epi)catechin(-gallate) was observed. Thus, a significant effect of the foaming agent could be ascertained. It is known that catechins cannot be foamed without additives (Thompson 2004) because of their hydrophilic character. Parlar et al. (2008) found that catechins can be foamed very well adding Tween20 to the initial solution. In contrast, all of the wood species in this study containing catechins could not be foamed with Tween20 but by using SDS. Some of the extractives components of these wood species must have inactivated Tween20, so it could not produce a sufficient amount of foam.

Regarding the quantified compounds, only minor differences between the spumates and the water extracts from BAN (triterpenes) and MER (flavonoids: i.a. fisitinidol/robinetinidol and isomers) were detected (Table 5). In general, the highest amounts were found in the spumates foamed with N2 (water extract and wood meal), while the lowest contents were found in the wood spumate foamed with CO2 (mentioned before in Figs. 3 and 5). Because spumate 2 of IPN cannot be compared to other spumates, the results are not mentioned in the table. However, slightly higher amounts of organic acids and triterpenes (e.g., $C_{30}H_{50}O_2$ like erythrodiol or derivatives) were detected in the spumate. Lapachone, which was detected in the water extract, could not be found in the spumate.

Regarding the "total amount of quantified compounds," the difference between the water extract and the spumates 1 and 2 is mainly due to the lower content of sugars in these spumates (see Fig. 6a and b). The content of sugar alcohols and reducing

Table 6 Results of GC/MS analysis of spumates after hydrolysis

	MS fragments (of silylated compound derivatives)	Identified compound/substance class
PEQ		
R_t 31.8	**497 [M-15]** (10) 394 (69) 203 (100) 189 (77) 73 (94)	Triterpene
R_t 32.7	**585 [M-15]** (10) 482 (20) 320 (30) 203 (100) 189 (37) 73 (78)	Oleanolic acid
R_t 34.7	**673 [M-15]** (5) 598 (2) 570 (10) 320 (30) 203 (60) 73 (100)	Hederagenin
R_t 38.3	**687 [M-15]** (3) 584 (10) 320 (15) 292 (7) 203 (92) 73 (100)	Triterpene
R_t 35.9	**601 [M-15]** (3) 498 (10) 391 (2) 320 (30) 203 (100) 73 (80)	Triterpene
R_t 37.6	**599 [M-15]** (5) 469 (20) 320 (30) 203 (100) 73 (90)	Triterpene
Rt 38.3	**687 [M-15]** (5) 584 (20) 495 (10) 455 (3) 320 (30) 203 (95) 147 (30) 73 (100)	Triterpene
Rt 38.7	**687 [M-15]** (5) 584 (10) 541 (8) 438 (15) 320 (35) 203 (100) 189 (35) 73 (89)	Triterpene
MAS		
Rt 29.8	**584 (2)** 479 (5) 425 (8) 397 (10) 307 (15) 245 (20) 187 (85) 145 (38) 131 (38) 73 (100)	n.i.
Rt 30.0	**584 (2)** 551 (5) 494 (10) 463 (20) 333 (15) 217 (20) 201 (41) 187 (93) 133 (36) 73 (100)	n.i.
Rt 31.9	**584 (8)** 477 (5) 391 (5) 347 (5) 307 (5) 227 (10) 178 (26) 173 (22) 119 (18) 73 (100)	n.i.
R_t 32.7	**585 [M-15]** (8) 482 (15) 393 (25) 320 (25) 203 (70) 73 (100)	Ursolic acid
R_t 33.1	**671 [M-15]** (10) 583 (15) 493 (8) 451 (6) 375 (10) 320 (15) 261 (20) 203 (35) 147 (25) 73 (100)	Triterpene
R_t 33.5	**687 [M-15]** (5) 612 (5) 581 (15) 510 (3) 481 (5) 305 (15) 260 (35) 73 (100)	Triterpene
R_t 33.8	**671 [M-15]** (8) 581 (15) 508 (4) 464 (8) 418 (10) 320 (15) 261 (25) 203 (30) 147 (25) 73 (100)	Triterpene
R_t 34.7	**673 [M-15]** (5) 570 (15) 320 (30) 203 (60) 73 (100)	Hederagenin
R_t 35.2	**687 [M-15]** (5) 612 584 320 302 73 (100)	Quillaic acid

Contents with substantial increase which are due to a certain bubble gas, as well as absolute masses of detected compounds are given in bold

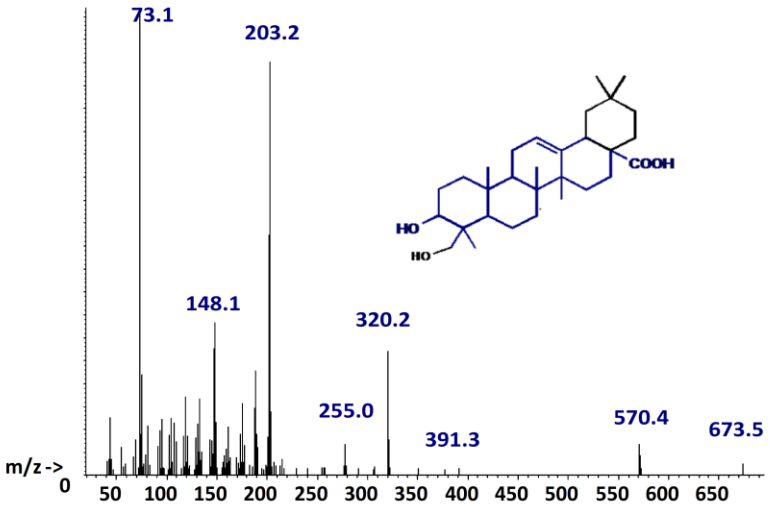

Fig. 8 PEQ: MS of compound (r_t = 34.7 min), identified as TMS-hederagenin

sugars were quantified together. Comparing device 1 and device 2, no differences could be detected via GC/MS. Table 5 shows obviously that only a minor part of the compounds is detectable via GC/MS, and moreover, the main peaks in the chromatograms are composed of carbohydrates. Although alternative derivatizations were performed, all other detected components were only found in traces and could not be identified beyond doubt by now. This is due to limitation of GC/MS when analyzing very high molecular compounds. Some of the compounds already described in different solvents were not detected here, like stigmasterol (Kilic and Niemz 2012) as well as quercetin and ellagic acid in MUI (da Silva et al. 2011) and the stilbenes laevifonol and laevifosid (Sakai et al. 2003) in BAN and stilbenes in MER (Hillis and Yazaki 1973).

Separation and quantification of the tenside SDS

Different methods were tested to separate the tenside SDS from the spumate. Precipitation of SDS with KCl as reported by Carro et al. (1994) at low temperature and acidic pH value was achieved, but the results are not satisfying as a considerable amount of the extractives was also precipitated. The potassium dodecyl sulfate sediment is clearly colored and FTIR also showed distinct absorbances that derive from the extract. Furthermore, relatively high amounts of KCl are needed, making the method not suitable to obtain SDS free extract to a larger extent.

Using an amphoteric resin, good separation results were obtained. FTIR showed no SDS derived absorbance after 15 min contact time between resin and extract. However, for instance, small amounts of extractives were removed from the solution as well and could not be recovered by washing the resin sufficiently. Furthermore, it was not possible to regenerate the resin and it is therefore too expensive to provide a suitable method to purify the extractives at a larger scale. Semi-quantitative

determination was achieved by means of energy-dispersive X-ray spectroscopy that shows a content of SDS at about 4.8% in MER wood spumate foamed with N_2.

Conclusion

The foam fractionation method was applied to wood for the first time, and the results principally support an applicability to wooden materials. However, the conditions of the experiments have to be adjusted to the respective wood species, because the nature and composition of the extractives varies in-between the species. Compared to conventional water extraction, the method achieved a specific isolation of saponins in a fraction of time. However, FTIR spectra analysis revealed that it could not isolate the total of water extractable constituents. In addition, the total mass of the spumates was considerably lower than in the water extract. Therefore, the experiment must be repeated several times to provide the same masses.

In its pure version, the method is limited to wood species that have "self-foaming" extractives (like saponins). However, poor foaming capacity and foam stability can be improved by adding suitable surfactants (tensides). Optimization of various process parameters, such as pH value of the initial solution, gas flow rate, gas type and the size of the foaming device offers potentials to adapt the foaming process to the specific wood-extractive chemistry.

In further investigations, other foaming agents or the tweezing technique should be tested to improve the separation and accumulation of wood extract components. A cost-efficient and applicable way to separate and recover added surfactants has to be developed. Enrichment factors of the separated components should be determined to be able to estimate the ecologic and economic benefit of the method when applied to wooden material and to direct possible future use of these compounds in biorefinery processes.

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Direct bioautography for the screening of selected tropical wood extracts against basidiomycetes

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Abstract: To understand the reasons for the high durability of tropical wood species, the chemistry of the extractives needs to be elucidated. As these extractives consist of a great variety of components differing in quantity and composition, the analysis is often time-consuming. To focus on the key bioactive substances, bioassay-guided fractionation is helpful, but the established bioassay methods cannot be readily adapted to basidiomycete fungi that are commonly used for the respective durability tests, because they do not sporulate easily in laboratory settings. The research therefore aims at developing a direct bioautography using homogenized hyphae from basidiomycetes, to overcome this restriction. Extracts from four tropical wood species were analyzed regarding their potential bioactivity on two selected basidiomycete fungi. To this end, the chemically complex mixtures and extract constituents were resolved by a two-dimensional planar chromatography and the metabolites were located by characteristic zones of fungal growth inhibition, which was accentuated by a color reaction. The bioactive fractions were analyzed by gas chromatography/mass spectrometry (GC/MS). Potentially responsible compounds could be identified, such as the alkaloid bicuculline from *Mezilaurus itauba*, which has not been described in this species yet. The presented bioassay method can be used as a rapid screening method for bioactive components from wood.

Keywords: basidiomycetes, bioassay, GC/MS, heartwood extracts, hyphae agar

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Introduction

The natural durability of wood is a measure of how well the material is able to resist microbial attacks. According to EN 113 (Deutsches Institut für Normung e.V. 1996) and EN 350-1 (Deutsches Institut für Normung e.V. 1994), it is determined by exposing wood samples to certain fungal species and measuring the loss of mass (Råberg et al. 2005). Two important groups of wood-degrading fungi specified in EN 113 are brown rots and white rots with *Rhodonia placenta* and *Trametes versicolor*, respectively, as two well-known representatives. Both fungi have already been used in earlier studies testing the activity of the tropical wood species *Tectona grandis* Linn. F. and *Dipteryx odorata* (Aubl) Willd. (Wanschura et al. 2016) as well as *Handroanthus* spp. (formerly Tabebuia spp.), *Mezilaurus itauba* (Meissn.) Taub. ex Mez. and *Manilkara bidentata* (A.DC.) A. Chev. (Wanschura et al. 2017).

Many tropical timbers stand out due to their high durability against wood-degrading fungi (Schwager und Lange 1998). However, the mechanism behind this phenomenon has not been fully understood yet. The high content and the composition of extractives in tropical wood compared to species from colder climates might be one reason (Schultz and Nicholas 2000). It is an accepted fact that the extractives contribute to the natural durability of wood to a considerable extent, which was already shown in a number of studies (Rutiaga-Quinones 2001 and literature cited therein, Kirker et al. 2013).

Natural extracts were already utilized in ancient times, and in recent years, the chemistry of extractives has retrieved growing interest due to ecological concerns of synthetic substances (Singh T and Sing 2012; Snow et al. 2019) and basically the working principles of nature might open totally new bioactive structures. Applications to pharmaceutics and food and for the production of platform chemicals are partly implemented (Willför et al. 2003, 2005; Smeds et al. 2012). Prominent examples are sitosterol as a cholesterol inhibitor (AbuMweis et al. 2008) or xylitol in chewing gums (Mäkinen 2014), but many other potentially bioactive wood extractives (e.g. polyphenols, stilbenes, lignans) remain unused so far (Yang 2009), which might be useful, i.e. as wood protection agents.

However, unless high volume by-product material flows from conventionally used processes like in the pulp and fiber industry could be tapped (Yang 2009), the commonly low extractive concentration in wood poses a major challenge. A possible approach, the adsorptive bubble separation was found to be an effective and potentially cost-efficient method to extract and accumulate extractives from wood (Wanschura et al. 2018). The bubble separation method was already brought to a semi-technical scale using other plant sources such as rosemary (*Rosmarinus officinalis*) or hops (*Humulus lupulus*) (Gabel 2008). However, regarding the current market requirements on wood preservatives (low-cost, long-term efficacy), competition with approved synthetic agents is an economical challenge (Yang 2009).

Generally, extracts of wood, like any other plant extracts, are complex mixtures of compounds. Analysis of their bioactive key substances is often time-consuming and necessitates sophisticated laboratory equipment. However, rapid and efficient detection of biologically active compounds is an important aspect of many analytical processes and led to the development of various bioassays for screening purposes (Hostettmann 1991).

Planar chromatography combined with a microbial detection method, termed bioautography, is considered an efficient assay for the detection of antimicrobial compounds (Dewanjee et al. 2015). It allows for the localization of the activity even in a complex matrix and therefore permits a target-guided isolation of the active components (Rahalison et al. 1991).

Conventional bioautography methods to test (wood) extractives are mostly designed for spore-producing fungi (Favre-Godal et al. 2013). The spores are easily harvestable and inoculated on agar. However, these standard antifungal assays cannot be readily adapted to basidiomycetes, like, in particular *T. versicolor* and *R. placenta*. The production of basidiospores *in vitro* was subject of several studies in the past (Croan and Highley 1991), and even if it was improved over the last few years (Choi et al. 2001, 2002; Stirling et al. 2016), the work with hyphae is still standard in many laboratories (Deklerck et al. 2017).

The main objective was to develop a direct bioautography using homogenized hyphae from basidiomycetes. Therefore, it was investigated whether the bioautographic agar overlay method (Rahalison et al. 1991) and the hyphae agar (Kawamura et al. 2004) can be combined. Combining the two techniques should provide a possibility to benefit from the advantages of a direct bioautography (rapid, easy to implement) while testing wood extractives against commonly used basidiomycetes, which do not readily sporulate. Separating the compounds thoroughly should be achieved by performing the thin-layer chromatography as a two-dimensional development (2D-TLC) as suggested by Wedge and Nagle (2000). Furthermore, the vitality of the mixed hyphae should be determined by using the viability stain 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). By indicating the vitality of the mixed hyphae through a stain, the judgment of whether a compound (-mixture) causes fungal inhibition or not should be improved.

Consequently, the presented technique should provide a rapid method to evaluate bioactive components in complex extract mixtures, but also applicable to fractions of them or single extractives, to focus the analytical efforts on the possibly causative key compounds, trying to better understand the increased durability of the tested tropical wood species.

Materials and methods

Wood samples and test organisms: The tropical wood species used in this research were *Astronium graveolens*, *Intsia* spp., *Shorea laevis* and *M. itauba* that come from South and Central America. The heartwood is classified as durability class 1 according to EN 350-2, and the raw density ranges between 0.65 and 1.16 g cm^{-3} (Gérard et al. 2011). The boards used to cut the specimens were purchased from local lumberyards. Wood blocks of 1 cm^3 were sawn out of the heartwood, subsequently milled in a cross hammer mill (Retsch) together with dry ice and sieved with a mesh size of 0.315–0.05 mm. The sample moisture content was determined via oven drying at 105°C as double determination. The sample weight is referred to as absolutely dry. Constant mass according to this test is reached, when the mass decrease after re-drying is less than 0.5%.

Two fungi, the white-rot fungus *T. versicolor* BAM 116 (CTB 863 A) and the brown-rot fungus *R. placenta* BAM 113 (FPRL 280) were chosen as test organisms (according to EN 113), in order to detect a possible bioactivity of the wood extractives in the four species.

Even though all examined wood species are angiosperms, a brown-rot fungus was selected, too, because the investigation refers to the extracts and not to the degradation in outdoor exposure.

Solvent extraction: Determination of the extract content in organic solvents was done in a Soxhlet extractor using petroleum ether, acetone and methanol (all supplied by Merck, Darmstadt, Germany, SupraSolv quality) successively for 6 h each as double determination with a relative deviation of less than 10%. The Soxhlet extractor was filled with 5 g of wood flour and extracted with 170 ml of the respective organic solvent in a 250-ml round-bottomed flask. Extract contents were determined gravimetrically after drying and are given as % of dry substance.

Thin-layer chromatography (TLC): TLC was performed as a two-dimensional chromatography (2D) on preparative glass plates (stationary phase: silica 60; 2 mm; 20×20 cm; Merck, Darmstadt, Germany)

without a fluorescence indicator for bioautography and for gas chromatography/mass spectrometry (GC/MS) analysis. Contrary to the findings by Osmonova (2011), who reported that the silica TLC plates from Merck were not suitable, because the coating was not stable when covered with warm agar, this kind of plate worked well under the tested conditions.

For a proper detection of the substance spots, the separation was also performed on analytical aluminum TLC plates (stationary phase: silica 60; 2 mm; 20×20 cm) as well. The plates were subjected to derivatization by dipping the plates for 10 s in a mixture of $(NH_4)_6Mo_7O_{24}$ (5%) and $Ce(SO_4)_2$ (0.2%) in a 5% sulfuric acid solution. Afterward, they were dried with a heat gun.

For the bioassays, 30 µl of extract solution with a concentration of 100 g l^{-1} (resp. 3 mg) was applied onto the plates. The acetone extracts were solved in a mixture of 75% acetone/25% methanol, and the methanol extracts were solved in a mixture of 75% methanol/25% H_2O. The solvents used for the mobile phases are given in Table 1. For the acetone and the methanol extracts, the same solvent mixtures were used. After performing chromatography, the plates were left to dry overnight in a vacuum oven at 60°C. By doing so, the organic solvents, which cause the inactivation of enzymes or death of living organisms, were completely removed prior to biological detection.

Bioassays were performed as duplicates. Therefore, 12 plates per wood species were prepared:

(1) two TLC plates with acetone extract and
(2) two TLC plates with the respective methanol extract per fungi for the following bioassay analysis.
(3) one TLC plate per fungi and wood species without extractives as a reference.

Furthermore, one TLC plate per extract and wood species was prepared for the following GC/MS analysis.

Inoculation of fungal cultures (hyphae agar) and bioactivity assay: Hyphae agar

Homogenized hyphae solution was prepared according to Kawamura et al. (2004): for each experiment, 5.1 g malt extract (4%) was mixed with 125 ml dH_2O using a stir bar until the solution was clear. Then, the bouillon was poured into Erlenmeyer flasks and autoclaved (sterilization temperature 121°C for 20 min; autoclave: HMC Europe, Tüßling, Germany). Three inoculation blocks (0.5×0.5 cm) were cut from the fungal film of an agar plate (fungal cultures were grown on a 4% malt agar and were inoculated to fresh media every 6–8 weeks) of either *R. placenta* or *T. versicolor*. The inoculation blocks were put into the malt extract medium and the liquid cultures were incubated in an incubator (Memmert) for approximately 2 weeks at 22°C, 70% relative humidity.

Two hundred and fifty milliliters of water was added to 9.75 g of potato dextrose agar (PDA), which was then autoclaved as mentioned earlier. The malt extract medium was decanted from the liquid cultures of *R. placenta* (19 000 rpm) or *T. versicolor* (10 750 rpm) and the hyphae were homogenized using a homogenizer (VWR, Ismaning, Germany). Then, the suspension was centrifuged (4 min), the supernatant was discarded and the hyphae were washed with 10 ml of sterile 0.9% sodium chloride solution. Afterward, the hyphae were suspended in 2.5 ml of 0.9% sodium chloride.

Five milliliters of hyphae solution was added to 250 ml PDA with a temperature of >50°C (hyphae concentration of approximately 2%). Instead of spraying an aqueous MTT solution onto the already solidified agar overlay on the TLC plates (Rahalison et al. 1991; Osmonova 2011), 1 ml of the solution was mixed directly into the agar with a concentration of 10 mg ml^{-1}.

Bioassay

To generate a 1-mm-thick agar layer on the TLC plate, the plate was locked into a suitable form and overlaid with 120 ml of the hot hyphae agar-MTT mixture. After drying (for 1–2 min), the plate was disassembled from the template form, stored in a bioassay dish (245×245×25 mm, Corning Life Science, Lowell, MA, USA) and incubated in an incubator at 22°C and 70% relative humidity.

To determine the location of the compounds on the TLC designated for GC/MS analysis, the bioassay plates were photographed after incubation. On each photo, the inhibition zones were marked. Together with the detection of the substance spots by ultraviolet (UV) light, these pictures were used as an overlay to mark the corresponding spots on the "GC/MS-plate", which were then scratched from the plate. Furthermore, the TLC plates were compared with a respective chromatogram developed under identical conditions, which was revealed with the oxidative dying reagent (see Coloring of the hyphae through the vital stain MTT). This compound visualization provided helpful information about their localization in the plate.

GC/MS: The substance spots from the TLC plates were dissolved in 5 ml methanol and were separated from the silica via microfiltration. After evaporating the solvent, the residue was dissolved in 100 µl dimethylformamide (DMF) and 30 µl N,O-bis(trimethylsilyl) trifluoroacetamide/chlorotrimethylsilane (BSTFA/TMCS; Sigma-Aldrich).

The samples were placed in a thermoblock at 80°C for 1 h. One microliter of the sample was injected at an injector temperature of 300°C with a split of 30:1 (39 ml min^{-1}). The carrier gas was He. GC was heated at 10°C min^{-1} from 100°C to 320°C (held isothermally for 15 min). A SGE-BPX 5 separation column was used: 15 m×0.25 mm;

Table 1: Solvent mixtures used for the separation of the extract components.

	1D	2D
Shorea laevis	Butanone:methanol:dH_2O (20:3:4) + 10 drops of formic acid to 300 ml solvent mixture	Butanone:propanol:dH_2O (6.5:2.5:1) + 10 drops of formic acid to 300 ml solvent mixture
Mezilaurus itauba	Cyclohexane:ethyl acetate:ethanol (1:1:19.5) + 10 drops of NH_3 to 300 ml solvent mixture	Chloroform:methanol:dH_2O (35:18:1) + 15 drops of NH_3 to 300 ml solvent mixture
Intsia spp.	Cyclohexane:acetone:ethanol (3:6:1) + 10 drops of formic acid to 300 ml solvent mixture	Chloroform:acetone:methanol (1:2:2) + 10 drops of formic acid to 300 ml solvent mixture
Astronium graveolens		

0.25 µm film; ionization: –70 eV. Detector temperature was set to 150°C and MS source to 230°C and MS was recorded in a mass range from 40 to 800 amu. Identification of compounds was done via comparison of spectra with a NIST08 database or measurement of reference compounds.

Results and discussion

Coloring of the hyphae through the vital stain MTT

Based on the reduction of the yellow tetrazolium salt, actively growing cells produce a blue/purple, insoluble formazan product (Mosmann 1983). This tetrazolium dye reduction is generally assumed to be dependent on Nicotinamidadenindinucleotidphosphat (NAD(P)H)-dependent oxidoreductase enzymes in the cytosolic compartment of the cell (Berridge and Tan 1993; Berridge et al. 2005). MTT has been utilized successfully to quantify the effects of antifungal agents on cell viability or as a method of colorimetric determination of fungal cell densities on a number of fugal species (Hidore et al. 1991; Meshulam et al. 1995; Freimoser et al. 1999; Etexeberria et al. 2011; Patel et al. 2013; Stockert et al. 2018).

Preliminary tests showed that the best results concerning the homogenized hyphae were achieved using cultures that were incubated for 14 days in a liquid broth and adjusted to 2% hyphae in the PDA. It was found that it is possible to combine the homogenized hyphae from *T. versicolor* and *R. placenta* with MTT as an activity indicator, with a concentration of 40 µg ml^{-1} for the best optical results. Figure 1 shows the results of a diffusion test to evaluate the best conditions for the tests using β-thujaplicin, an established fungicide (Rennerfelt 1948) at a concentration of 0.2 g l^{-1} that produced clear zones of fungal inhibition.

Determination of antifungal activity of the extract fractions via 2D-TLC agar overlay method

Extract content of the tested species

The extracts of the wood species were obtained through successive extraction with three solvents (Table 2). Preliminary results revealed that the lipid fractions of the extracts showed no bioactivity in agar dilution tests. Therefore, these parts were not further tested, although they contributed depending on the extract amounts to a considerable extent (*M. itauba*: 4.1%).

2D-TLC Bioassay

Figure 2 (I–IV) shows the bioassay results from *Astronium graveolens* acetone and methanol extracts tested against *T. versicolor* and *R. placenta*. Zones of fungal growth inhibition could be clearly recognized by an absence of fungal growth and a bright beige coloring of the agar (unless the agar is not colored by the extract compound; zones on the plates are marked with an arrow), surrounded by the blue agar colored by the MTT reaction.

As a completely homogeneous dispersion of the hyphae is difficult, the optical determination of the bioactive zones

Table 2: Percentage of extract amounts referring to the dry weight of the wood species.

Species/solvents	Petroleum ether [%]	Acetone (%)	Methanol (%)	Σ
Astronium graveolens	1.6	1.5	3.0	6.1
Intsia spp.	0.6	8.3	10.6	19.5
Shorea laevis	0.4	8.9	1.7	10.9
Mezilaurus itauba	4.2	1.4	2.5	8.2

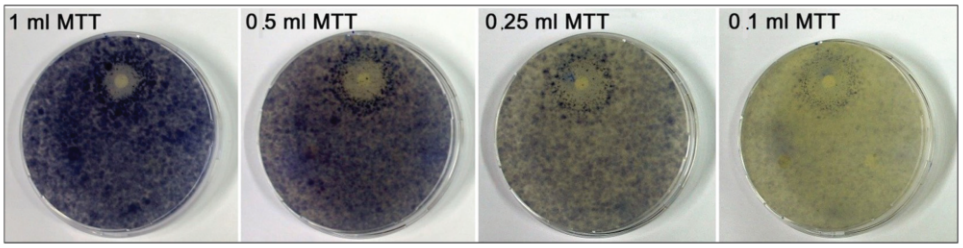

Figure 1: Different volumes (1 ml; 0.5 ml; 0.25 ml; 0.10 ml) of MTT in 250 ml of hyphae-PDA from *R. placenta*, after 8 days. β-Thujaplicin (concentration: 0.2 g l^{-1}) was used as a positive control.

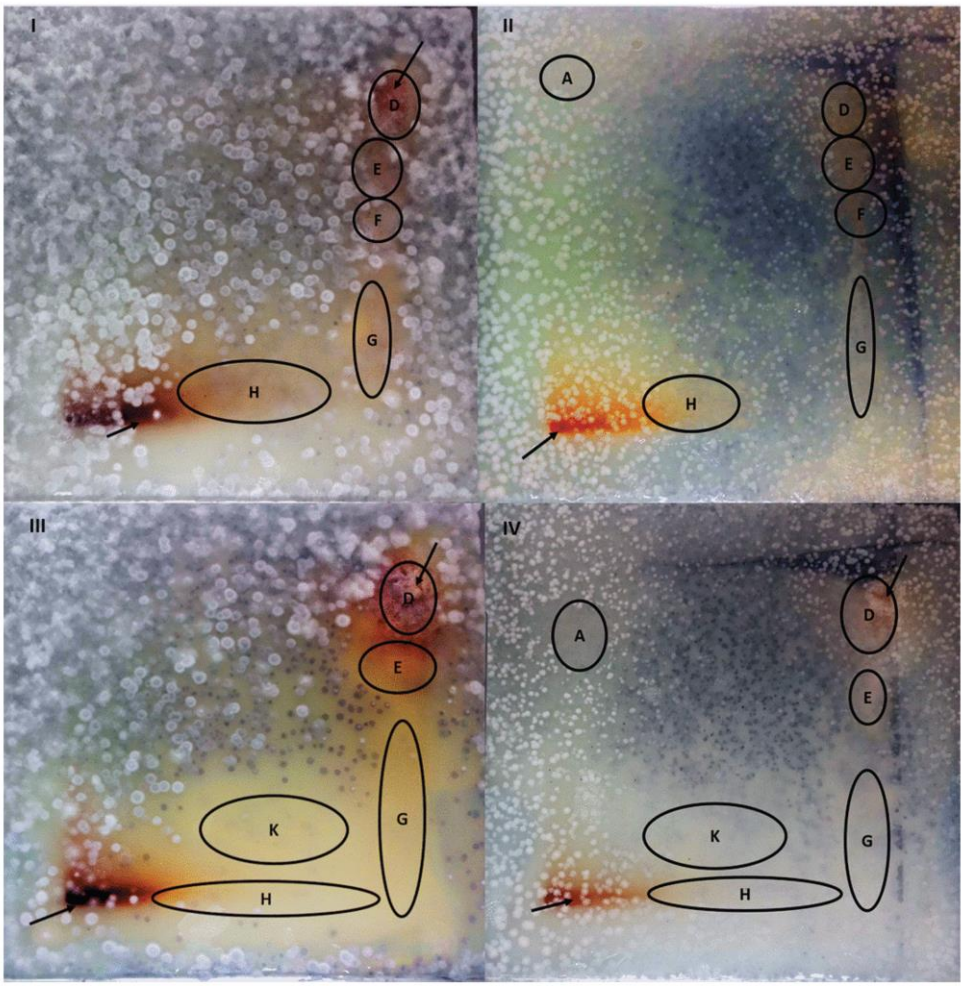

Figure 2: 2D-TLC direct bioautography of *Astronium graveolens* acetone (I/II) and methanol (III/IV) extract: bioactivity response from *T. versicolor* (left) and *R. placenta* (right) after 3 days.

was much more exact using the MTT vitality stain than an evaluation by judging the hyphal growth alone. Hyphae that were located close to the surface rapidly formed white spots of hyphal growth on the agar surface. This took more time for hyphae located deeper in the agar. Consequently, parts of the plate were almost overgrown, while in other parts, less growth was visible, which may lead to misinterpretations of the bioactive zones (false positives). Even though the intensity of the blue coloring varies (due to unequally distributed hyphae), the optical determination of the bioactive zones is highly improved.

For a better visualization of the bioactive zones on the photos shown in Figure 2 (I–IV), the zones were marked by a circle. Inside the circles, the partitioned antifungal constituents of the extract can be found.

It could be observed that both fungi are not able to distinguish between compounds that are located very close together on the TLC. Instead, they formed inhibition zones with no or less fungal activity. On the one hand, this could be due to the fungal sensitivity, but more on the other hand, the substances very close together are more likely to get mixed during the diffusion process from the silica phase into the agar overlay, although the agar layer is very thin. Therefore, the detected fractions are aggregated to zones of fungal growth inhibition.

On the plates with the acetone extract, five zones with bioactivity against both fungi could be detected: D, E, F, G and H, while fraction A only inhibited the growth of *R. placenta*. In the methanol extract, five zones were also detected. However, instead of zone F, zone K showed

bioactivity on these plates. Again, zone A only inhibited the growth of *R. placenta*. Compared to compounds in the other active zones, the compound(s) in zone A may have a smaller microbiologically effective spectrum.

The response to the tested extracts was more pronounced in tests with *T. versicolor*. The inhibition zones were brighter, more differentiated and it took longer for the fungi to overgrow the inhibition zones.

Both fungi recovered from the hyphae-mixing procedure in a short period: However *T. versicolor* seemed to recover slightly faster than *R. placenta*. The reaction time until the blue coloring of the MTT appeared was varying from 3 to 8 days, whereby the response time for *R. placenta* cultures was longer.

This was the case for all tested wood extracts (not all bioassay data shown). All zones of the separated extracts showed clear inhibition zones and demonstrated that the method can be applied successfully to wood extracts. Moreover, the agar overlay technique may also be applied to undeveloped TLC plates with directly spotted extracts or substances, for example, with different concentrations. An additional finding was that the hyphae solution (in 0.9% NaCl) could be stored in the refrigerator for up to 3 weeks. Activity tests showed that the hyphae were still vital after this period and showed activity in the hyphae agar. Therefore, it is also possible to prepare the hyphae solution prior to the preparation of the TLC plates.

Determination of the separated components by GC/MS

All spots, from locations with or without bioactivity in the bioassay, were subjected to GC/MS. The measurement revealed a surprising variety of compounds, namely fatty acids, carboxylic acids, alkanes, alcohols (mainly glycerol) and sugars as well as phenolic compounds, flavonoids, triterpenes and alkaloids. TLC only involves a certain amount of extractives and the method cannot deal with concentrations.

Some compounds were found in all wood samples: palmitic acid and stearic acid as well as different other fatty acids were detected in all zones. Together with varying contents of glycerol, it is supposed that they are present as triglycerides in the extracts, which are partly degraded during the sample preparation. Furthermore, most of the detected sugars were also found in every zone: sometimes as a single component, sometimes associated with other compounds. To test whether the sugars were spread over the plate during the development, samples were taken from places with no substance spot. As these samples were negative to sugar compounds, it is suggested that many of the compounds in the extract are present as glycosides.

It was observed that the acetone and the methanol extracts of each wood sample have zones in common that were located on the same places on the plates. For the shown example in Figure 2 (I–IV), these were the zones D, E, G and H. GC/MS revealed that in these zones the same, or even chemically related compounds, could be detected. Catechin in zone D or gallic acid in zone E are examples for this finding (Table 3). This localization was due to the use of the same solvent mixtures for the separation of the extracts. Furthermore, it seems as if the successive extraction with raising polarity of the solvents did not create the intended "pre-separation" effect and therefore many of the compounds were found in both extracts.

Compounds detected in the inhibition zones of *Astronium graveolens*

As already mentioned, the extracts from the acetone and the methanol extract shared the bioactive zones D, E, G and H, while K was only present in the methanol extract and zone A was only active against *R. placenta*. In zone A, GC/MS revealed high amounts of a fatty acid, identified as caprylic acid. Fatty acids are known as fungicidal food additives (Pohl et al. 2011) and several studies showed the fungicidal ability of fatty acid formulations like a caprylic acid-based formulation (Clausen et al. 2010) or a caprylic-pelargonic-capric acid formulation (Liu et al. 2014) against several different fungi. A possible hypothesis for the mode of action is that the fatty acids cause an increase in membrane fluidity, which will result in the leakage of the intracellular components and cell death (Clausen et al. 2010; Pohl et al. 2011). Therefore, an influence of caprylic acid on the growth of *R. placenta* hyphae may be also possible.

Catechin at R_t 20.13 and an unidentified sterol at R_t 23.32 were observed in zone D on the plate with the acetone as well as on the plate with the methanol extract (Table 3). Two compounds that are probably (epi-)gallocatechin gallates (R_t 30.54 and 32.73) could only be detected in the methanol extract. This is consistent with the fact that zone D on the methanol plates is more intense compared to the respective acetone plate (Figure 2A–D).

It is known that *Astronium* species contain different hydrolyzable tannins like gallotannins and ellagitannins together with different phenolic derivates (Da Silva et al. 2011). Other already known components found in *Astronium* species are gallic acid, caffeic acid, chlorogenic acid, china acid, quercetin and rutin (Imai et al. 2008;

Table 3: Compounds detected on the TLC that were developed similar to the bioassay plates.

Wood species				*Astronium graveolens*										*Intsia* spp.										*Shorea laevis*						*Mezilaurus itauba*					
Tested extract		**a**	**m**	**a**	**m**	**a**	**m**	**a**	**m**	**a**	**m**	**m**		**a**	**m**	**a**	**m**	**m**	**a**	**m**	**a**	**a**		**a**	**m**	**m**	**a**	**m**	**a**		**m**	**a**	**m**	**a**	**m**	**m**
Zone on TLC			**A**	**D**		**E**		**G**		**H**		**K**		**C**		**D**		**E**	**F**		**G**	**H**		**B**		**D**	**E**		**F**		**E**	**F**		**G**		**K**
Compound	RT (min)																																		
Caprylic acid	5.46	x	x																																
n.i. compound	12.40																		x																
Gallic acid	12.63					x	x				x											x													
Dehydroabietic acid	16.80													x													x	x	x						
Bicuculline	18.61																													x	x	x			
Methylcoclaurine	18.73																																x		
Laurotetanine	18.93																															x			
Catechin isomer	19.08													x	x	x																			
Resveratrol	19.15																								x										
Catechin isomer	19.18													x	x		x																x	x	
n.i. alkaloid	19.22																																x	x	
Coclaurine	19.30																													x	x	x	x	x	x
Catechin isomer	19.33													x	x	x		x																	
Catechin isomer	19.34														x		x	x																	
Reticuline	19.40																																x	x	
Norbicuculline	19.48																													x	x	x		x	
n.i. alkaloid	19.58																																		x
n.i. alkaloid	19.63																																	x	
n.i. flavonoid	19.86					x	x				x	x	x																						
Catechin	20.13			x	x																														
n.i. alkaloid	20.14																																		x
Boldine	20.71																															x			
Boldine isomer	20.83																														x	x			
n.i. compound	20.85													x																					
Boldine derivate	20.99																																x	x	
Boldine isomer	21.22																																x	x	x
n.i. triterpene	21.50																															x			
n.i. triterpene	21.53																																		x
n.i. alkaloid	22.49																																	x	
n.i. alkaloid	22.63																															x			x
Isoboldine	22.66																																	x	
n.i. triterpene	22.74								x																										
n.i. triterpene	22.96								x																										
n.i. sterol	23.32			x	x																														
n.i. triterpene	24.84																																	x	
n.i. triterpene	25.27																								x										
n.i. triterpene	26.98																						x		x										
n.i. triterpene	27.21																						x	x	x										
(Epi-)gallocatechin gallate	30.54				x																														
(Epi-)gallocatechin gallate	32.73				x																														

Zones are located in comparison to the bioactive zones of the acetone (a) and the methanol (m) extract. Only the possibly bioactive compounds are shown. Determination was realized via GC/MS.

Brito Pereira Bezerra Martins et al. 2018). In zone E and in the methanol zones H and K, an unidentified flavonoid (R_t 19.86) and gallic acid (R_t 12.63) were detected, as well as two triterpenes (R_t 22.74 and R_t 22.69) in methanol zone G. The bioactive potential of the acetone zones G and H cannot be explained sufficiently at this moment.

Compounds detected in the inhibition zones of *Intsia* spp.

As can be seen in Table 3, catechin isomers like robinetinidol or fisitinidol and some more flavonoids were detected in zones C, D and E. Furthermore, the reduced hyphal growth in zone H could be explained by a higher content of gallic acid. Gallic acid has already been detected in an *Intsia* species by Kilic and Niemz (2012). Robinetin was reported as the main flavonoid in *Intsia bijuga*. Further constituents mentioned in this study were dihydromyricetin, myricetin, naringenin and leucocyanidin (Hillis and Yazaki 1973).

Compounds detected in the inhibition zones of *Shorea laevis*

In the bioactive zones B and D, some fungicidal triterpenes were detected and additionally resveratrol in zone D. Hadi (2009) identified hopeaphenol, a resveratrol tetramer, in *Shorea ovalis*. Bisset et al. (1971) detected triterpenes like ursolic aldehyde, dipterocarpol, shoreic acid and dammarenolic acid in *Shorea roxburghii*. The bioactivity of zones E and F might be related to a diterpenic compound, which has a fragment pattern that indicates the occurrence of dehydroabietic acid. Dehydroabietic acid has a well-studied bioactivity profile, including antiviral, antibiotic and antifungal activity (Helfenstein et al. 2017). Even though dehydroabietic acid, as a resin acid, is usually found in conifers like *Pinus* spp., it is known that *Shorea* species form resin ducts (Wagenführ and Scheiber 1974).

Some more compounds, already isolated from the heartwood of *Shorea* spp., such as the tannin-related compounds like gallic acid, flavogallonic acid dilactone, valoneic acid dilactone, ellagic acid and catechin (Schwager and Lange 1998; Hirano et al. 2003) were not detected on the TLC. One reason might be the relatively low concentration of some compounds on the TLC, which could therefore not be isolated properly. To address the concentration problems that do not allow correct identification of probably bioactive compounds, further preparative chromatography methods could provide higher amounts of the pure compounds, which would allow for additional analysis methods, such as nuclear magnetic resonance (NMR).

Compounds detected in the inhibition zones of *Mezilaurus itauba*

Like many Lauraceae species, *M. itauba* contains a portfolio of alkaloids (Gottlieb 1972). Already identified alkaloids from *Mezilaurus synandra* are coclaurine and corytuberine as well as some lactones (Silva et al. 1983) and neolignans from *M. itauba* (Yanez et al. 1988).

While the benzyltetraisochinoline coclaurine (R_t 19.30) was present in all bioactive zones (E, F, G, K), reticuline (R_t 19.40) and methylcoclaurine (R_t 18.73) (Figure S1A) could only be detected in zone G. With the exception of zone E, all zones contained aporphines like boldine (R_t 20.71) or one of its isomers isoboldine and corytuberine (R_t 20.83, R_t 21.22). Furthermore, its derivates laurotetanine (R_t 18.93) and methyllaurotetanine (Figure S1B) or its isomer isocorydine (R_t 20.99) (Figure S1B) could be identified. The structures of the identified compounds are given in the Supplementary material (Figure S1A and Figure S1B). Benzyltetraisochinolines as well as aporphines belong to the most frequently detected alkaloids in the family Lauraceae (Custódio and da Veiga Junior 2014).

In the zones E, F and K, two compounds were detected, which have not been described in *Mezilaurus* species yet: norbicuculline and bicuculline (structure given in Figure S), of which the latter is known as a GABA (4-aminobutanoic acid) receptor antagonist (Starke 2013), the chief inhibitory neurotransmitter in the central nervous system (Watanabe et al. 2002). Furthermore, a triterpene was found in zone G (R_t = 24.84).

As the results show, a part of the natural durability of the tested tropical wood species can certainly be attributed to the detected substances causing the inhibition zones on the TLC plates. Because this inhibition is also dependent on the substance concentrations, the minimal inhibitory concentrations should be determined in a next step. Furthermore, these concentrations must then be related to the concentrations that prevail in the wood. Some of the compounds may meet the requirements in potency and concentration to play an outstanding role for natural durability. Nevertheless, consideration should be given to the entire extractive mixture of the wood species. Some of them play a role as an elicitor, regulator or water repellent and may have important influence on the natural durability.

Conclusions

Due to multiple additional properties of extractives (e.g. antioxidant, water repelling, metal chelating), bioactivity tests containing solid wood or sawdust need to be used to determine their full capacity for wood protection. However, the agar overlay bioautography method is a possibility to examine the direct fungicidal activity and was successfully implemented for basidiomycete fungi using *R. placenta* and *T. versicolor* in media seeded with homogenized hyphae. The added vital stain MTT provided a rapid and clear assignment of the zones containing antifungal agents. The chemically complex mixtures of semipolar and polar tropical wood extracts from *A. graveolens*, *Intsia* spp., *S. laevis* and *M. itauba* were resolved by 2D-TLC and analyzed by GC/MS. In most of the bioactive zones, major substance classes including several known fungicidal compounds could be identified. Although the concentrations of the single compounds cannot be determined via this method, it could be shown that the method represents a straightforward analysis tool to test extracts from wood for bioactive substances even in complex mixtures. This can be rated as a proof of principle, with prominent examples like gallic acid, catechin and resveratrol. Furthermore, the isoquinoline alkaloids bicuculline and norbicuculline were detected, which have not been described in *M. itauba* yet.

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